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Putative carboxylesterase gene identification and their expression patterns in *Hyphantria cunea* (Drury)

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Olfactory system is important for behavioral activities of insects to recognize internal and external volatile stimuli in the environment. Insect odorant degrading enzymes (ODEs) including antennal-specific carboxylesterases (CXEs) are known to degrade redundant odorant molecules or to hydrolyze olfactorily important sex pheromone components and plant volatiles. Compared to many well-studied Type-I sex pheromone-producing Lepidopteran species, the molecular mechanisms of the olfactory system of Type-II sex pheromone-producing *Hyphantria cunea* (Drury) remain poorly understood. In current study, we first identified a total of ten CXE genes based on our previous *H. cunea* transcriptomic data. We constructed a phylogenetic tree, compared motif-patterns between Lepidopteran CXEs, and used quantitative PCR to investigate the gene expression of *H. cunea* CXEs (HcunCXEs). Our results indicated that HcunCXEs are highly expressed in antennae, legs and wings, suggesting a potential function in degrading sex pheromone components, host plant volatiles, and other xenobiotics. This study not only provides a theoretical basis for subsequent olfactory mechanism studies on *H. cunea,* but also offers some new insights into functions and evolutionary characteristics of CXEs in lepidopteran insects. From a practical point of view, these HcunCXEs might represent meaningful targets for developing behavioral interference control strategies against *H. cunea*.

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Abstract

Olfactory system is important for behavioral activities of insects to recognize internal and external volatile stimuli in the environment. Insect odorant degrading enzymes (ODEs) including antennal-specific carboxylesterases (CXEs) are known to degrade redundant odorant molecules or to hydrolyze olfactorily important sex pheromone components and plant volatiles. Compared to many well-studied Type-I sex pheromone-producing Lepidopteran species, the molecular mechanisms of the olfactory system of Type-II sex pheromone-producing *Hyphantria cunea* (Drury) remain poorly understood. In current study, we first identified a total of ten CXE genes based on our previous *H. cunea* transcriptomic data. We constructed a phylogenetic tree, compared motif-patterns between Lepidopteran CXEs, and used quantitative PCR to investigate the gene expression of *H. cunea* CXEs (HcunCXEs). Our results indicated that HcunCXEs are highly expressed in antennae, legs and wings, suggesting a potential function in degrading sex pheromone components, host plant volatiles, and other xenobiotics. This study not only provides a theoretical basis for subsequent olfactory mechanism studies on *H. cunea,* but also offers some new insights into functions and evolutionary characteristics of CXEs in lepidopteran insects. From a practical point of view, these HcunCXEs might represent meaningful targets for developing behavioral interference control strategies against *H. cunea*.

Introduction

A complete insect olfactory process requires the participation and cooperation of various olfaction-related proteins (*Scott et al., 2001*; *Vogt, 2003*; *Leal, 2013*). During the process, external liposoluble odor molecules first pass through the polar pores on the sensillum surface, then enter the lymph under the integument where they further combine with odorant binding proteins (OBPs) before being transferred to the dendritic membrane of olfactory receptor neurons (ORNs) (*Tegoni, Campanacci & Cambillau, 2004*; *Leal, 2013*; *Pelosi et al., 2018*). The molecule-bound odorant receptors (ORs) then convert the chemical signals into electrical signal that transmits to the central nervous system through axons of the ORNs (*Song et al., 2008*). This whole process guides insects to make different relevant physiological responses and behavioral decisions. Once the signal transmission is completed, redundant odorant molecules need to be degraded or inactivated by odorant degrading enzymes (ODEs) in the antennal sensilla, otherwise, the odorant receptors will remain in a stimulated state, which may lead to disorders of the nervous system and pose fatal hazards to the insects (*Vogt & Riddiford, 1981*; *Steinbrecht, 1998*; *Durand et al., 2010b*; *Leal, 2013*). ODEs degrade redundant odorant molecules in the lymph of antennal sensilla and within the cells. Based on the structural difference of various target substances, ODEs can generally be divided into five categories: carboxylesterase (CXE), cytochrome P450 (CYP), alcohol dehydrogenase (AD), aldehyde oxidase (AOX) and glutathione-*S*-transferase (GST) (*Rybczynski, Reagan & Lerner, 1989*; *Ishida & Leal, 2005*; *Pelletier et al., 2007*; *Durand et al., 2010a*; *Yang et al., 2019*).

As primary metabolic enzymes, CXEs are widely distributed among insects, microbes and plants (*Guo & Wong, 2020*). CXEs play an essential role in insect physiology and metabolism, as well as in herbicide activation (*Enayati Ranson & Hemingway, 2005*; *Li, Schuler & Berenbaum, 2007*; *Guo & Wong, 2020*). In addition to the metabolism and detoxification of endobiotics and xenobiotics, another important role of CXEs is to maintain the sensitivity of ORNs. The way to play its role is to rapidly degrade stray odors so as to prevent vulnerable ORNs from being continuously invaded by harmful volatile xenobiotics (*Li et al., 2013*). So far, a large number of genes encoding CXEs been identified and their functions in insect olfaction have also been investigated in various insects, including *Mamestra brassicae*, *Antheraea polyphemus; Sesamia nonagrioides*, *Popillia japonica*, *Spodoptera littoralis*, *Epiphyas postvittana*, *Agrilus planipennis*, *S. litura*, *S. exigua*. (*Vogt, Riddiford & Prestwich, 1985*; *Maïbèche-Coisne et al., 2004*; *Ishida & Leal, 2005*; *Merlin et al., 2007*; *Ishida & Leal 2008*; *Jordan et al., 2008*; *Durand et al., 2010b*; *Mamidala et al., 2013*; *He et al., 2014a*; *He et al., 2014b*; *He et al., 2014c*; *He et al., 2015*). For instance, the *A. polyphemus* pheromone-degrading enzyme CXE (*Apol*PDE) could effectively degrade its sex pheromone acetate component (*Maïbèche-Coisne et al., 2004*; *Ishida & Leal, 2005*). In *P. japonica* and *D. melanogaster*, the purified native or recombinant antennal CXEs were found to degrade their sex pheromone constituents (*Ishida & Leal, 2008*; *Younus et al., 2014*). In addition, some of CXEs from *S. exigua*, *S. littoralis* and *S. litura* were also found to degrade both their sex pheromones and the plant volatiles (*Gomi, Inudo & Yamada, 2003*; *Durand et al., 2011*).

The fall webworm, *Hyphantria cunea* (Drury) (Lepidoptera; Erebidae), native to North America, is a worldwide quarantine pest insect. This moth has now spread to most European countries (except the Nordics), South Korea, North Korea and China, and lately to Central Asia (*Itô & Miyashita, 1968*; *Gomi, 2007*). As an invasive pest, *H. cunea* was first found in Dandong (Liaoning province, China); it has rapidly spread to Hebei and adjacent provinces in China (*Gomi, 2007*; *Yang et al., 2008*; *Tang, Su & Zhang, 2012a*). In 2012, the State Forestry Administration's Forest Pest Inspection and Identification Center identified the first outbreak of *H. cunea* in Sanshan district, Wuhu City, Anhui Province, which was the southernmost known outbreak of *H. cunea*. Its invasion has caused serious damage to the local forests, agricultural crops and landscaping/ornamental trees, resulting in great economic and ecological losses. Thus, effective quarantine programs and environmentally safe pest management solutions are needed to combat this serious invasive pest insect. More importantly, a better understanding of its chemical ecology may facilitate more effective pest management strategies. However, compared to many well-studied Type-I sex pheromone-producing moth species, the molecular mechanisms of olfaction in the Type-II sex pheromone-producing *H. cunea* are poorly understood. In the current study, a total of 10 CXE genes were identified based on our previous *H. cunea* transcriptomic data (Zhang et al., 2016). To understand the potential physiological roles of these HcunCXEs, we constructed a phylogenetic tree, compared motif-patterns between different Lepidopteran CXEs and used reverse transcription-quantitative PCR and reverse transcription PCR to investigate the expression of these genes. We found that HcunCXEs displayed a

antennae- or leg/wing-biased expression, suggesting a potential function in degrading sex pheromones, host plant volatiles, and/or other xenobiotics.

Materials and Methods

Insect rearing and tissue collection

Insect cages were used for rearing *H. cunea* pupae at 25°C, 70-80% RH and 14L:10D photoperiod. These *H. cunea* pupae were collected from a first-generation population at Baimao Town, Jiujiang District, Wuhu City, Anhui province. Several body parts/tissues: antennae, thoraxes, abdomens, legs, and wings, of virgin males and females were dissected under the microscope and pooled by sex and body parts. Male and female pupae and fourth instar larvae ware also sampled. Dissected body parts or whole-body samples were flash frozen in liquid nitrogen and stored at -80℃ until use.

Analysis of gene expression level

The *H. cunea* antennal transcriptome (SUB6944247) (*Zhang et al., 2016*) was used as a reference sequence for mapping clean reads for each tested sample. RSEM software package for quantifying transcript abundances from RNA-Seq data was used to obtain the read count number of each sample (*Li & Dewey, 2011*). To investigate the expression patterns and levels of these genes, FPKM (fragments per kilobase of exon model per million mapped reads) value was used (*Trapnell et al., 2010*).

Homologous search of CXE genes in *H. cunea*

The *H. cunea* CXE genes were identified according to the BLAST results on NCBI. The Open Reading Frame finder (OFR Finder) was used to search for the open reading frame of these CXE genes. To calculate their theoretical isoelectric points (pI) and molecular weights (MW) of the full-length HcunCXEs gene candidates, an ExPASy tool (http://web.expasy.org/compute_pi/) (*Petersen et al., 2011*). Therefore, SignalP-4.0 was used to predict signal peptides of the CXE genes (*Petersen et al., 2011*).

Phylogenetic analysis of CXE genes in *H. cunea*

Genes related to the ODEs of *H. cunea* and other reported insects were subjected to multi-sequence alignment on ClustalX2.0 (*Larkin et al., 2007*), and the phylogenetic tree was constructed using MEGA5.0 (*Larkin et al., 2007*; *Tamura et al., 2011*) software and neighbor-joining method (1000 repetitions) for systematic evolution analysis. The genes of insect ODEs required for the phylogenetic tree were shown in Supplementary Table S1.

Motif analysis of CXEs

A total of 44 CXEs from *H. cunea* (10 HcunCXEs), *S. inferens* (15 SinfCXEs) and *S. litura* (19 SlitCXEs) were used for identification of conserved motifs and pattern analysis. The online program Multiple Em for Motif Elicitation (MEME, version 4.11.1) was used to obtain the motif

141 in all CXEs genes (*Bailey et al., 2015*). MEME was done with the following parameters: the
142 width between the range of 6 -10, and the number of motifs was below 8.
143
144 **RNA extraction and synthesis of the first-strand cDNA**
145 The sampled body tissues were grounded using Tissue-Tearor which rapidly homogenized the
146 samples in DEPC-treated sterile water. Extraction and purification of total RNA from each
147 sample were done using TRIzol reagent (Invitrogen, USA). The degradation and contamination
148 of RNA product were monitored on 1% agarose gels, and purity was checked using a
149 NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). First-stranded cDNA templates
150 were synthesized using 1 μg of RNA templates with the PrimeScript™ RT reagent Kit according
151 the manufacturer instruction (TaKaRa, Japan).
152
153 **RT-qPCR and RT-PCR analysis**
154 Expression profiles of the identified *H. cunea* CXE genes in different body parts of adults
155 (antennae, legs, wings, thoraxes, abdomens) and two other life stages (pupae and larvae) were
156 analyzed.
157 RT-qPCR and RP-PCR assays were employed for the multiple copies of DNA production.
158 RT-qPCR reaction was conducted in a 25μL reaction mixture system containing 12.5μL of
159 SYBR® Premix Ex Taq II (Tli RNaseH Plus), 1μL of each primer, 2μL of sample cDNA, and
160 8.5μL of sterilized H2O.

The RT-qPCR cycles were set at: 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 60°C for 30 sec. Each experiment was carried out in a CFX96 real-time PCR detection instrument (Bio-rad, USA) using 8-strip PCR tubes (Bio-rad). The reaction data were recorded, and the dissolution curves were appended. The reproducibility confirmation of each RT-qPCR reaction was replicated three times for each sample (*Xu et al., 2018*).

The variability of each gene expression in different body tissues was tested by using Q-Gene method (*Muller et al., 2002*; *Simon, 2003*). The relative expressions of mRNA of each gene (mean ± SD) were analyzed using one-way ANOVA (SPSS22.0 for Windows, IBM, USA), followed by LSD and Duncan's tests at $\alpha = 0.05$. Graphical plotting/mapping was done by GraphPad prism v5.0 Software (GraphPad Software Inc, CA, USA). The RT-qPCR primers of CXE gene in *H. cunea* are listed in Supplementary Table S2.

RT-PCR analysis was performed as follows: 94°C for 2 min of initiation, and 29 cycles of 94°C for 30 sec, 52°C for 30 sec, 72°C for 15 sec, and 2 min at 72°C for final extension. Elongation factor-1 alpha (EF1-a) gene of *H. cunea* was used as an internal reference. In addition, instead of template cDNA, RNase-free water was used as the blank control. A total of 25μL reaction mixture containing 12.5μL of 2x Ex Taq MasterMix (CWBIO, China), 1μL of each primer, 1μL of sample cDNA, and bring up to 25μL of sterilized H2O. 10μL aliquot of each reaction product was taken to obtain agarose gel electrophoresis detection results. The RT-PCR primer sequences of CXE genes in *H. cunea* are listed in Supplementary Table S3.

Results

Identification of CXE genes from *H. cunea*

Based on a comparative analysis of the *H. cunea* antennal transcriptome using Blastx databases (*Zhang et al., 2016*), a total of 10 HcunCXE genes were identified, which were further compared with the CXE genes in *S. inferens*. As shown in Table 1, six HcunCXEs (HcunCXE1, HcunCXE3-5 and HcunCXE7-8) had complete ORFs. According to the prediction of the web server (Table 2), the molecular weights of these HcunCXEs ranged from 10.52 to 62.23 kDa. The signal peptide predictions showed that only HcunCXE7 and HcunCXE9 have predicted signal peptide sites (Table 2).

Phylogenetic analysis of *H. cunea* CXEs

To evaluate the relationship of HcunCXEs with other insects' CXEs, a phylogenetic tree was constructed (Fig. 1). As shown in Fig. 1, the published CXE genes could be divided into three subclasses: extracellular genes, intracellular genes and neural signaling genes (*Durand et al., 2010b*). In the current study, HcunCXE1, HcunCXE7 and HcunCXE9 were clustered in the extracellular gene subclass, suggesting that these HcunCXEs might have relatively similar sequences of amino acids. The other 7 HcunCXEs including HcunCXE2-6, HcunCXE8 and HcunCXE10 fell into the intracellular gene subclass. In addition, the clade of intracellular gene subclass formed by HcunCXEs was found most closely related to those formed by *D. melanogaster, S. inferens* and *S. litura* CXEs, suggesting that the intracellular CXEs in *H. cunea*

shared a more recent common ancestor with the CXEs in *D. melanogaster, S. inferens* and *S. litura* than with the CXEs in other insect species.

Motif pattern analysis of *H. cunea* CXEs

To compare the motif-pattern of CXEs in different families of Lepidoptera, a total of 44 CXEs from *H. cunea* (10 HcunCXEs), *S. inferens* (15 SinfCXEs) and *S. litura* (19 SlitCXEs) were used for identification of conserved motifs and pattern analysis. As shown in Fig. 2, eight relatively common motifs with 40 CXEs were obtained. The most common pattern of motifs with 15 homologous CXEs (HcunOCXE5/9, SinfCXE3/5/10/11/14/16 and SlitCXE3/4/5/10/11/14/16) had a motif order of 6-5-3-3-1-8-2-7-4. In addition, 11 homologous CXEs (HcunCXE1/4/9, SinfCXE1/6/18/20 and SlitCXE6/8/12/17) had seven motifs with an order as 5-3-1-8-2-7-4; 5 homologous CXEs (HcunCXE7 (2) (1) (1) HcunCXE7, SinfCXE3 and SlitCXE2/13/15) had a motif order of 6-5-3-1-8-2-7. Interestingly, CXEs of *H. cunea* and *S. inferens* shared the same pattern with a motif order as 5-3-1-8-2 and 7–4.

Tissue distribution of HcunCXEs

To explore the possible physiological functions of the HcunCXEs, we examined tissue expressions of all the 10 HcunCXEs using RT-qPCR with primers specific for each of the 10 HcunCXEs genes (Table S2). As shown in Fig. 3, eight HcunCXEs (HcunCXE1, 3, 4, 5, 6, 8, 9 and 10) were expressed in the antennae. Among which, two HcunCXEs (HcunCXE1 and 3) were

female-biased and 3 HcunCXEs (HcunCXE4, 9 and 10) were male-biased; however, the other 3 HcunCXEs (HcunCXE5, 6 and 8) were equally expressed in both sexes. On the other hand, expression of HcunCXE2 and HcunCXE7 in the legs or wings was higher than that in the antennae. This result suggested that these two HcunCXEs may be participated in the degrading of host plant volatiles, and/or other xenobiotics.

To investigate whether these HcunCXEs are also expressed in the other body parts or life stages, RT-PCR experiment was carried out using total RNA samples taken from *H. cunea* adults and other life stages (pupae and larvae). As shown in Fig. 4, gel electrophoresis bands were generated from HcunCXE2 products from the adult thoraxes and abdomens. In addition, faint/light bands of HcunCXE7 and HcunCXE8 were detected in both thoraxes and abdomens, as well as the pupae. Interestingly, nine out of 10 HcunCXEs (HcunCXE1-5 and 7-10) were also detected in the larvae, indicating that HcunCXEs are widely expressed in the larval stage.

Discussion

H. cunea adults reportedly showed strong electrophysiological (antennal) responses to their host plant odors, especially to green leaf volatiles (*Tang, Su & Zhang, 2012a*; *Tang, Zhang & Zhang, 2012b*). In this case, the ODEs in the moth antennae would quickly remove or degrade the plant odor molecules from activated receptors after the electroantennogram (EAG) responses for odor recognitions were completed. In the current study, 10 putative CXE genes were identified based on our previous *H. cunea* transcriptomic data. All these 10 *H. cunea* CXE genes showed a very

high homology to the CXE genes identified in *S. inferens*. We speculated that these *H. cunea* CXE genes mainly degrade sex pheromone components and host plant volatiles. Unlike many well-studied Type-I sex pheromone-producing lepidopteran insects (>75% moth species), the *H. cunea* sex pheromone is consisted of Type II pheromone components (*Ando & Inomata, 2004*). Till now, most of the published moth ODEs are from the Type I sex pheromone producing lepidopterans; thus, our study represents the first report of ODE genes from a Type II sex pheromone-producing moth species. *H. cunea* is an extremely polyphagous species with a great fecundity (several hundred eggs/female) and a quick dispersal capacity. *H. cunea* larvae are generalists, capable of feeding on over 170 species of host plants, including many broad-leaved tree species. To cope with such diverse host plant species, this moth must have developed a series of olfactory receptor neurons to recognize diverse plant volatiles. Surprisingly, the number (n=10) of CXE genes we identified from *H. cunea* was much lower than those of other reported lepidopterans species: 19 in *Chilo suppressalis*, 35 in the tea geometrid *Ectropis obliqua* Prout and 76 in *B. mori* (*Yu et al., 2009*; *Liu et al., 2015*; *Sun et al., 2017*).

The phylogenetic tree analysis showed that HcunCXE1, 7 and 9 belonged to the extracellular gene subclass, including the secretory enzymes that likely act on hormones and pheromones (Fig. 1). The remaining 7 CXE genes fell into the intracellular gene subclass (Fig. 1), including intracellular enzymes that mostly play roles in dietary metabolism and detoxification. HcunCXE2, 3, 4, 5, 6, 8 and 10 were homologous to those (e.g. DmelCG10175 and DmelCG6414) in *D. melanogaster.* Chertemps et al. (2012) demonstrated that an extracellular

CXE of *D. melanogaster*, esterase-6 (Est-6), is responsible in or related to the sensory physiological and behavioral responses to its pheromone. Thus, these *H. cunea* CXE genes (HcunCXE2, 3, 4, 5, 6, 8 and 10) may also affect the mating and courtship competitions in *H. cunea* through degradation of some ester kairomones or plant allelochemicals.

Antennal-specific or highly expressed esterases belong to the CXE type in the carboxy/cholinesterases (CCEs) family. The first ODE was identified form *A. polyphemus* (ApolSE) as an antenna-specific esterase, with a high ability to degrade the acetate component (*E*6*Z*11-16: AC) of its pheromone blend (*Vogt & Riddiford, 1981*). Since then, antennal-specific esterases have been cloned from *A. polyphemus* (*Ishida & Leal, 2002*) and *Mamestra brassicae* Linnaeus (*Maïbèche-Coisne et al., 2004*). Recent studies show that many insect CXEs are expressed specifically in antennae, and their major functions in olfactory process are to degrade odor molecules and to metabolize toxic substances. Interestingly, the expressions of some HcunCXEs in the legs and wings were found to be higher than those in the antennae. The ten *H. cunea* CXEs genes we identified through the gene expression analysis had a low level of expressions in different body tissues of *H. cunea* adults (Fig. 3). However, they were widely expressed in the larvae, which may be related to their extremely broad host plant range that needs more CXEs to degrade large amount of carboxylic acid esters. Our quantitative PCR results indicated (Fig. 3) that some *H. cunea* genes were highly expressed in both male and female antennae, likely for degradation of sex-pheromones and/or plant volatiles both from hosts

or non-hosts, whereas the genes highly expressed in the legs and wings might be related to the degradations of some non-volatile substances for contact signals.

CXEs play multiple key roles in the hydrolysis of carboxylic acids esters. CXEs also include some metabolic enzymes that are associated with insecticide resistance (*Li, Schuler & Berenbaum, 2007*). Many previous studies in insect CXEs were focused on their functions in mediating insecticide resistance (*Hemingway & Karunaratne, 1998*; *Li, Schuler & Berenbaum, 2007*). In contrast, the mechanisms underlying degradation of plant allelochemicals are still unclear. It has been found that *Papilio Canadensis* CXEs could be induced by phenolic glycosides. Moreover, in *Lymantria dispar*, the activities of CXEs were positively correlated with the larval survival, indicating that these esterases might be involved in the glycoside metabolism (*Lindroth, 1989*; *Lindroth & Weisbrod, 1991*). In addition, a significant increase of CXE activity in the midguts of *S. litur* was observed while uptake of plant glycoside rutin (Ghumare, Mukherjee & Sharma, 1989). The CXEs in *Sitobion avenae* might participate in the gramine detoxification (*Cai et al., 2009*). Quercetinrutin and 2-tridaconone were also found to induce the activities of CXEs in *Helicoverpa Armigera* (*Gao et al., 1998*; *Mu, Pei & Gao, 2006*).

Little is known about *H. cunea* olfaction mechanisms at molecular levels, especially on how CXEs degrade various semiochemicals in its chemical communication system. Further research is needed to 1) understand the functions of antennal-specific CXEs in *H. cunea* via cloning, expression and purification of these CXEs and enzymatic kinetic analysis; 2) determine the locations/distributions of related CXEs by *in-situ* hybridization; 3) evaluate the potential

300 correlations between CXE transcription levels and their corresponding electrophysiological and
301 behavioral responses by silencing CXEs via RNA interference (*Caplen, 2004*), and 4) ultimately
302 discover the mode of action or functionality of CXEs in the olfactory signal conduction (signal
303 inactivation).
304

305 Conclusions

306 In summary, we identified 10 CXE genes in *H. cunea* by analyzing its antennal transcriptomic
307 data. These HcunCXEs displayed an antennae- or leg/wing-biased expression. The ubiquitous
308 expression of these HcunCXEs in different tissues and life stages, implicated their multiple roles,
309 *i.e*, degradation of odor molecules, metabolism and detoxification of dietary and environmental
310 xenobiotics. Our findings provide a theoretical basis for further studies on the olfactory
311 mechanism of *H. cunea* and offer some new insights into functions and evolutionary
312 characteristics of CXEs in lepidopteran insects. From a practical point of view, these HcunCXEs
313 might represent meaningful targets for developing behavioral interference control strategies
314 against *H. cunea*.
315

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321

Competing Interests

323 Dr. Qing-He Zhang is an employee of Sterling International, Inc., Spokane, WA, USA.

324

References

326 **Ando T, Inomata SI, Yamamoto M. 2004.** Lepidopteran sex pheromones. Topics in Current
327 Chemistry **239**:51-96 DOI 10.1007/b95449.

328 **Bailey TL, Johnson J, Grant CE, Noble WS. 2015.** The MEME Suite. *Nucleic Acids Research*
329 **43(W1)**:W39-W49 DOI 10.1093/nar/gkv416.

330 **Cai QN, Han Y, Cao YZ, Hu Y, Zhao X, Bi JL. 2009.** Detoxification of gramine by the cereal
331 aphid *Sitobion avenae*. *Journal of Chemical Ecology* **35(3)**:320-325 DOI
332 10.1007/s10886-009-9603-y.

333 **Caplen N. 2004.** Gene therapy progress and prospects. Downregulating gene expression: the
334 impact of RNA interference. *Gene therapy* **11(16)**:1241-1248 DOI 10.1038/sj.gt.3302324.

335 **Chertemps T, François A, Durand N, Rosell G, Dekker T, Lucas P, Maïbèche-Coisne M.**
336 **2012.** A carboxylesterase, Esterase-6, modulates sensory physiological and behavioral
337 response dynamics to pheromone in *Drosophila*. *BMC biology* **10(1)**:56 DOI
338 10.1186/1741-7007-10-56.

339 **Durand N, Carot-Sans G, Bozzolan F, Rosell G, Siaussat D, Debernard S, Chertemps T,**
340 **Maïbèche-Coisne M. 2011.** Degradation of pheromone and plant volatile components by

a same odorant-degrading enzyme in the cotton leafworm, *Spodoptera littoralis*. *PLOS ONE* **6(12)**:e29147-688 DOI 10.1371/journal.pone.0029147.

Durand N, Carot-Sans G, Chertemps T, Bozzolan F, Party V, Renou M, Debernard S, Rosell G, Maïbèche-Coisne M. 2010a. Characterization of an antennal carboxylesterase from the pest moth *Spodoptera littoralis* degrading a host plant odorant. *PLOS ONE* **5(11):**e15026 DOI 10.1371/journal.pone.0015026.

Durand N, Carot-Sans G, Chertemps T, Montagné N, Jacquin-Joly E, Debernard S, Maïbèche-Coisne M. 2010b. A diversity of putative carboxylesterases are expressed in the antennae of the noctuid moth *Spodoptera littoralis*. *Insect Molecular Biology* **19(1)**:87-97 DOI 10.1111/j.1365-2583.2009.00939.x.

Enayati AA, Ranson H, Hemingway J. 2005. Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology* **14(1)**:3-8 DOI 10.1111/j.1365-2583.2004.00529.x.

Gao XW, Zhao Y, Wang X, Dong X, Zheng B. 1998. Induction of carboxylesterasein *Helicoverpa Armigera* by insecticides and plant allelohemicals. *Acta Entomologica Sinica* **41**:5-11 DOI 10.16380/j.kcxb.1998.s1.002.

Ghumare S, Mukherjee S, Sharma R. 1989. Effect of rutin on the neonate sensitivity, dietary utilization and mid-gut carboxylesterase activity of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae). *Proceedings: Animal Sciences* **98(6)**:399-404 DOI 10.1007/bf03179652.

360 **Gomi T. 2007.** Seasonal adaptations of the fall webworm *Hyphantria cunea* (Drury)
361 (Lepidoptera: Arctiidae) following its invasion of Japan. *Ecological Research* **22(6)**:855-
362 861 DOI 10.1007/s11284-006-0327-y.
363 **Gomi T, Inudo M, Yamada D. 2003.** Local divergence in developmental traits within a
364 trivoltine area of *Hyphantria cunea* Drury (Lepidoptera: Arctiidae). *Entomological*
365 *Science* **6**:71-75.
366 **Guo S, Wong SM. 2020.** A Conserved Carboxylesterase Inhibits Tobacco mosaic virus (TMV)
367 Accumulation in Nicotiana benthamiana Plants. *Viruses* **12(2)**:195 DOI
368 10.3390/v12020195.
369 **He P, Li ZQ, Liu CC, Liu SJ, Dong SL. 2014a.** Two esterases from the genus *Spodoptera*
370 degrade sex pheromones and plant volatiles. *Genome* **57(4)**:201-208 DOI 10.1139/gen-
371 2014-0041.
372 **He P, Zhang J, Li ZQ, Zhang YN, Yang K, Dong SL, He P. 2014b.** Functional
373 characterization of an antennal esterase from the noctuid moth, *Spodoptera exigua*.
374 *Archives of Insect Biochemistry and Physiology* **86(2)**:85-99 DOI 10.1002/arch.21164.
375 **He P, Zhang YN, Yang K, Li ZQ, Dong SL. 2015.** An antenna-biased carboxylesterase is
376 specifically active to plant volatiles in *Spodoptera exigua. Pesticide Biochemistry*
377 *Physiology* **123**:93-100 DOI 10.1016/j.pestbp.2015.03.009.
378 **He P, Zhang YN, Li ZQ, Yang K, Zhu JY, Liu SJ, Dong SL. 2014c.** An antennae-enriched
379 carboxylesterase from *Spodoptera exigua* displays degradation activity in both plant

volatiles and female sex pheromones. *Insect Molecular Biology* **23(4)**:475-486 DOI 10.1111/imb.12095.

Hemingway J, Karunaratne S. 1998. Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Medical and Veterinary Entomology* **12(1)**:1-12 DOI 10.1046/j.1365-2915.1998.00082.x.

Ishida Y, Leal WS. 2002. Cloning of putative odorant-degrading enzyme and integumental esterase cDNAs from the wild silkmoth, *Antheraea polyphemus*. *Insect Biochemistry and Molecular Biology* **32(12)**:1775-1780 DOI 10.1016/S0965-1748(02)00136-4.

Ishida Y, Leal WS. 2005. Rapid inactivation of a moth pheromone. *Proceedings of the National Academy of Sciences* **102(39)**:14075-14079 DOI 10.1073/pnas.0505340102.

Ishida Y, Leal WS. 2008. Chiral discrimination of the Japanese beetle sex pheromone and a behavioral antagonist by a pheromone-degrading enzyme. *Proceedings of the National Academy Sciences* **105(26)**:9076-9080 DOI 10.1073/pnas.0802610105.

Itô Y, Miyashita K. 1968. Biology of *Hyphantria cunea* Drury (Lepidoptera: arctiidae) in Japan. V. Preliminary life tables and mortality data in urban areas. *Population Ecology* **10(2)**:177-209.

Jordan M, Stanley D, Marshall S, De Silva D, Crowhurst R, Gleave A, Greenwood D, Newcomb R. 2008. Expressed sequence tags and proteomics of antennae from the tortricid moth, *Epiphyas postvittana*. *Insect Molecular Biology* **17(4)**:361-373 DOI 10.1111/j.1365-2583.2008.00812.x.

Larkin MA. Blackshields G, Brown N, Chenna R, McGettigan P, McWilliam H. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23(21)**:2947–2948 DOI 10.1093/bioinformatics/btm404.

Leal WS. 2013. Odorant reception in insects: roles of receptors, binding proteins, and degrading enzymes. *Annual Review of Entomology* **58**:373-391 DOI 10.1146/annurev-ento-120811-153635.

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12(1)**:323 DOI 10.1186/1471-2105-12-323.

Li X, Schuler MA, Berenbaum MR. 2007. Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology* **52**:231-253 DOI 10.1146/annurev.ento.51.110104.151104.

Li Y, Farnsworth CA, Coppin CW, Teese MG, Liu JW, Scott C, Zhang X, Russell RJ, Oakeshott JG. 2013. Organophosphate and pyrethroid hydrolase activities of mutant esterases from the cotton bollworm *Helicoverpa armigera*. *PLOS ONE* **8(10)** DOI 10.1371/journal.pone.0077685.

Lindroth RL. 1989. Host plant alteration of detoxication activity in *Papilio glaucus*. *Entomologia Expermentalis et Applicata* **50(1)**:29-35.

418 **Lindroth RL, Weisbrod AV. 1991.** Genetic variation in response of the gypsy moth to aspen
419 phenolic glycosides. *Biochemical Systematics and Ecology* **19(2)**:97-103 DOI
420 10.1016/0305-1978(91)90031-T.
421 **Liu S, Gong ZJ, Rao XJ, Li MY, Li SG. 2015.** Identification of putative carboxylesterase and
422 glutathione S-transferase genes from the antennae of the *Chilo suppressalis* (Lepidoptera:
423 Pyralidae). *Journal of Insect Science* **1**:1 DOI 10.1093/jisesa/iev082.
424 **Maïbèche-Coisne M, Merlin C, FrançoisMC, Queguiner I, Porcheron P, Jacquin-Joly E.**
425 **2004.** Putative odorant-degrading esterase cDNA from the moth *Mamestra brassicae*:
426 cloning and expression patterns in male and female antennae. *Chemical Senses*
427 **29(5)**:381-390 DOI 10.1093/chemse/bjh039.
428 **Mamidala P, Wijeratne AJ, Wijeratne S, Poland T, Qazi SS, Doucet D, Cusson M, Beliveau**
429 **C, Mittapalli O. 2013.** Identification of odor-processing genes in the emerald ash borer,
430 *Agrilus planipennis*. *PLOS ONE* **8(2)** DOI 10.1371/journal.pone.0056555.
431 **Merlin C, Rosell G, Carot-Sans G, François MC, Bozzolan F, Pelletier J, Jacquin-Joly E,**
432 **Guerrero A, Maïbèche-Coisne M. 2007.** Antennal esterase cDNAs from two pest moths,
433 *Spodoptera littoralis* and *Sesamia nonagrioides*, potentially involved in odourant
434 degradation. *Insect Molecular Biology* **16(1)**:73-81 DOI 10.1111/j.1365-
435 2583.2006.00702.x.

436 **Mu SF, Pei L, Gao XW. 2006.** Effects of quercetin on specific activity of carboxylesteras and
437 glutathione S-transferase in *Bemisia tabaci*. *Journal of Applied Entomology* **43(004)**:491-
438 495 DOI 10.3969/j.issn.0452-8255.2006.04.014.
439 **Muller P, Janovjak H, Miserez A, Dobbie Z. 2002.** Processing of gene expression data
440 generated by quantitative real-time RT PCR. *Biotechniques* **32(6)**:1372-1374.
441 **Pelletier J, Bozzolan F, Solvar M, François MC, Jacquin-Joly E, Maïbèche-Coisne M. 2007.**
442 Identification of candidate aldehyde oxidases from the silkworm *Bombyx mori* potentially
443 involved in antennal pheromone degradation. *Gene* **404(1-2)**:31-40 DOI
444 10.1016/j.gene.2007.08.022.
445 **Pelosi P, Iovinella I, Zhu J, Wang G, Dani FR. 2018.** Beyond chemoreception: diverse tasks of
446 soluble olfactory proteins in insects. *Biological Reviews* **93(1)**:184-200 DOI
447 10.1111/brv.12339.
448 **Petersen TN, Brunak S, Heijne G, Nielsen H. 2011.** SignalP 4.0: discriminating signal
449 peptides from transmembrane regions. *Nature Methods* **8(10)**:785 DOI
450 10.1038/nmeth.1701.
451 **Rybczynski R, Reagan J, Lerner MR. 1989.** A pheromone-degrading aldehyde oxidase in the
452 antennae of the moth *Manduca sexta*. *Journal of Neuroscience* **9(4)**:1341-1353 DOI
453 10.1523/JNEUROSCI.09-04-01341.1989.

Scott K, Brady Jr B, Cravchik B, Morozov P, Rzhetsky A, Zuker C, Axel R. 2001. A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila*. *Cell* **104(5)**:661-673 DOI 10.1016/S0092-8674(01)00263-X.

Simon P. 2003. Q-Gene: processing quantitative real-time RT–PCR data. *Bioinformatics* **19(11)**:1439-1440 DOI 10.1093/bioinformatics/btg157.

Song HG, Young Kwon J, Soo Han H, Bae YC, Moon C. 2008. First contact to odors: our current knowledge about odorant receptor. *Sensors* **8(10)**:6303-6320 DOI 10.3390/s8106303.

Steinbrecht RA. 1998. Odorant-binding proteins: expression and function. *Annals of the New York Academy of Sciences* **855**:323-332.

Sun L, Wang Q, Wang Q, Zhang Y, Tang M, Guo H, Fu J, Xiao Q, Zhang Y, Zhang Y. 2017. Identification and expression patterns of putative diversified carboxylesterases in the tea geometrid *Ectropis obliqua* Prout. *Frontiers in Physiology* **8**:1085 DOI 10.3389/fphys.2017.01085.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28(10)**:2731-2739 DOI 10.1093/molbev/msr121.

Tang R, Su M, Zhang Z. 2012a. Electroantennogram responses of an invasive species fall webworm (*Hyphantria cunea*) to host volatile compounds. *Chinese Science Bulletin* **57(35)**:4560-4568 DOI 10.1007/s11434-012-5356-z.

Tang R, Zhang JP, Zhang ZN. 2012b. Electrophysiological and behavioral responses of male fall webworm moths (*Hyphantria cunea)* to herbivory-induced mulberry (Morus alba) leaf volatiles. *PLOS ONE* **7(11)** DOI 10.1371/journal.pone.0049256.

Tegoni M, Campanacci V, Cambillau C. 2004. Structural aspects of sexual attraction and chemical communication in insects. *Trends in Biochemical Sciences* **29(5)**:257-264 DOI 10.1016/j.tibs.2004.03.003.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Van Baren MJ, Salzberg SL, Wold BJ, Pachter L. 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nature Biotechnology* **28(5)**:511 DOI 10.1038/nbt.1621.

Vogt R. 2003. 14-Biochemical diversity of odor detection: OBPs. ODEs and SNMPs. Insect *Biochemistry and Molecular Biology* 397-451.

Vogt RG, Riddiford LM. 1981. Pheromone binding and inactivation by moth antennae. *Nature* **293(10)**:161-163.

Vogt RG, Riddiford LM, Prestwich GD. 1985. Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of *Antheraea polyphemus*. *Proceedings of the National Academy of Sciences* **82(24)**:8827-8831 DOI 10.1073/pnas.82.24.8827.

Xu LT, Zhang YQ, Zhang SH, Deng JD, Lu M, Zhang LW, and Zhang J. 2018. Comparative analysis of the immune system of an invasive bark beetle, *Dendroctonus valens*, infected by an entomopathogenic fungus. *Developmental & Comparative Immunology* **88**:65-69 DOI 10.1016/j.dci.2018.07.002.

Yang YC, Li WB, Tao J, Zong SX. 2019. Antennal transcriptome analyses and olfactory protein identification in an important wood-boring moth pest, *Streltzoviella insularis* (Lepidoptera: Cossidae). *Scientific Reports* **9:**17951 DOI 10.1038/s41598-019-54455-w.

Yang Z, Wang X, Wei J, Qu H, Qiao X. 2008. Survey of the native insect natural enemies of *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) in China. *Bulletin of Entomological Research* **98(3)**:293-302 DOI 10.1017/S0007485308005609.

Younus F, Chertemps T, Pearce SL, Pandey G, Bozzolan F, Coppin CW, Russell RJ, Maïbèche-Coisne M, Oakeshott JG. 2014. Identification of candidate odorant degrading gene/enzyme systems in the antennal transcriptome of *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology* **53**:30-43 DOI 10.1016/j.ibmb.2014.07.003.

Yu QY, Lu C, Li WL, Xiang ZH, Zhang Z. 2009. Annotation and expression of carboxylesterases in the silkworm, *Bombyx mori*. *BMC genomics* **10(1)**:553 DOI 10.1186/1471-2164-10-553.

Zhang LW, Kang K, Jiang SC, Zhang YN, Wang TT, Zhang J, Sun L, Yang YQ, Huang CC, Jiang LY. 2016. Analysis of the antennal transcriptome and insights into olfactory

genes in *Hyphantria cunea* (Drury). *PLOS ONE* **11(10):**e0154729 DOI 10.1371/journal.pone.0164729.

Figure legends

Figure 1 Molecular phylogeny comparing HcunCXEs with CXEs from seven insect species. 10 CEXs (HcunCXE1-10) from *H. cunea* (Hyph) and CXEs from *A. mellifera* (Amel), *A. polyphemus* (Apol), *B. mori* (Bmor), *D.melanogaster* (Dmel), *H. virescens* (Hvir), *M. sexta* (Msex), *S. inferens* (Sinf), *S. litura* (Slit) were used to construct the phylogenetic tree. See Materials and Methods for details of the phylogenetic analysis.

Figure 2 Motif analysis of CXEs in *H. cunea*. The upper parts list the eight motifs discovered in the 44 CXEs using MEME online server (http://meme. nbcr.net/meme/). The lower parts indicate approximate locations of each motif on the protein sequence. The numbers in the boxes correspond to the numbered motifs in the upper part of the figure, where small number indicates high conservation. The numbers on the bottom showed the approximate locations of each motif on the protein sequence, starting from the N-terminal. This figure only listed the most common 8 motif-patterns presented in 44 CXEs.

Figure 3 Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues. FA, female antennae; MA, male antennae; L, legs; W, wings. The relative mRNA levels were normalized to those of the *EF1-a* gene and analyzed using the Q-gene method. All values are shown as the mean ± SEM. The data were analyzed by the least significant difference (LSD) test after one-way analysis of variance (ANOVA). Different letters indicate significant differences between means ($P < 0.05$).

Figure 4 RT-PCR analysis of HcunCXEs gene expression in tissues taken from *H. cunea* adults and other life stages. EF1-a was used as an internal control; NC, negative control with no template in the reaction.

Figure 1

Molecular phylogeny comparing HcunCXEs with CXEs from seven insect species.

10 CEXs (HcunCXE1-10) from *H. cunea* (Hyph) and CXEs from *A. mellifera* (Amel), *A. polyphemus* (Apol), *B. mori*(Bmor), *D.melanogaster* (Dmel), *H. virescens* (Hvir), *M. sexta* (Msex), *S. inferens* (Sinf), *S. litura* (Slit) were used to construct the phylogenetic tree. See Materials and Methods for details of the phylogenetic analysis.

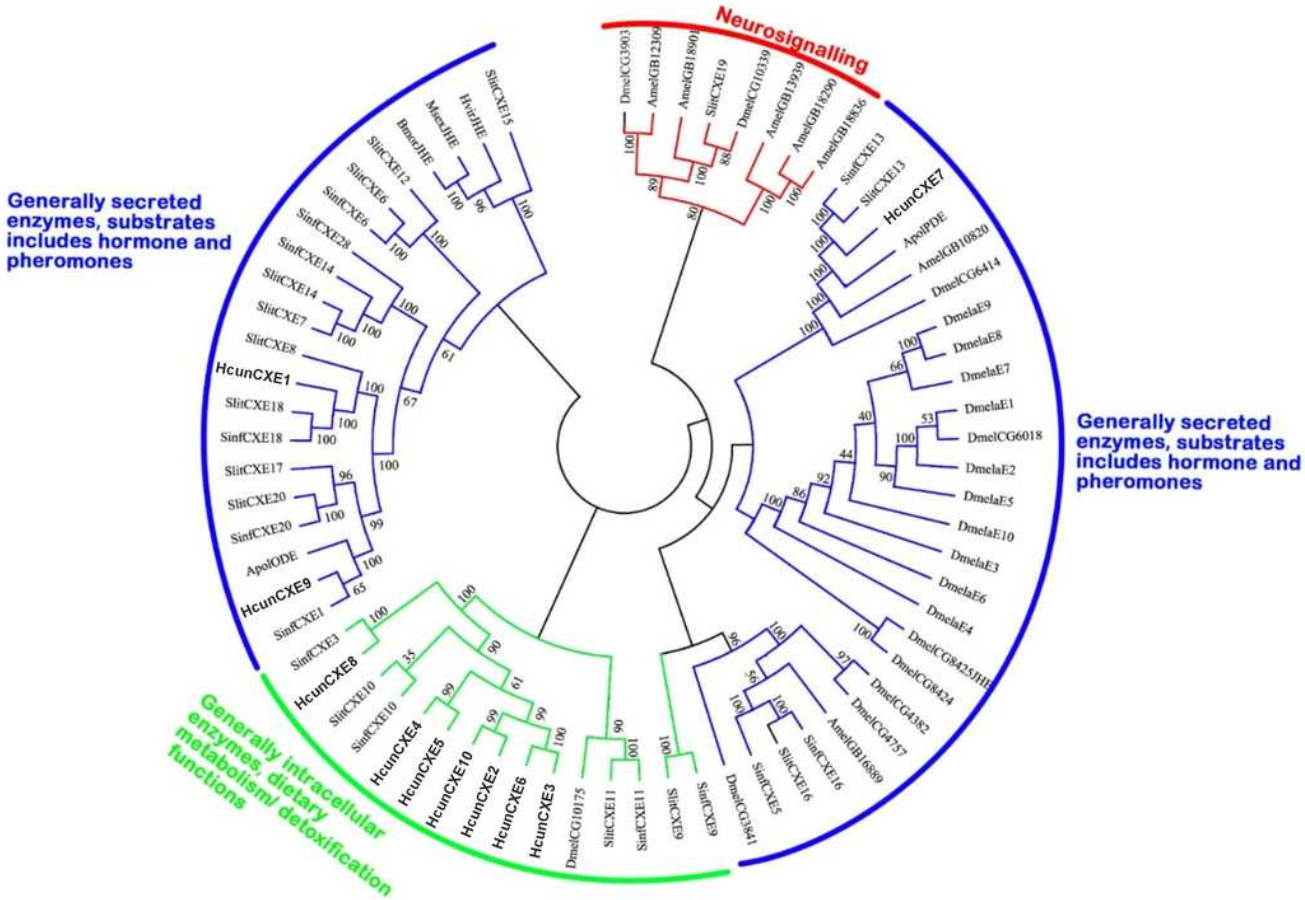
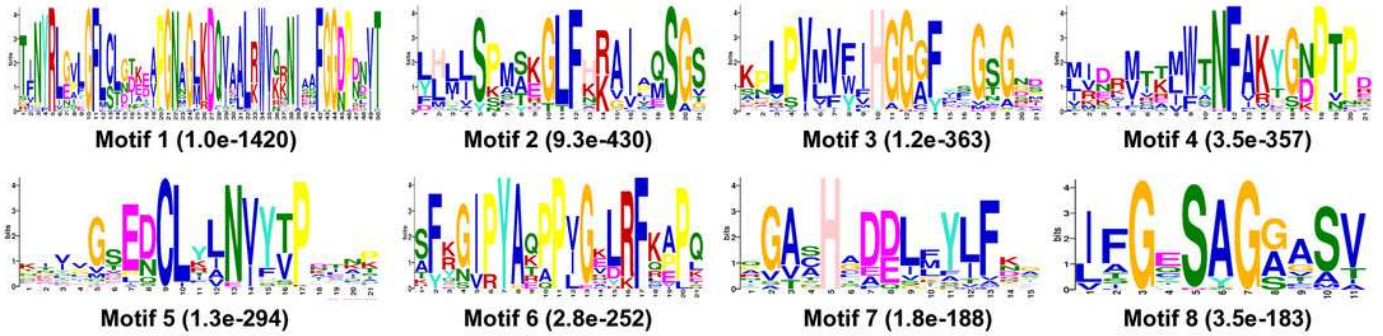


Figure 2

Motif analysis of CXEs in *H. cunea*.

The upper parts listed the eight motifs discovered in the 44 CXEs using MEME online server (http://meme. nbcr.net/meme/). The lower parts indicate approximate locations of each motif on the protein sequence. The numbers in the boxes correspond to the numbered motifs in the upper part of the figure, where small number indicates high conservation. The numbers on the bottom showed the approximate locations of each motif on the protein sequence, starting from the N-terminal. This figure only listed the most common 8 motif-patterns presented in 44 CXEs.


Motif 1 (1.0e-1420)
Motif 2 (9.3e-430)
Motif 3 (1.2e-363)
Motif 4 (3.5e-357)
Motif 5 (1.3e-294)
Motif 6 (2.8e-252)
Motif 7 (1.8e-188)
Motif 8 (3.5e-183)

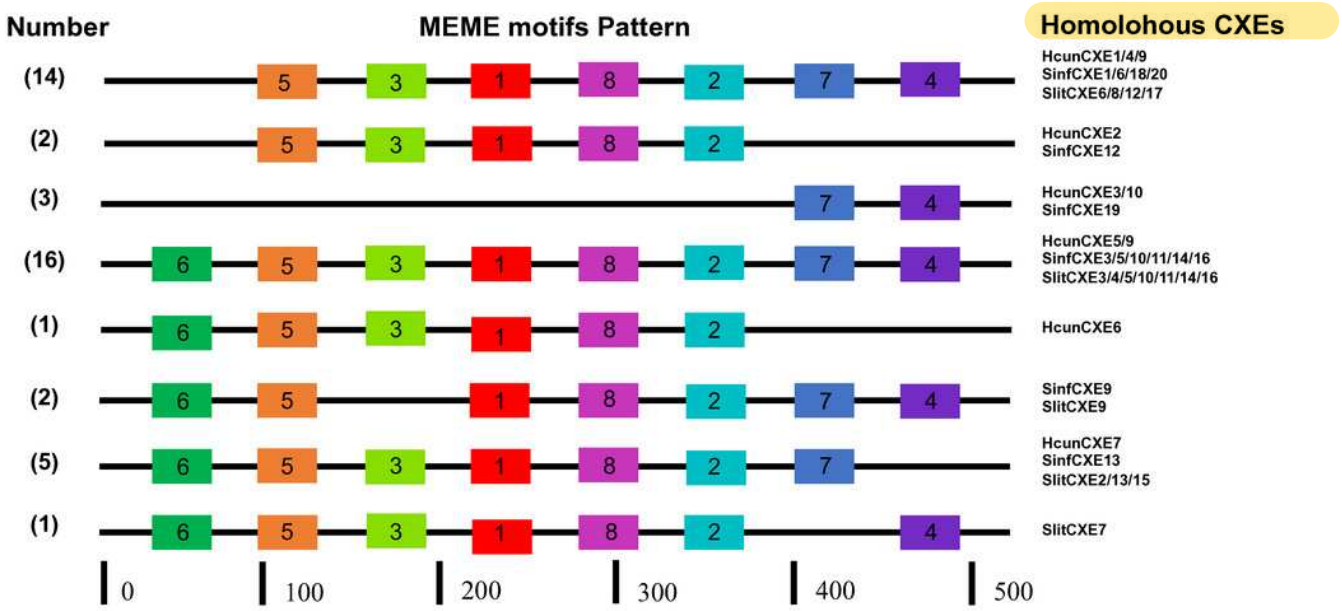

Number
MEME motifs Pattern
Homolohous CXEs
(14)
(2)
(3)
(16)
(1)
(2)
(5)
(1)
HcunCXE1/4/9
SinfCXE1/6/18/20
SlitCXE6/8/12/17
HcunCXE2
SinfCXE12
HcunCXE3/10
SinfCXE19
HcunCXE5/9
SinfCXE3/5/10/11/14/16
SlitCXE3/4/5/10/11/14/16
HcunCXE6
SinfCXE9
SlitCXE9
HcunCXE7
SinfCXE13
SlitCXE2/13/15
SlitCXE7
0
100
200
300
400
500

Figure 3

Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues.

FA, female antennae; MA, male antennae; L, legs; W, wings. The relative mRNA levels were normalized to those of the *EF1-a* gene and analyzed using the Q-gene method. All values are shown as the mean ± SEM. The data were analyzed by the least significant difference (LSD) test after one-way analysis of variance (ANOVA). Different letters indicate significant differences between means ($P < 0.05$).

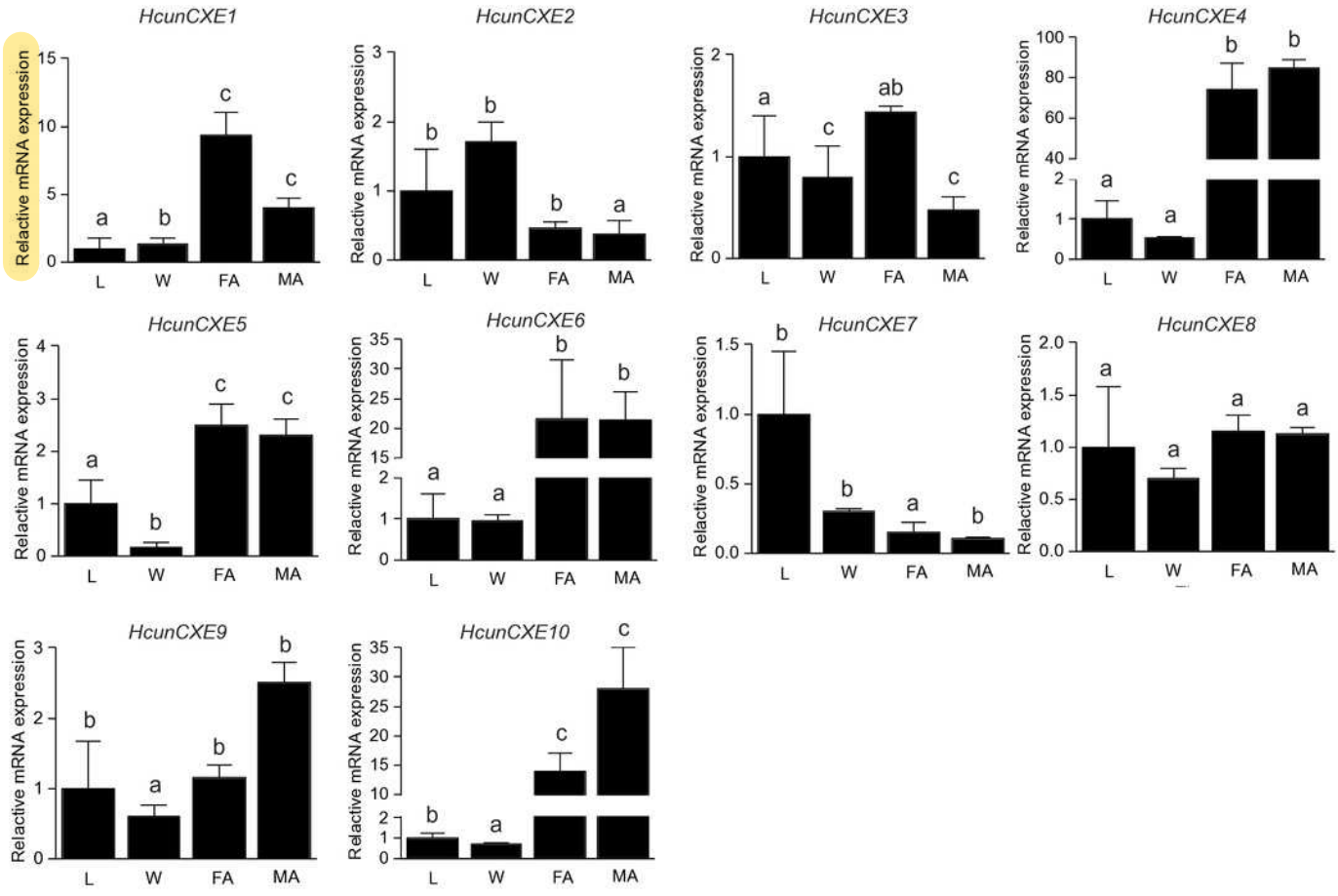


Figure 4

RT-PCR analysis of HcunCXEs gene expression in tissues taken from *H. cunea* adults and other life stages.

EF1-a was used as an internal control; NC, negative control with no template in the reaction.

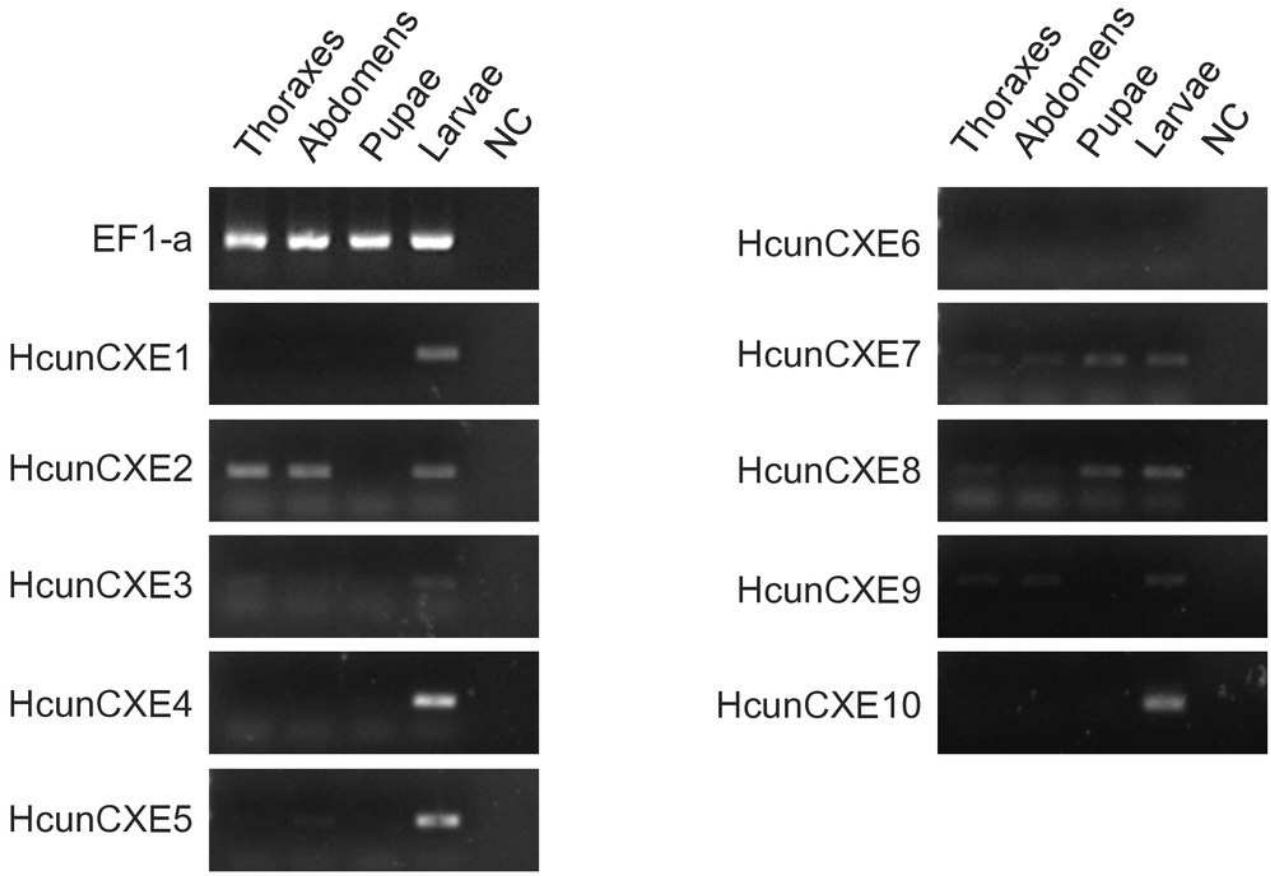


Table 1(on next page)

Gene name, information of open reading frame and Blastx match of the 10 putative HcunCXEs identified in this study.

Table 1:

Gene name, information of open reading frame and Blastx match of the 10 putative HcunCXEs identified in this study.

Gene Name	ORF Length (bp)	Complete ORF	FPKM value	Best Blastx Match			
				Species	Acc.number	E - value	Identity (%)
HcunCXE1	1668	YES	4.9	*S. inferens*	AII21990.1	0.0	73
HcunCXE2	777	NO	3.77	*S. inferens*	AII21980.1	3e-135	73
HcunCXE3	375	YES	3.26	*S. inferens*	AII21980.1	2e-105	60
HcunCXE4	1389	YES	61.01	*S. inferens*	AII21984.1	0.0	59
HcunCXE5	1593	YES	143.14	*S. inferens*	AII21984.1	0.0	62
HcunCXE6	1161	NO	17.04	*S. inferens*	AII21984.1	4e-174	62
HcunCXE7	1677	YES	13.18	*S. inferens*	AII21987.1	0.0	75
HcunCXE8	1608	YES	12.64	*S. inferens*	AII21980.1	0.0	66
HcunCXE9	1653	YES	6.13	*S.inferens*	AII21978.1	0.0	71
HcunCXE10	273	NO	21.32	*S. inferens*	AII21984.1	8e-39	64

Note: ORF, open reading frame.

Table 2(on next page)

Gene name and characteristics including molecular weight, isoelectric point and signal peptide of the 10 putative HcunCXEs with open reading frames.

Table 2:
Gene name and characteristics including molecular weight, isoelectric point and signal peptide of the 10 putative HcunCXEs with open reading frames.

Gene Name	MW (Kda)	PI	SP
HcunCXE1	62.23	7.56	NO
HcunCXE2	28.44	5.67	NO
HcunCXE3	13.98	4.85	NO
HcunCXE4	52.2	5.31	NO
HcunCXE5	59.52	5.41	NO
HcunCXE6	43.17	5.09	NO
HcunCXE7	61.71	6.32	1-17
HcunCXE8	60.68	5.75	NO
HcunCXE9	62.18	8	1-16
HcunCXE10	10.52	8.89	NO

Note: MW, Molecular weight; pI, isoelectric point; SP, signal peptide.