Putative carboxylesterase gene identification and their expression patterns in *Hyphantria cunea* (Drury) (#49228)

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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

The 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 olfactory 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* antennal 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 indicate 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 is transmitted 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 poor spatio-temporal resolution of the odor signal, 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 (*He et al., 2014a*). Traditionally, ODEs can be divided into five categories based on the structural difference of various target substances: 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*).

However, ODEs of different categories have been shown to catalytically interact with odor molecules of the same type and structure. It is currently believed that the different enzyme families of ODEs may work together in degradation and clearing of the same type of odor molecule (*Steiner et al. 2019*).

As primary metabolic enzymes, CXEs are widely distributed among insects, microbes and plants (*Guo & Wong, 2020*). The active site contains several conserved serines, which promote the cleavage and formation of ester bonds (*Bornscheuer, 2002*) and play an important role in the metabolism of heterologous substances, pheromone degradation, neurogenesis, development regulation and many other functions (*Yu et al., 2009*). In addition to the metabolism and detoxification of endobiotics and xenobiotics, another important role of CXEs is to maintain the sensitivity of ORNs. The CXEs enable rapid degradation of stray odors and 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 *Drosophila melanogaster, 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; Chertemps et al. 2015*). For instance, the *A. polyphemus* pheromone-degrading enzyme CXE (*Apol*PDE) was shown to 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, as well as hydrolyze volatile esters released from their natural food sources *(Gomi, Inudo & Yamada, 2003; Durand et al., 2011; Chertemps et al. 2015).*

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) and 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. Previous studies

have described four sex pheromone components, including two straight chain aldehydes, (9*Z*,12*Z*)-octadecadienal (Z9, Z12-18Ald) and (9*Z*,12*Z*,15*Z*)-octadecatrienal (Z9, Z12, Z15-18Ald), and two epoxides, (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-3,6-heneicosadiene (Z3, Z6-9S, 10R-epoxy-21Hy) and (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxy-1,3,6-heneicosatriene (1, Z3, Z6-9S, 10R-epoxy-21Hy), which are produced by female *H. cunea* (*M. et al., 1989*). There are two major groups of moth sex pheromones: Type I pheromones and Type II pheromones (*M. et al., 1989*; *Millar, 2000; Ando et al., 2004*). Type I pheromones mostly contain C_{10}-C_{18} unsaturated hydrocarbons and a terminal functional group (>75% moth species). Type II pheromones lack a terminal functional group and contain C_{17}-C_{23} unsaturated hydrocarbons and epoxy derivatives (*Millar, 2000, Ando et al., 2004*). 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* antennal 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 (RT-qPCR) and reverse transcription PCR (RT-PCR) to investigate the expression of these genes. We found that HcunCXEs displayed either antennae- or leg/wing-biased expression. The differential expression pattern of HcunCXEs suggests a potential function in degrading pesticides and/or other xenobiotics.

Materials and Methods

Insect rearing and tissue collection

H. cunea pupae were collected from a first-generation population at Baimao Town, Jiujiang District, Wuhu City, Anhui province. Insect cages were used for rearing *H. cunea* pupae at 25°C, 70-80% RH and 14L:10D photoperiod. After eclosion, adults were provided with 1% honey water. In the fourth hour of the second dark period, antennae, thoraxes, abdomens, legs, and wings of virgin males and females were dissected under the microscope and pooled by sex and body part. Male and female pupae and fourth instar larvae were also sampled. Five samples were taken for each body part with the exception of antennae, of which 30 pairs were collected by pulling out from the base of the antennae with tweezers. Dissected body parts or whole-body samples were flash frozen in liquid nitrogen and stored at -80℃ until use.

Gene annotation

The *H. cunea* antennal transcriptome (PRJNA605323) (*Zhang et al., 2016*) was used as a reference sequence for mapping clean reads for each tested sample. Genetic annotation was carried out using Nr (NCBI non-redundant protein sequences), Nt (NCBI nucleotide), Pfam (Protein family), KOG/COG (Clusters of Orthologous Groups of proteins/enKaryotic Ortholog Groups), Swiss-Prot (A manually annotated and reviewed protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) databases (Fig. S3-6). Based on the results of gene annotation and Blast comparison, the candidate genes of HcunCXE

141 were determined and named according to the identification order from the antennal
142 transcriptomic data.
143

144 **Homologous search and sequencing analysis of CXE genes in *H. cunea***

145 The *H. cunea* CXE genes were identified according to the BLAST results on NCBI. The Open
146 Reading Frame finder (OFR Finder) (https://www.ncbi.nlm.nih.gov/orffinder/) was used to
147 search for the open reading frame of these CXE genes. An ExPASy tool
148 (http://web.expasy.org/compute_pi/) (*Petersen et al., 2011*) was used to calculate their
149 theoretical isoelectric points (pI) and molecular weights (MW) of the full-length HcunCXEs
150 gene candidates, and SignalP-5.0 (https://services.healthtech.dtu.dk/service.php?SignalP) was
151 used to predict signal peptides of the CXE genes (*Petersen et al., 2011*).
152

153 **Phylogenetic analysis of CXE genes in *H. cunea***

154 Genes related to the CXEs of *H. cunea* and other reported insects of *Seasamia inferens,*
155 *Spodoptera littoralis, Spodoptera exigua, Cnaphalocrocis medinalis, Bombyx mori, Drosophila*
156 *melanogaster* and *Tribolium caastaneum* were subjected to multi-sequence alignment on
157 MAFFT (*Wong et al., 2008*). The phylogenetic tree was constructed using MEGA-X (*Tamura et*
158 *al., 2011*) software and maximum likelihood method (1000 bootstrap repetitions) for systematic
159 evolution analysis. Lastly, the phylogenetic tree was edited on the website iTOL

(https://itol.embl.de/). The genes of insect ODEs required for the phylogenetic tree were shown in Supplementary Table S1.

Motif analysis of CXEs

According to the relationship of CXEs in the phylogenetic analysis, a total of 43 CXEs from *H. cunea* (10 HcunCXEs), *S. inferens* (15 SinfCXEs) and *S. littoralis* (18 SlitCXEs) were used for identification of conserved motifs and pattern analysis (*Durand et al., 2010b; Zhang et al., 2014*). The online program Multiple Em for Motif Elicitation (MEME, version 5.1.1) (http://meme-suite.org/tools/meme) was used to obtain the motif in all CXEs genes (*Bailey et al., 2015*). MEME was done with the following parameters: the width between the range of 6 -10, and the number of motifs was below 8.

RNA extraction and synthesis of the first-strand cDNA

The sampled body tissues were ground using Tissue-Tearor which rapidly homogenized the samples in DEPC-treated sterile water. Extraction and purification of total RNA from each sample were done using TRIzol reagent (Invitrogen, USA) according to manufacturer instructions. The degradation and contamination of RNA product were monitored on 1% agarose gels, and purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). First-stranded cDNA templates were synthesized using 1 μg of RNA templates with the PrimeScript™ RT reagent Kit according the manufacturer instructions (TaKaRa, Japan).

180

181 **RT-qPCR and RT-PCR analysis**

182 Expression profiles of the identified *H. cunea* CXE genes in different body parts of adults and
183 two other life stages were analyzed. Tissues included antenna of 30 adults, legs of 5 adults of
184 each sex, wings of 5 adults of each sex, thoraxes and abdomens of 5 adults of each sex, 5 whole
185 pupae of each sex and 5 larvae (fourth instar).

186 RT-qPCR and RT-PCR assays were employed for production of multiple copies of DNA.
187 RT-qPCR reaction was conducted in a 25μL reaction mixture system containing 12.5μL of
188 SYBR® Premix Ex Taq II (Tli RNaseH Plus) (TaKaRa, Japan), 1μL of each primer, 2μL of
189 sample cDNA, and 8.5μL of sterilized H_2O.

190 The RT-qPCR cycles were set at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec,
191 60°C for 30 sec. Each experiment was carried out in a CFX96 real-time PCR detection
192 instrument (Bio-rad, USA) using 8-strip PCR tubes (Bio-rad, USA). The reaction data were
193 recorded, and the dissolution curves were appended. Both Elongation factor-1 alpha (EF1-a) and
194 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal reference. Three
195 biological replicates were performed, and the reproducibility confirmation of each RT-qPCR
196 reaction was replicated three times for each sample (*Xu et al., 2018*).

197 The variability of each gene expression in different body tissues was tested by using Q-
198 Gene method (*Muller et al., 2002*; *Simon, 2003*). The relative expressions of mRNA of each
199 gene (mean ± SD) were analyzed using one-way ANOVA (SPSS22.0 for Windows, IBM, USA),

200 followed by LSD and Duncan's tests at α = 0.05. The MIQE guidelines: minimum information
201 for publication of quantitative real-time PCR experiments. Graphical plotting/mapping was done
202 by GraphPad Prism v5.0 Software (GraphPad Software Inc, CA, USA). The RT-qPCR primers
203 of CXE gene in *H. cunea* are listed in Supplementary Table S2.
204 RT-PCR analysis was performed as follows: 94°C for 2 min of initiation, and 29 cycles of
205 94°C for 30 sec, 52°C for 30 sec, 72°C for 15 sec, and 2 min at 72°C for final extension.
206 Elongation factor-1 alpha (EF1-a) gene of *H. cunea* was used as an internal reference. In addition,
207 instead of template cDNA, RNase-free water was used as the blank control. A total of 25μL
208 reaction mixture containing 12.5μL of 2x Ex Taq MasterMix (CWBIO, China), 1μL of each
209 primer, 1μL of sample cDNA, and bring up to 25μL of sterilized H2O. 10μL aliquot of each
210 reaction product was taken to obtain agarose gel electrophoresis detection results. The RT-PCR
211 primer sequences of CXE genes in *H. cunea* are listed in Supplementary Table S3.
212

213 ## Results

214 ### Identification of CXE genes from *H. cunea*

215 Based on a comparative analysis of the *H. cunea* antennal transcriptome using Blastx databases
216 (*Zhang et al., 2016*), a total of 10 HcunCXE genes were identified. Blastx comparison showed
217 that these 10 HcunCXE genes have high homology with CXE genes of *S. inferens*. As shown in
218 Table 1, six HcunCXEs (HcunCXE1, HcunCXE3-5 and HcunCXE7-8) had complete ORFs.
219 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. 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 *S. inferens, C. medinalis, S. exigua* and *S. littoralis* CXEs. Sequence alignments showed that the amino acid identities of HcunCXE1 and SinfCXE18, HcunCXE9 and SinfCXE1, HcunCXE7 and SinfCXE13, HcunCXE7 and CmedCXE5 were 73.9%, 71.3%, 74.6% and 65%, respectively (Fig. S2). These results suggest that the intracellular CXEs in *H. cunea* shared a more recent common ancestor with the CXEs in *S. inferens, C. medinalis, S. exigua* and *S. littoralis* 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 43 CXEs from *H. cunea* (10 HcunCXEs), *S. inferens* (15 SinfCXEs) and *S. litura* (18 SlitCXEs) were used for identification of conserved motifs and pattern analysis. As shown in Fig. 2, eight relatively common motifs with 43 CXEs were obtained. The most common pattern of motifs with 16 homologous CXEs (HcunOCXE5/8, SinfCXE3/5/10/11/14/16 and SlitCXE3/4/5/10/11/14/16/19) had a motif order of 6-5-3-1-8-2-7-4. In addition, 14 homologous CXEs (HcunCXE1/4/9, SinfCXE1/6/18/20/26 and SlitCXE6/8/12/17/18/20) had seven motifs with an order as 5-3-1-8-2-7-4; 5 homologous CXEs (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

We next examined the expression of HcunCXE genes in adult female and male antennae, legs and wings using RT-qPCR with primers specific for each of the 10 HcunCXEs genes (Table S2). As shown in Fig. 3 and Fig. S1, all HcunCXEs were expressed in the antennae. Among which, three HcunCXEs (HcunCXE4, 5, 8) were highly expressed in the antennae (Fig. S1 C and D). Two HcunCXEs (HcunCXE1 and 3) were female-biased (Fig. 3 A and C) and two HcunCXEs (HcunCXE 9 and 10) were male-biased (Fig. 3 I and J); although the sex-biased expression is not statistically significant, there is a clear numerical difference between expression level in the sexes. The other HcunCXEs, however, were equally expressed in both sexes. Comparing

expression across tissues, five HcunCXEs (2, 3, 5, 7 and 8) were highly expressed in the legs and wings (Fig. S1 A and B). HcunCXEs expression of HcunCXE2 and HcunCXE7 in the legs or wings was higher than that in the antennae (Fig. 3 B and G).

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

In the current study, 10 putative CXE genes were identified based on our previous *H. cunea* antennal transcriptomic data (*Zhang et al., 2016*). All 10 *H. cunea* CXE genes showed a high homology to the CXE genes identified in *S. inferens* (identity ≥59%, Fig. 1 and Table 1). 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 comprised of Type II pheromone components (*Ando & Inomata, 2004*). At present, most of the published moth ODEs are from the Type I sex pheromone producing lepidopterans; thus, our study represents the first report of

279 ODE genes from a Type II sex pheromone-producing moth species. *H. cunea* is an extremely
280 polyphagous species with a great fecundity (several hundred eggs/female) and a quick dispersal
281 capacity. *H. cunea* larvae are generalists, capable of feeding on over 170 species of host plants,
282 including many broad-leaved tree species. To cope with such diverse host plant species, this
283 moth must have developed a series of olfactory receptor neurons to recognize diverse plant
284 volatiles (*Zhang et al., 2016*). The number (n=10) of CXE genes we identified from *H. cunea*
285 was lower than those of other reported lepidopterans species: 19 in *Chilo suppressalis*, 35 in the
286 tea geometrid *Ectropis obliqua* Prout and 76 in *B. mori* (*Yu et al., 2009*; *Liu et al., 2015*; *Sun et*
287 *al., 2017*). These results suggest that *H. cunea* does not seem to require more CXEs, since the
288 other ODEs including cytochrome P450 (CYP), alcohol dehydrogenase (AD), aldehyde oxidase
289 (AOX) and glutathione-S-transferase (GST) are likely involved in odorant degradation in
290 olfactory processes. On the other hand, the difference in number of CXEs in various species
291 might result from differences in sample preparation and sequencing method/depth. In addition,
292 the ecological/evolutionary differences across species may also be a reason. Insects have to adapt
293 to their external environment, different environments lead to the formation of different
294 physiological and behavioral characteristics.

295 The phylogenetic tree analysis showed that HcunCXE1, 7 and 9 belong to the extracellular
296 gene subclass, including the secretory enzymes that likely act on hormones and pheromones (Fig.
297 1). The remaining 7 CXE genes fell into the intracellular gene subclass (Fig. 1), including
298 intracellular enzymes that mostly play roles in dietary metabolism and detoxification.

HcunCXE2, 3, 4, 5, 6, 8 and 10 are homologous to this (e.g. DmelCG10175) 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. A subsequent study found that EST-6 was able to degrade various volatile esters in vitro and function as expected for an ODE which plays a role in the response of the flies to esters (*Chertemps et al., 2012*). 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. On the other hand, based on the omnivorous nature of *H. cunea* and its species-specific sex pheromone, these CXE genes may be the unique category of *H. cunea* to degrade odor substances.

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. 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.*

319 *cunea* adults (Fig. 3 and Fig. S1). However, they were widely expressed in the larvae, which may
320 be related to their extremely broad host plant range that needs more CXEs to degrade large
321 amount of carboxylic acid esters. Our quantitative PCR results (Fig. 3 and Fig. S1) indicated that
322 some *H. cunea* genes were highly expressed in both male and female antennae, likely for
323 degradation of sex-pheromones and/or plant volatiles both from hosts or non-hosts, whereas the
324 genes highly expressed in the legs and wings might be related to the degradations of some non-
325 volatile substances for contact signals. In addition, a previous study of SexiCXE14 and
326 SexiCXE15 (antennae-enriched carboxylesterase genes in *Spodoptera exigua*) showed that
327 antenna bias expression plays a role in the degradation of volatile substances and sex
328 pheromones in plants (*He et al., 2015*). However, the expression of SexiCXE11 was much higher
329 level in abdomen and wings, and its activity in hydrolyzing plant volatile substances was
330 stronger than that in degrading ester sex pheromones (*He et al., 2019*). In the current study,
331 HcunCXE1, 3, 4, 5, 6, 8, 9, and 10 showed antenna-biased expression, while the expression of
332 HcunCXE2 and 7 in legs and wings was higher than that in antennae. These results suggested
333 that HcunCXEs have different functions and may participate in the degradation of host plant
334 volatiles and/or other xenobiotics.
335 CXEs play multiple key roles in the hydrolysis of carboxylic acids esters. CXEs also include
336 some metabolic enzymes that are associated with insecticide resistance (*Li, Schuler &*
337 *Berenbaum, 2007*). Many previous studies in insect CXEs were focused on their functions in
338 mediating insecticide resistance (*Hemingway & Karunaratne, 1998*; *Li, Schuler & Berenbaum,*

339 *2007*). In contrast, the mechanisms underlying degradation of plant allelochemicals are still
340 unclear. It has been shown that phenolic glycosides can induce expression of *Papilio canadensis*
341 CXEs. Moreover, in *Lymantria dispar*, the activities of CXEs were positively correlated with the
342 larval survival, indicating that these esterases might be involved in the glycoside metabolism
343 (*Lindroth, 1989*; *Lindroth & Weisbrod, 1991*). In the current study, nine out of 10 HcunCXEs
344 were found to express in the larvae (Fig. 4), indicating that the activities of HcunCXEs may
345 positively correlate with survival of *H. cunea* larval. In addition, a significant increase of CXE
346 activity in the midgut of *S. litura* was observed during uptake of the plant glycoside rutin
347 (*Ghumare, Mukherjee & Sharma, 1989*). The CXEs in *Sitobion avenae* have been suggested to
348 participate in the gramine detoxification (*Cai et al., 2009*). Quercetinrutin and 2-tridaconone
349 were also found to induce the activities of CXEs in *Helicoverpa Armigera* (*Gao et al., 1998*; *Mu,*
350 *Pei & Gao, 2006*). Although the gene expression of HcunCXEs in *H. cunea* midgut and some
351 other tissues are still unknown, based on these previous findings, it is reasonable to speculate that
352 HcunCXEs might also play multiple functions in *H. cunea* physiology and metabolism.
353 Understanding the specific function of HcunCXEs will require further analyses using in vitro and
354 in vivo methods.

355 Little is known about *H. cunea* olfaction mechanisms at molecular levels, especially
356 concerning how CXEs degrade various semiochemicals in its chemical communication system.
357 Further research is needed to 1) understand the functions of antennal-specific CXEs in *H. cunea*
358 via cloning, expression and purification of these CXEs and enzymatic kinetic analysis; 2)

359 determine the locations/distributions of related CXEs by *in-situ* hybridization; 3) evaluate the
360 potential correlations between CXE transcription levels and their corresponding
361 electrophysiological and behavioral responses by silencing CXEs via RNA interference (*Caplen,*
362 *2004*), and 4) ultimately discover the mode of action or functionality of CXEs in the olfactory
363 signal conduction (signal inactivation).
364

365 Conclusions

366 In summary, we identified 10 CXE genes in *H. cunea* by analyzing its antennal transcriptomic
367 data. These HcunCXEs displayed an antennae-or leg/wing-biased expression. The ubiquitous
368 expression of these HcunCXEs in different tissues and life stages suggest that they have multiple
369 roles, *i.e*, degradation of odor molecules, metabolism and detoxification of dietary and
370 environmental xenobiotics. Our findings provide a theoretical basis for further studies on the
371 olfactory mechanism of *H. cunea* and offer some new insights into functions and evolutionary
372 characteristics of CXEs in lepidopteran insects. From a practical point of view, these HcunCXEs
373 might represent meaningful targets for developing behavioral interference control strategies
374 against *H. cunea*.
375

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Competing Interests

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

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597
598
599
600 **Figure legends**
601 **Figure 1 Molecular phylogeny comparing HcunCXEs with CXEs from other insect species.**
602 10 CEXs (HcunCXE1-10) from *H. cunea* (Hcun) and CXEs from *S. exigua* (Sexi), *C. medinalis*
603 (Cmed), *B. mori* (Bmor), *D. melanogaster* (Dmel), *T. castaneum* (Tcas), *S. inferens* (Sinf), *S.*
604 *littoralis* (Slit) were used to construct the phylogenetic tree. The phylogenetic tree was aligned
605 by MAFFT, and constructed by MEGA-X using maximum likelihood method. The adopted
606 model is LG-G+I, and the model value is shown in table 4 of additional materials. The Bootstrap
607 value of this tree is 1000, which is to integrate the branch length tree with the Bootstrap value
608 tree and then beautify it. A: Genrally secreted enzymes, substrates include hormone and
609 pheromones; B: Generally intracellular enzymes, dietary metabolism/ detoxification functions; C:

610 JHE; D: Genrally secreted enzymes, substrates includes hormone and pheromones; E:
611 Nerouligins; F: ACHE.
612
613 **Figure 2 Motif analysis of CXEs in *H. cunea*.** (A-H) Eight motifs discovered in the 43 CXEs
614 using MEME online server (http://meme. nbcr.net/meme/). (I) Approximate locations of each
615 motif on the protein sequence. The numbers in the boxes correspond to the numbered motifs in
616 (A-H), where small number indicates high conservation. The numbers on the bottom showed the
617 approximate locations of each motif on the protein sequence, starting from the N-terminal. This
618 figure only listed the most common 8 motif-patterns presented in 43 CXEs.
619
620 **Figure 3 Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues.** (A-J) HcunCXEs
621 (HcunCXE1, 2, 3, 4, 5, 6, 7, 8, 9 and 10). FA, female antennae; MA, male antennae; L, legs; W,
622 wings. The relative mRNA levels were normalized to those of the *EF1-a* gene and analyzed
623 using the Q-gene method. All values are shown as the mean ± SEM. The data were analyzed by
624 the least significant difference (LSD) test after one-way analysis of variance (ANOVA).
625 Different letters indicate significant differences between means ($P < 0.05$).
626
627 **Figure 4 RT-PCR analysis of HcunCXEs gene expression in tissues taken from *H. cunea***
628 **adults and other life stages.** EF1-a was used as an internal control; NC, negative control with
629 no template in the reaction.
630

631 **Figure S1. Relative mRNA expression of *HcunCXEs* in *H. cunea* tissues.** The relative mRNA
632 levels were normalized to those of the *EF1-a* gene and analyzed using the Q-gene method. All
633 values are shown as the mean ± SEM. The data were analyzed by the least significant difference
634 (LSD) test after one-way analysis of variance (ANOVA). Different letters indicate significant
635 differences between means ($P < 0.05$).
636
637 **Figure S2. Comparison of the amino acid sequences of HcunCXEs with CXEs proteins**
638 **from different species.** A, HcunCXE1 with SinfCXE18; B, HcunCXE9 and SinfCXE1; C, HcunCXE7
639 with SinfCXE13 and CmedCXE5. The percentages on the right represent the amino acid identities.
640
641 **Figure. S3 Homology analysis of *H. cunea* unigenes. (A)** E-value distribution. **(B)** Similarity
642 distribution. **(C)** Species distribution. All unigenes that had BLASTX annotations within the
643 NCBI nr database with a cutoff *E*-value of 10^{-5} were analyzed. The first hit of each sequence
644 was used for analysis.
645
646 **Figure. S4 Gene ontology (GO) assignment of *H. cunea* unigenes.** The GO classification map
647 was done by uploading the GO ID numbers of genes for their involvement in biological
648 processes, cellular components, and molecular functions.
649

650 **Figure. S5 Clusters of Orthologous Groups (KOG) classification of *H. cunea*.** The abscissa is
651 the name of 26 groups of KOG, and the ordinate is the ratio of the number of genes annotated to
652 the group to the total number of genes annotated.

653

654 **Figure. S6 Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of *H. cunea***
655 **unigene.** The *x*-axis indicates the percentage of annotated genes, and the y-axis indicates the
656 KEGG categories. The capital letters against the colored bars indicate five main categories: **(A)**
657 cellular processes, **(B)** environmental information processing, **(C)** genetic information processing,
658 **(D)** metabolism, and **(E)** organism systems.

659

660

Figure 1

Molecular phylogeny comparing HcunCXEs with CXEs from other insect species.

10 CEXs (HcunCXE1-10) from *H. cunea* (Hcun) and CXEs from *S. exigua* (Sexi), *C. medinalis* (Cmed), *B. mori* (Bmor), *D. melanogaster* (Dmel), *T. castaneum* (Tcas), *S. inferens* (Sinf), *S. littoralis* (Slit) were used to construct the phylogenetic tree. The phylogenetic tree was aligned by MAFFT, and constructed by MEGA-X using maximum likelihood method. The adopted model is LG-G+I, and the model value is shown in table 4 of additional materials. The Bootstrap value of this tree is 1000, which is to integrate the branch length tree with the Bootstrap value tree and then beautify it. A: Genrally secreted enzymes, substrates include hormone and pheromones; B: Generally intracellular enzymes, dietary metabolism/ detoxification functions; C: JHE; D: Genrally secreted enzymes, substrates includes hormone and pheromones; E: Nerouligins; F: ACHE.

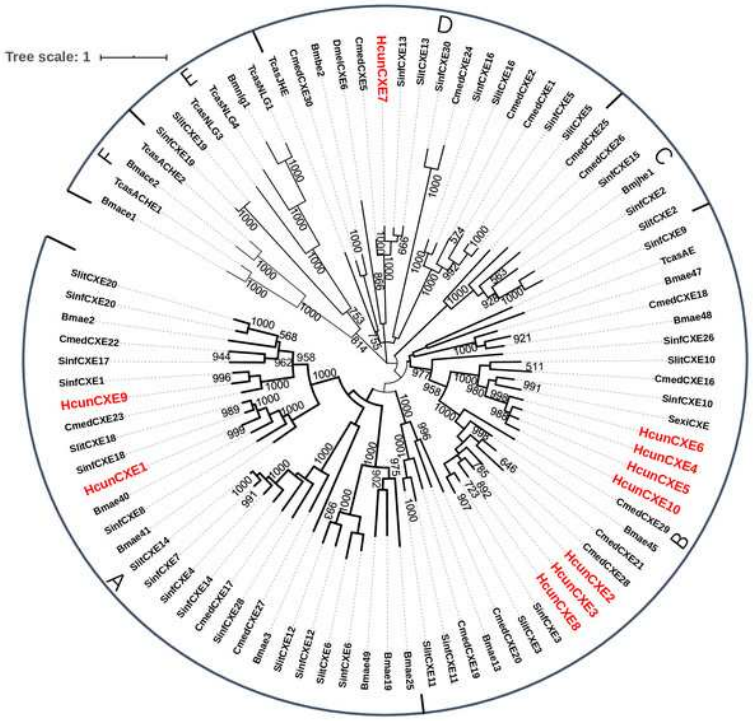
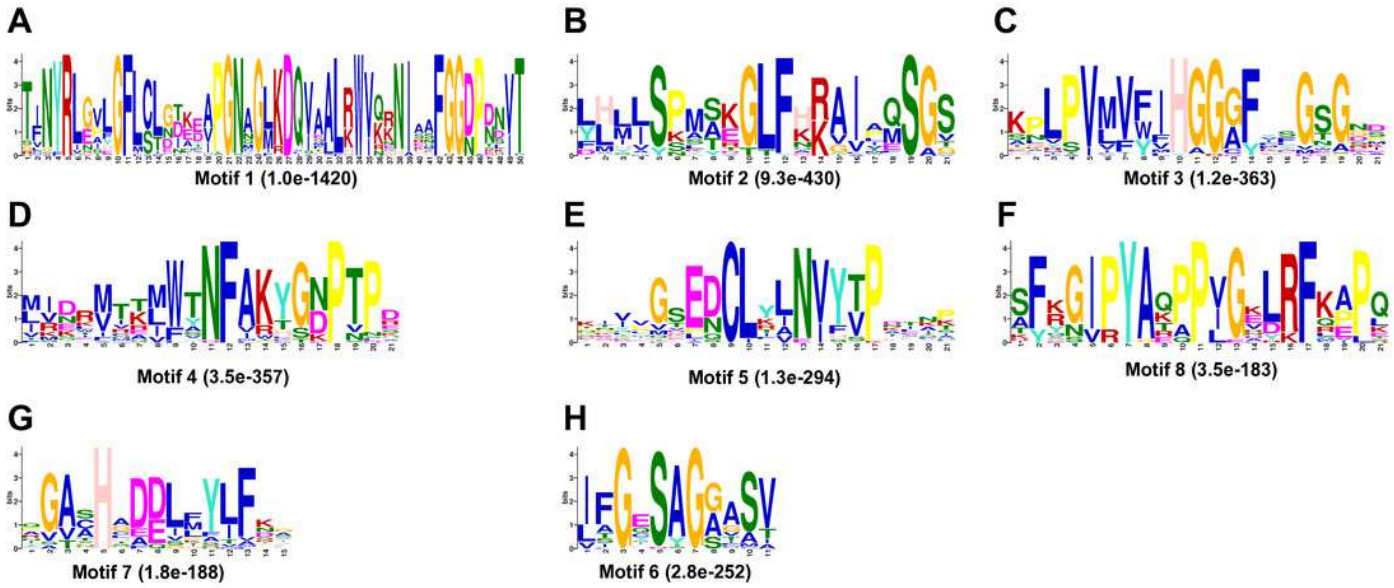

Tree scale: 1
A
B
C
D
E
F
HcunCXE7
HcunCXE9
HcunCXE1
HcunCXE6
HcunCXE4
HcunCXE5
HcunCXE10
HcunCXE2
HcunCXE3
HcunCXE8

Figure 2

Motif analysis of CXEs in *H. cunea*.

(A) Eight motifs discovered in the 43 CXEs using MEME online server (http://meme.nbcr.net/meme/). (B) Approximate locations of each motif on the protein sequence. The numbers in the boxes correspond to the numbered motifs in (A), 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 43 CXEs.


A
Motif 1 (1.0e-1420)
B
Motif 2 (9.3e-430)
C
Motif 3 (1.2e-363)
D
Motif 4 (3.5e-357)
E
Motif 5 (1.3e-294)
F
Motif 8 (3.5e-183)
G
Motif 7 (1.8e-188)
H
Motif 6 (2.8e-252)

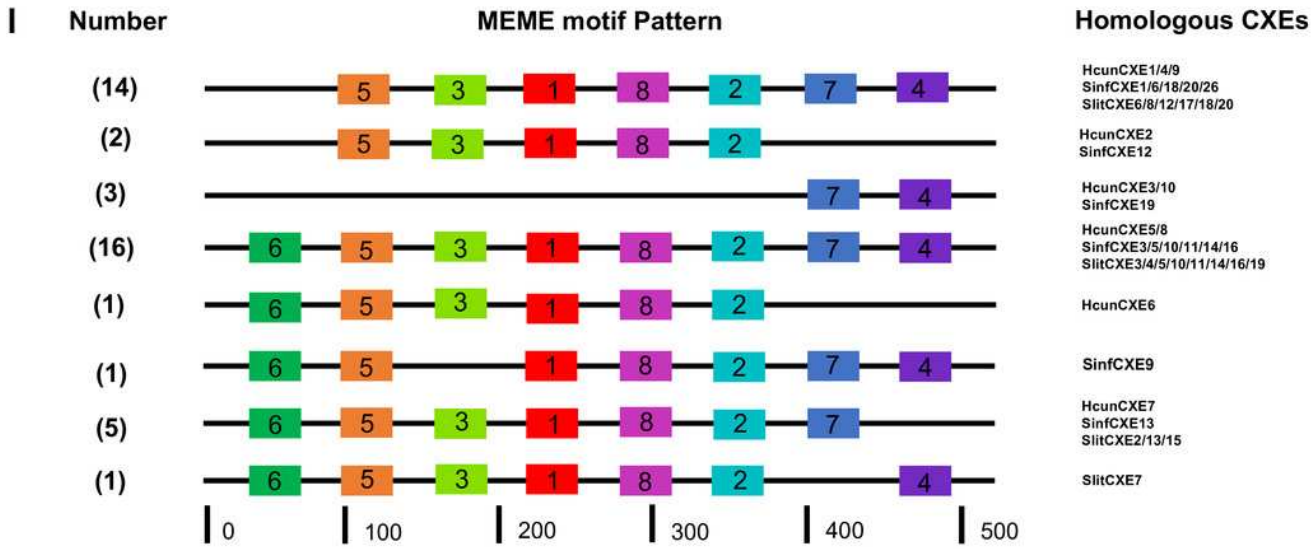

I
Number
MEME motif Pattern
Homologous CXEs
(14)
(2)
(3)
(16)
(1)
(1)
(5)
(1)
0
100
200
300
400
500
HcunCXE1/4/9
SinfCXE1/6/18/20/26
SlitCXE6/8/12/17/18/20
HcunCXE2
SinfCXE12
HcunCXE3/10
SinfCXE19
HcunCXE5/8
SinfCXE3/5/10/11/14/16
SlitCXE3/4/5/10/11/14/16/19
HcunCXE6
SinfCXE9
HcunCXE7
SinfCXE13
SlitCXE2/13/15
SlitCXE7

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 ($P < 0.05$).

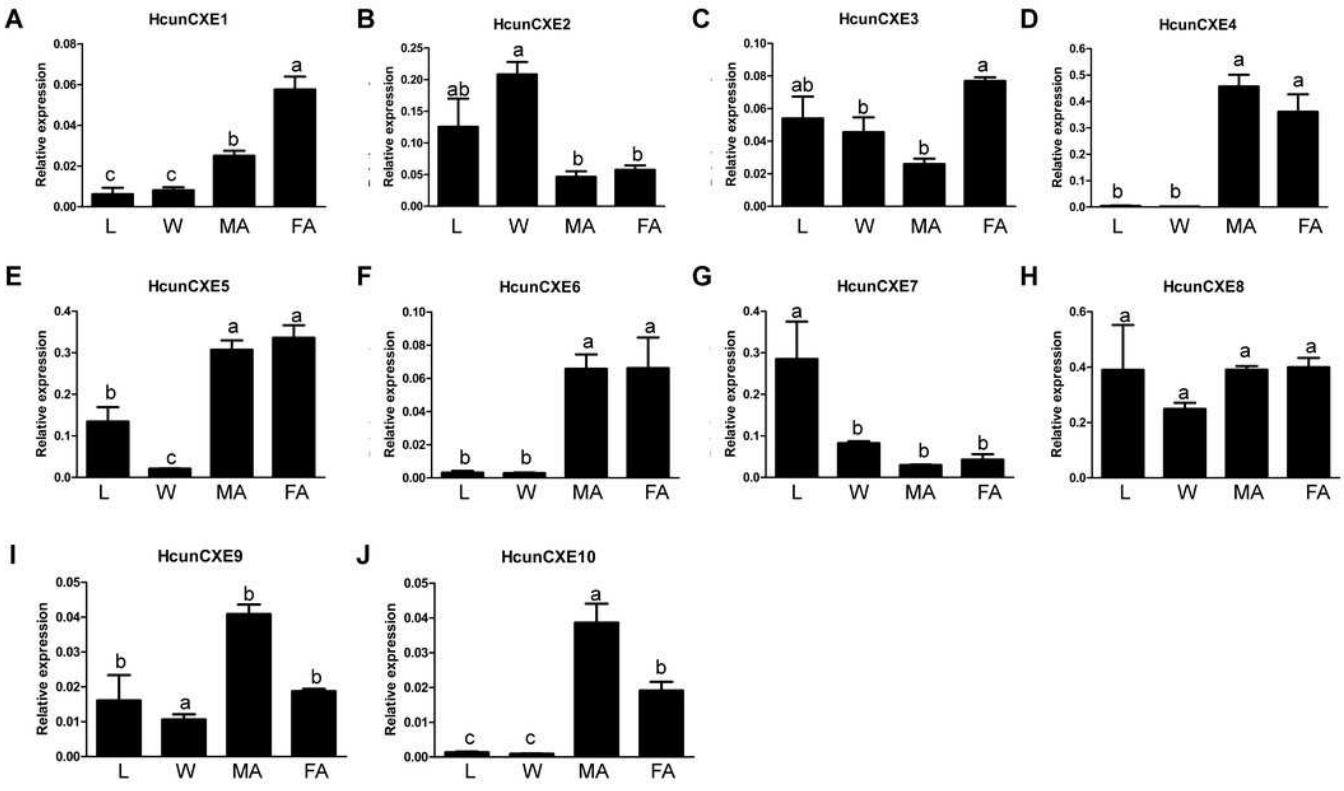


Figure 4

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.

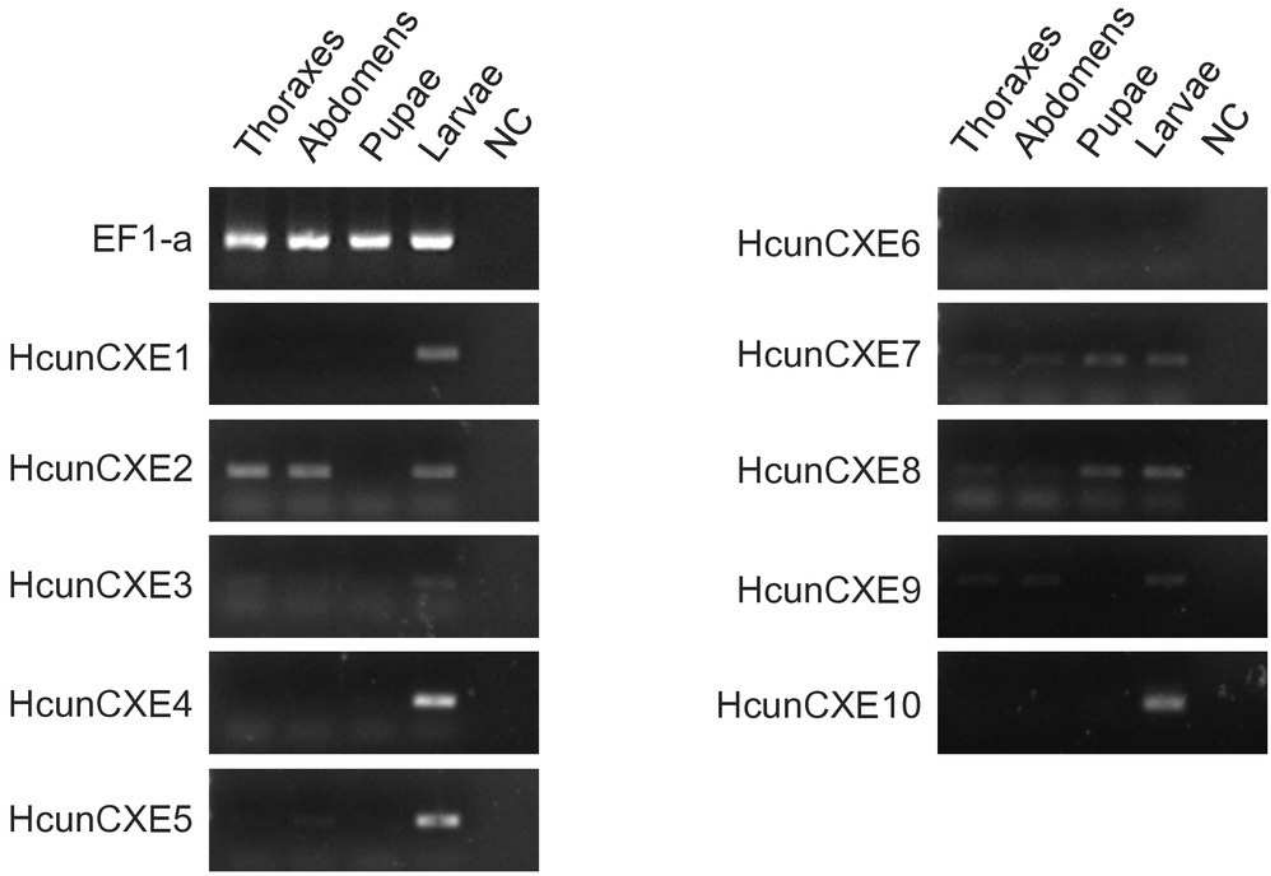


Table 1(on next page)

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

Note: ORF, open reading frame . *S. inferens, Sesamia inferens.*

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. *S. inferens, Sesamia inferens.*

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.

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

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