Identification and expression patterns of chemosensory proteins in the black-back prominent moth, *Clostera restitura* (Lepidoptera: Notodontidae)

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Abstract. Insects have evolved highly specific and sensitive olfactory sensory systems to detect plant hosts and mates. Chemosensory proteins (CSPs) play an important role in this process, but in this respect there is limited information on *Clostera restitura*, one of the most destructive defoliators of poplars in China. In the present study, we first identified seven candidate CSPs in *C. restitura*. Sequence alignment and phylogenetic analysis showed that these candidate proteins possessed typical characteristics of the insect CSP family and were similar to those of other Lepidoptera. These genes were expressed in different developmental stages and tissues, and the levels of expression differed after mating. Some CresCSPs were more associated with development and others with mating. They may play an important role in host recognition, egg development and mating behaviour. Furthermore, the CSPs were ubiquitously detected in all tissues and most of them were highly expressed in antennae, especially female antennae. We suggest the CresCSPs may contribute to female oviposition site recognition. CresCSPs that are highly transcribed in wings and legs, may function in gustation. This study provides a better understanding of the molecular mechanisms of olfaction in *C. restitura* and environmentally friendly pest management strategy for controlling *C. restitura*.

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INTRODUCTION

*Clostera restitura* Walker (Lepidoptera: Notodontidae) is one of the most destructive defoliators of poplar trees in China. This pest is widespread in southern China, especially in forest-rich provinces, such as Anhui, Jiangsu, Zhejiang, Shanghai, Fujian, Guangxi, Guangdong, Hunan and Hainan (Zhang, 1997; Liu et al., 2016; Fang et al., 2018; Xin et al., 2018). It also occurs widely in other Asian countries including India, Indonesia, Malaysia and Vietnam (Wu & Fang, 2003; Schintlmeister, 2008). An outbreak of *C. restitura* usually causes severe economic damage in a surprisingly short period. More and more scientists are exploring the biological characteristics, behaviour and control strategies for *C. restitura* (Jing et al., 2007; Tang et al., 2008).

Insects have evolved highly specific and sensitive olfactory sensory systems to perceive chemical information from the environment and transform this information into electrical signals. This process is essential for insect feeding, courtship, defence and migration (Zwiebel & Takken, 2004). Diverse kinds of proteins, odour binding proteins (OBPs), chemosensory proteins (CSPs), odour receptors (ORs), odour degrading enzymes (ODEs), ion receptors (IRs) and sensory neuron membrane proteins (SNMPs), participate in chemical perception (Leal, 2013; Cao et al., 2014; El fekih et al., 2016, Fleischer et al., 2018). Volatile chemical signals and other stimuli of lipophilic compounds cannot be directly transported to chemosensory receptors across hydrophilic lymph and must be bound by OBPs and CSPs (Yi et al., 2014a). Therefore, OBPs and CSPs, which are known as carrier proteins, play key roles in insect olfaction (Dani et al., 2011).

CSPs and well-studied OBPs, are low-molecular-mass and soluble proteins used in insect chemoreception (Pelosi et al., 2006). CSPs are characterized by four cysteine residues (C₁-X₆₋₈-C₂-X₁₆₋₂₁-C₃-X₂-C₄) that form two disulfide bridges (Tomasselli et al., 2006). Similar to OBPs, the rigid hydrophobic pocket in CSPs can capture and transport external chemical cues to receptors. CSPs were first discovered in the antennae of *Drosophila melanogaster* (Mckenna et al., 1994). Further studies revealed that CSPs were expressed not only in antennae but also in other insect
tissues, including legs (Picimbon et al., 2001), pheromone glands (Dani et al., 2011), wings (Zhu et al., 2015), proboscises (Liu et al., 2014), labial palps and maxillae (Angeli et al., 1999), which differs from OBPs.

The multi-tissue expression pattern of CSPs indicate they may also have other functions, apart from chemosensation (Tegoni et al., 2004). In fact, with the development of genome and transcriptional sequencing, recent studies have demonstrated that CSPs do contribute to other physiological processes (Zhu et al., 2015; Kang, 2016; Zhang et al., 2017; Ting et al., 2018; Zeng et al., 2018). As carrier proteins, CSPs bind with small molecules, for instance nutrients, toxic compounds, hormones and semiochemicals, (Pelosi et al., 2017). Most CSPs identified in Bombyx mori (Dani et al., 2011; Qiao et al., 2013), Plutella xylostella (Liu et al., 2010) and Sesamia inferens (Zhang et al., 2013) are widely expressed not only in antenna but also the female sex pheromone gland, which indicates a role in insect mating. In addition, CSP6 of Helicoverpa armigera is highly transcribed in sensory organs and pheromone glands, and has high binding affinity for pheromone components, which reveals that HarmCSP6 is probably involved in transporting female sex pheromones in H. armigera (Li et al., 2015). A decrease in CSPs is associated with reduced survival and fecundity in females of Spodoptera exigua, which demonstrates that female survival and reproduction is closely associated with CSPs transcription (Gong et al., 2012). Xin et al. (2017) reports that the mid gut expressed CSPs in Spodoptera litura may be responsible for its ability to adapt to different ecosystems. Populations of insecticide-resistant Diaatraea saccharalis have a higher CSPs transcription than susceptible populations, which indicates that CSPs participate in this insect’s immune response. A similar phenomenon is reported in Tribolium castaneum (Guo et al., 2012; Gao et al., 2018). Moreover, CSPs are essential for the development of the embryonic integument of Apis mellifera, behavioural phase change in migratory locust and insect tissue regeneration (Pelosi et al., 2006; Maleszka et al., 2007; Guo et al., 2011). Notably, RNAi reduction in NlugCSP8 transcript abundance causes a decrease in the behavioural response to particular attractants, which is likely to result in more effective and eco-friendly control strategies for the brown plant hopper (Muhammad et al., 2018).

This laboratory previously constructed a cDNA library for C. restituta and characterized the expression profiles of OBPs. It is likely that OBPs play a key role in foraging, seeking mates and host recognition in C. restituta (Gu et al., 2019). In addition, we cloned the full-length of the cDNA encoding CresCSP3 and analyzed its tissue specific expression pattern. The CresCSP3 transcripts detected in heads, antennae, wings and legs indicate they may contribute to insect development, mating behaviour and host location (Li et al., 2018). In this study, sequence cloning and analysis of the patterns of expression of CresCSPs are used to determine the physiological roles of 7 other CSPs in C. restituta, which will help in the development of eco-friendly techniques to be used in the control C. restituta in the future.

MATERIALS AND METHODS

Insect rearing and sample collection

Clostera restituta eggs were collected from the leaves of 7-year-old Populus euphrasiana cv. L-72 trees in an agricultural afforestation area, Pukou District, Nanjing (32°180´N, 118°28´E), Jiangsu Province, China and kept in an incubator (26 ± 0.5°C, 70 ± 5% relative humidity, 16L : 8D photoperiod) at the Laboratory of Entomology at Nanjing Forestry University. On hatching, larvae were transferred to 15-cm-diameter sterilized Petri dishes and reared on fresh leaves of Populus deltoides. Pupae were placed in individual tubes. On emergence the adults were put into plastic boxes (15 × 10 × 10 cm) and fed ad libitum a 10% honey solution. The second generation eggs, larvae, pupae and adults were used in the experiments.

For the analysis of the pattern of expression, eggs (N = 30), 1st to 5th instar larvae (N = 3), pupae (N = 3), antennae of 1–6 day old virgin males and females (N = 30) and antennae of mated and virgin adults (N = 30) were collected. The antennae, heads (without antennae, N = 3), legs (N = 30) and wings (N = 6) were dissected from newly emerged male and female adults. All these samples were immediately frozen in liquid nitrogen and stored at −80°C.

RNA extraction, cloning and sequencing

Total RNA was isolated using TRIzol Reagent (Ambion, State of Texas, USA) following the manufacturer’s instructions, before checking the quantity of RNA. First-strand cDNA for RT-PCR and RT-qPCR were synthesized from 1 μg total RNA using 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Seven pairs of specific oligonucleotide primers (Table S1) were designed based on the transcriptome database for C. restituta (Gu et al., 2019) and used to amplify the complete open reading frames (ORFs). We performed PCRs to amplify the specific genes, following the manufacturer’s protocol (Zoman, Beijing, China). Amplification products were purified using a DNA purification system (Tiangen, Beijing, China) and cloned into a pEasy-T1 cloning vector (TransGen Biotech, Beijing, China). Seven randomly selected positive clones per construct were sequenced.

Sequences and phylogenetic analyses

The ORFs of the putative chemosensory genes were identified using ORF Finder (http://www.ncbi.nlm.nih.gov/orf/gorf/gorf.html) (Min et al., 2005). Similarity searches were performed using NCBI-BLAST (http://blast.ncbi.nlm.nih.gov/). Sequence alignments were performed using DNAMAN version 6.0. Signal peptides were identified using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). The molecular weights and isoelectric points of mature proteins were calculated using the ExPaSy server program (http://www.expasy.ch/cgi-bin/pi_tool).

Phylogenetic trees were constructed based on the cDNA sequences of CSPs from C. restituta and CSPs in other Lepidoptera in the UniGene database at NCBI. Maximum-likelihood phylogenetic trees were constructed using MEGA 6 and bootstrapping of 1,000 replicates (Tamura et al., 2011).

Real-time quantitative PCR

To determine the potential functions of the CSPs, we measured the relative levels of expression of these genes in the different developmental stages, virgin and mated individuals and different tissues using qPCR. We performed RT-qPCR with the previously mentioned cDNA templates. Reactions of each sample of the three biological replicates were run in triplicate. The qRT-PCR primers (Table S1) were designed online (https://www.genscript.
com/tools/real-time-pcr-tagman-primer-design-tool). The RPS13 gene was used as a reference gene (Gu et al., 2019). qRT-PCRs were performed in an Applied Biosystem 7500 System (USA) using SYBR Premix Ex Taq II (TaKaRa, Dalian, China), according to the manufacturer’s protocol. The cycling conditions were (1) 95°C, 30 s; (2) 95°C, 5 s; (3) 60°C, 34 s; (4) go to (2) for 40 cycles, and this procedure was followed by an analysis of melting curves ranging from 60 to 95°C to verify the presence of a single discrete peak for each reaction product. The cDNA templates in 10-fold dilution series were used to construct a relative standard curve to determine the PCR efficiency. In all experiments, all primers achieved amplification efficiencies of 95–100% (Table S1).

Statistical analysis
All data was processed using SPSS Statistics V20.0 (IBM). A one-way analysis of variance (ANOVA) with least significant difference (LSD) was used to analyze the patterns in the expression of genes in the various samples.

RESULTS
Identification of CSP genes in C. restitura
Based on the unigenes of the CSPs annotated in the C. restitura antennal transcriptome database (Gu et al., 2019), we used PCR to clone seven CSP genes from antennae of C. restitura, and the sequences of these genes were deposited in GenBank with accession numbers presented in Table S2. All of these CSPs had full-length ORFs with four conserved cysteines in the same corresponding positions according to multiple alignments of amino acid sequences of the seven CresCSPs (Fig. 1). The ORFs of CresCSP1-7 ranged from 321 to 387 bp in length and the predicted molecular weight varied from 11.81 to 14.73 kDa. In addition, they were all predicted to have signal peptides of 16 to 18 amino acids in length and isoelectric points from 5.35 to 8.58 (Table S2). The results of a BLASTX search showed that CresCSP1 to CresCSP7 were very similar to the CSPs in other Lepidoptera (> 64%), especially the CSPs in S. litura and B. mori, whereas CresCSP8 was not similar to the CSPs of these organisms (39%).

Phylogenetic analyses of the CSP genes in C. restitura
A phylogenetic tree was constructed based on the cDNA sequences of CSPs from C. restitura and other Lepidoptera, including B. mori, Eogystia hippocaeoculus, Grapholita molesta, H. armigera, Heliothis virescens, Lobesia botrana, Ostrinia furnacalis, Papilio xuthus, Plutella xylostella, S. exigua and S. litura (Fig. 2). The Maximum-Likelihood (ML) tree indicated that these seven Cres CSPs occur on different branches. CresCSP1 and CresCSP6 were clustered with CSPs from P. xuthus (PxutCSP1 and PxutCSP7) in one subbranch, while CresCSP2 and CresCSP8 occurred on separate branches along with SexiCSP1-2 and SexiCSP6, respectively. CresCSP5 was very similar to LboCSP13, whereas CresCSP4 was on a branch with CSPs from Noctuidae (Fig. 2).

Patterns of expression of CSP in C. restitura
Stage-specific expression of CSPs in C. restitura
All seven CresCSPs were expressed throughout the life cycle of C. restitura (Figs 3–4). However, the expression patterns of CSPs in C. restitura differed in different stages. CresCSP7 was expressed in eggs at relatively high levels. In the first instar, CresCSP4, CresCSP5 and CresCSP6 were transcribed significantly. mRNAs of CresCSP1 and CresCSP2 were especially dominant in the second and third instar, while that of CresCSP8 was strongly detected in the fourth and last instar.

The expression patterns of CSP genes after adult emergence were shown in Fig. 4. CresCSP1 and CresCSP8 were expressed mainly in 3-day-old females and males. CresCSP2 showed high levels of expression in 4-day-old males, while CresCSP4 and CresCSP5 were abundant in 4-day-old females. In addition, in 1-day-old C. restitura females there were many transcriptomes of CresCSP6, CresCSP7 and CresCSP8.
Fig. 3. Relative levels of expression of CresCSPs in eggs, larvae of different instars and pupae determined using qPCR. Data presented are the means of three replicates. Different lower case letters indicate significant differences (P < 0.05).

Fig. 4. Relative levels of expression of CresCSPs in 1 to 6-day-old female and male adults determined using qPCR. 1 - 1-day-old, 2 - 2-day-old, 3 - 3-day-old, 4 - 4-day-old, 5 - 5-day-old and 6 - 6-day-old. Data presented are the means of three replicates. Different lower case letters indicate significant differences (P < 0.05).
Mating-specific expression of CSPs in *C. restitura*

The expression levels of CSP genes associated with mating status were shown in Fig. 5. Adult males had less mRNA of CresCSP1, CresCSP4, CresCSP6 and CresCSP7 after mating, but there were many transcripts of CresCSP2, CresCSP5 and CresCSP8 in mated males. There was an increase in the expression raised of CresCSP8 in mated females of *C. restitura* and decrease in the expression of CresCSP1 and CresCSP4 after mating.

Tissue-specific expression of CSPs in *C. restitura*

The qRT-PCR results revealed that seven genes in *C. restitura* were expressed at different levels in a wide range of tissues (Fig. 6). All seven CresCSPs, however, were mainly detected in antennae, with higher transcription levels of almost all the CresCSPs except for CresCSP2 in female antennae than in male antennae. We also recorded an enriched level of transcripts of CresCSP1, CresCSP7 and CresCSP8 in the wings of *C. restitura* and a recordable amount of those of CresCSP1, CresCSP2 and CresCSP8 in the legs.

DISCUSSION

We have compiled a library of the cDNA and thirteen CSPs in the antennae of *C. restitura* in order to identify the olfactory-related genes expressed in its antennae (Gu et al., 2019). Currently, eight CSPs with high levels of transcription among the thirteen CresCSPs from the antennae of *C. restitura* were cloned, which is fewer than that reported in other Lepidoptera. The number of CSPs varied among Lepidoptera. According to previous studies, there were twenty candidate CSPs in *B. mori* (Gong et al., 2007), 14 in third instar larvae of *E. obliqua* (Sun et al., 2017) and 24 in *H. armigera* (Li et al., 2015). These results indicated that the number of CSPs genes varied in insects and was associated with different ligands that enabled them to adapt to changing environmental conditions. BLASTX results indicated that CresCSPs were very similar to the CSPs in other Lepidoptera, e.g., CSPs in *S. litura* and *B. mori*, which implied that CSPs in insects were highly conserved.

In addition, the bioinformatics analysis showed that CresCSPs have the same signature as other CSPs, low molecular weight, an N-terminal signal peptide sequence and four conserved cysteine residues, which supported the hypothesis that CSPs were highly conserved (Wanner et al., 2014).

The phylogenetic analysis showed that different CresC-SPs had a distant genetic relationship. Similar results were reported for many Lepidoptera (Liu et al., 2010; Li et al., 2015; Zhu et al., 2015; Sun et al., 2017). The diversification of CSP-encoding genes in *C. restitura* might be correlated with the various functions of CresCSPs. Similarly, Gong et al. (2015) reported six of seven CsupCSP genes were in each branch with Papilionidae CSPs. We also found that
six of eight CresCSPs form a branch with CSPs from other Lepidoptera, especially the Noctuidae, which suggests that CresCSP genes may have evolved similarly to that of CSP genes in other moths. Although these species belong to different families, the CSPs in these insects were conserved, indicating that the diversification of CSPs within a family might by duplication (Zhu et al., 2015).

Investigations of the expression patterns of chemosensory protein genes in the different development stages, sexes, individuals of different mating status and tissues in *C. restitura*, might provide new insights into the functions of CSPs. We found transcripts of all CresCSPs in all the developmental stages, but the levels of expression differed in each stage. For example, CresCSP7 was recorded mainly in eggs and CresCSP4, CresCSP5 and CresCSP6 mainly in 1st-larvae. Therefore, we speculated that CresCSP7 might be involved in the development of the eggs of *C. restitura* and CresCSP4, CresCSP5 and CresCSP6 in the searching for food after hatching. Our results were consistent with the study of CSP5 in *Apis mellifera* using RNAi, which shows that CSPs were involved in embryonic development (Maleszka et al., 2007). In addition, we found that CresCSP1, CresCSP2 and CresCSP8 were abundant in 2nd to 4th instar larvae. Under natural conditions, 2nd instar larvae of *C. restitura* rapidly consumed leaves and moved from defoliated trees to other trees in the vicinity in search of food (Sangha et al., 2005). These CresCSPs might contribute to perception by larvae of polar volatiles. An increasing number of studies indicated that insect CSPs were responsive to host plant volatiles (Liu et al., 2010; Hua et al., 2013; Yi et al., 2014b), thus, the CresCSPs might be responsible for the perception by larvae of polar volatiles.

After emergence, *C. restitura* males and females mated with each other when they were 3 to 4 days old (Sangha et al., 2005). Interestingly, most of CresCSPs, such as CresCSP1, CresCSP2, CresCSP4, CresCSP5 and CresCSP8 were mainly expressed in 3 to 4-day-old adults and, therefore, might be associated with mating behavior, in particular the secretion of sex-pheromone by females of *C. restitura* and their location by males. Many studies revealed that CSPs had a close connection with insect mating and egg-laying (Gong et al., 2012, 2015; Ju et al., 2014). Ligand-binding assays of CSPs in *Plutella xylostella* and *Sesamia inferens* reported higher binding to non-volatile oviposition deterrents and pheromone components, which supported the above suggestion (Liu et al., 2010; Zhang et al., 2014). Our results accorded with these studies and confirm the participation of CresCSPs in mating behaviour.

Sower et al. (1973) certified that insect males and females produced sex-pheromones in order to attract each
other and for release before mating (Sower et al., 1973). Recently, many studies suggested high levels of transcription of CSPs in the sex pheromone gland of females (Dani et al., 2011; Gu et al., 2013; Zhang et al., 2013). In this research, the low expression of CresCSP1 and CresCSP4 in mated adults indicated that CSP proteins could store pheromone components before release. The abundance of transcripts of CresCSP2, CresCSP5 and CresCSP8 in mated males reflected their role in mate seeking behaviour. It was worth noting that these three CresCSPs were more associated with mating behaviour than with ageing. Females of *C. restituta* normally couldn’t automatically oviposit at spawning sites, but first examined them using their tarsal sensillae (Thompson, 1988; Singh & Sangha, 2012). The post mating up-regulation of the expression of CresCSP8 in females of *C. restituta* revealed that this gene might be involved in the search for oviposition sites.

Analysis of the patterns in the expression of chemosensory genes in insects might contribute to predicting their functions. Like OBPs, CSPs were mainly expressed in insect antennae. In our study, most CresCSPs were highly expressed in the antennae, especially in female antennae (except for CresCSP2), which manifested that CresCSPs might participate in chemosensory processes. These results were similar to patterns of expression of CSP18 in *Athetis lepigone* and CSP14 and CSP15 in *H. armigera* (Li et al., 2015; Zhang et al., 2017). Our previous studies discovered that OBPs might contribute to recognition of pheromone molecules, whereas most CresCSPs play an important role in the recognition of spawning sites or binding host volatiles in females of *C. restituta*.

CresCSPs were widely distributed in chemosensory tissues in the head, antennae, wings and legs, which suggested they might be associated with physiological processes other than olfaction (Yang et al., 2014). Some CresCSPs (CresCSP1, CresCSP7 and CresCSP8) were highly transcribed in wings, which revealed they might be associated there with gustatory functions (Xu et al., 2009). In addition, there were high expressions of CSPs in the legs of many insects, such as, *S. littura*, *S. exigua* and *Cytorkhinus lividipennis* (Zhang et al., 2012; Zhu et al., 2015; Wang et al., 2017). A certain amount of CresCSP1, CresCSP2 and CresCSP8 was also recorded in the legs of *C. restituta*, which based on the conclusions of Kitabayashi et al. (1998), might indicate they were involved in regeneration or help in the regeneration of their legs. Some reports indicated the CresCSPs in the legs might have a gustatory function. To some extent, the expression of CresCSPs in legs could be associated with the creeping behavior occasionally related to feeding in adult *C. restituta*. This chemosensory function remained to be verified.

In this study, seven CresCSPs were identified in the antennae of *C. restituta*. Different levels of expression of the CresCSPs and a phylogenetic analysis indicated that these genes had particular functions. These genes were expressed in all the developmental stages and tissues tested, and the levels of expression changed after mating. It was possible they had an important role in olfaction and other physiological processes. We aim to further the understanding of the biological functions of these CSP genes in the future by ligand-binding, RNAi and CRISPR/Cas9 experiments. This study provides valuable information on the molecular mechanisms of olfaction in *C. restituta* and a possible novel way of controlling *C. restituta* and other Lepidoptera pest.

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Supplementary material follows (Tables S1–S3, Fig. S1).
### Table S1. Oligonucleotide primers used for cloning ORFs and in the expression Analyses of CresCSPs.

| Primer name  | Primer sequence (5'→3') |
|-------------|--------------------------|
| Specific primers for cloning CSP ORFs |
| CSP1F       | ATGAATCTACTAGTTTTATG     |
| CSP1R       | TTAGTCTACAGGCTAGGA       |
| CSP2F       | ATGAAGTCCGATTTCTCTTG     |
| CSP2R       | TTATACGTGTCTTAGATCAC     |
| CSP4F       | ATGAAGGTACTAGCTATTTC     |
| CSP4R       | TTAATTTTGGACTGCTTGCA     |
| CSP5F       | ATGAAGTTCTTCATATAAGC     |
| CSP5R       | TTAGTCGTCGCCAGAAGGA      |
| CSP6F       | ATGAAGGTTTTATTACTCAC     |
| CSP6R       | TTAATTCCTTCCAAATAATG     |
| CSP7F       | ATGAACACATTACTAGCTT      |
| CSP7R       | TTACCTTGATCCCTTTAATG     |
| CSP8F       | ATGAAGGTCAGATTCTGCGT     |
| CSP8R       | TTAATCTTCTTCTAATCT      |
| Specific primers to determine expression patterns |
| RPS13-F     | CGCACCTGTAAGGTATTT       |
| RPS13-R     | CACCAATTTGTGAGGGGAGTG    |
| CSP1qRT-F   | AGCGACAGGCACAATTGTGAA    |
| CSP1qRT-R   |CACCGGCTAGGAAGCTTGCA    |
| CSP2qRT-F   | CTTTGCGAATCTACGACTGC    |
| CSP2qRT-R   | CTTGTGCCAGGGCCTCTGT      |
| CSP4qRT-F   | GTGCCAGTGTAACGCAAACC     |
| CSP4qRT-R   | AGGAATGCACTGGGCTTTTG     |
| CSP5qRT-F   | TGCCGAGTTCAGAAAGCTGT     |
| CSP5qRT-R   | TGGTTAGGCGCTTGACAGT      |
| CSP6qRT-F   | CTTGTCACCTGGTACACCTT     |
| CSP6qRT-R   | GCAGTACAGGCCCTTGAAAC     |
| CSP7qRT-F   | AGCGACAGGCACATTGTGGA     |
| CSP7qRT-R   | CACCGGCTAGGAAGCTTGCA     |
| CSP8qRT-F   | AAGTTAGGACGGCGCTGGA      |
| CSP8qRT-R   | GGCAGTTGCTTCAAGTACA      |

| Amplification efficiencies | R2   |
|---------------------------|------|
| 95%                       | 0.997|
| 95%                       | 0.996|
| 96%                       | 0.995|
| 95%                       | 0.996|
| 97%                       | 0.996|
| 98%                       | 0.996|
| 96%                       | 0.996|
| 95%                       | 0.996|

### Table S2. Summary of CSP genes identified in C. restitura.

| Gene name | Acc. no. | ORF (bp) | Signal peptide | Isoelectric point | Molecular weight (kDa) | Species | Gene Acc. no. | E-value | Identity (%) |
|-----------|----------|----------|----------------|-------------------|------------------------|---------|---------------|---------|---------------|
| CSP1      | MG518396 | 372      | 18             | 5.72              | 14.39                  | Spodoptera litura | ALJ30214.1 | 5e-56         | 64      |
| CSP2      | MG518397 | 378      | 17             | 8.58              | 14.16                  | Spodoptera litura | ABM67688.1 | 1e-50         | 70      |
| CSP5      | MG518400 | 369      | 18             | 5.17              | 13.87                  | Spodoptera exigua | AKT26491.1 | 5e-62         | 72      |
| CSP6      | MG518401 | 366      | 16             | 5.35              | 13.68                  | Bombyx mori      | AFF18035.1 | 7e-46         | 65      |
| CSP7      | MG518402 | 387      | 17             | 8.21              | 14.73                  | Athetis dissimilis | AND82451.1 | 3e-56         | 68      |
| CSP8      | MG518403 | 321      | 18             | 5.85              | 11.81                  | Helicoverpa armigera | AEX07268.1 | 2e-10         | 39      |
| Gene | Name | Species | Accession number |
|------|------|---------|-----------------|
| CrecCSP1 | chemosensory protein 1 | Clostera restitura | MG518396 |
| CrecCSP2 | chemosensory protein 2 | Clostera restitura | MG518397 |
| CrecCSP3 | chemosensory protein 3 | Clostera restitura | MG518398 |
| CrecCSP4 | chemosensory protein 4 | Clostera restitura | MG518399 |
| CrecCSP5 | chemosensory protein 5 | Clostera restitura | MG518400 |
| CrecCSP6 | chemosensory protein 6 | Clostera restitura | MG518401 |
| CrecCSP7 | chemosensory protein 7 | Clostera restitura | MG518402 |
| CrecCSP8 | chemosensory protein 8 | Clostera restitura | MG518403 |
| BmorCSP7-2 | chemosensory protein CSP7 | Bombyx mori | AF509239.1 |
| BmorCSP1 | chemosensory protein 1 | Bombyx mori | DQ855507.1 |
| BmorCSP10 | chemosensory protein 10 | Bombyx mori | AB243753.1 |
| BmorCSP10 | chemosensory protein 10 | Bombyx mori | DQ855516.1 |
| BmorCSP11 | chemosensory protein 11 | Bombyx mori | DQ855517.1 |
| BmorCSP11-2 | chemosensory protein CSP11 | Bombyx mori | AB243754.1 |
| BmorCSP13 | chemosensory protein 13 | Bombyx mori | DQ855519.1 |
| BmorCSP14 | chemosensory protein 14 | Bombyx mori | DQ855520.1 |
| BmorCSP15 | chemosensory protein 15 | Bombyx mori | DQ855521.1 |
| BmorCSP16 | chemosensory protein 16 | Bombyx mori | DQ855522.1 |
| BmorCSP2-2 | chemosensory protein CSP2 | Bombyx mori | AF509238.1 |
| BmorCSP2 | chemosensory protein 2 | Bombyx mori | DQ855508.1 |
| BmorCSP3 | chemosensory protein 3 | Bombyx mori | AB243746.1 |
| BmorCSP4 | chemosensory protein 4 | Bombyx mori | DQ855510.1 |
| BmorCSP5-2 | chemosensory protein CSP5 | Bombyx mori | AB243748.1 |
| BmorCSP6 | chemosensory protein 6 | Bombyx mori | DQ855511.1 |
| BmorCSP6 | chemosensory protein 6 | Bombyx mori | AB243749.1 |
| BmorCSP6-2 | chemosensory protein CSP6 | Bombyx mori | AB243750.1 |
| BmorCSP7 | chemosensory protein 7 | Bombyx mori | DQ855513.1 |
| BmorCSP8-2 | chemosensory protein CSP8 | Bombyx mori | AB243751.1 |
| BmorCSP8 | chemosensory protein 8 | Bombyx mori | DQ855514.1 |
| BmorCSP9-2 | chemosensory protein CSP9 | Bombyx mori | AB243752.1 |
| BmorCSP9 | chemosensory protein 9 | Bombyx mori | DQ855515.1 |
| EhipCSP13 | chemosensory protein 13 | Eogystia hippophaeaeolus | KX655948.1 |
| EhipCSP14 | chemosensory protein 14 | Eogystia hippophaeaeolus | KX655949.1 |
| EhipCSP15 | chemosensory protein 15 | Eogystia hippophaeaeolus | KX655950.1 |
| EhipCSP17 | chemosensory protein 17 | Eogystia hippophaeaeolus | KX655952.1 |
| EhipCSP18 | chemosensory protein 18 | Eogystia hippophaeaeolus | KX655953.1 |
| EhipCSP2 | chemosensory protein 2 | Eogystia hippophaeaeolus | KX655937.1 |
| EhipCSP3 | chemosensory protein 3 | Eogystia hippophaeaeolus | KX655938.1 |
| EhipCSP4 | chemosensory protein 4 | Eogystia hippophaeaeolus | KX655939.1 |
| EhipCSP6 | chemosensory protein 6 | Eogystia hippophaeaeolus | KX655941.1 |
| EhipCSP7 | chemosensory protein 7 | Eogystia hippophaeaeolus | KX655942.1 |
| GmoCSP11 | chemosensory protein 11 | Grapholitha molesta | KR003783.1 |
| GmoCSP3 | chemosensory protein 3 | Grapholitha molesta | KR003780.1 |
| GmoCSP6 | chemosensory protein 8 | Grapholitha molesta | KR003781.1 |
| GmoCSP9 | chemosensory protein 9 | Grapholitha molesta | KR003782.1 |
| HarmCSP21 | chemosensory protein 21 | Helicoverpa armigera | KY810185.1 |
| HarmCSP22 | chemosensory protein 22 | Helicoverpa armigera | KY810186.1 |
| HarmCSP23 | chemosensory protein 23 | Helicoverpa armigera | KY810187.1 |
| HarmCSP25 | chemosensory protein 25 | Helicoverpa armigera | KY810502.1 |
| HarmCSP26 | chemosensory protein 26 | Helicoverpa armigera | KY810502.1 |
| HassCSP | chemosensory protein | Helicoverpa assulta | DQ285667.1 |
| HassCSP20 | chemosensory protein 20 | Helicoverpa assulta | KY810189.1 |
| HassCSP21 | chemosensory protein 21 | Helicoverpa assulta | KY810190.1 |
| HassCSP22 | chemosensory protein 22 | Helicoverpa assulta | KY810191.1 |
| HassCSP23 | chemosensory protein 23 | Helicoverpa assulta | KY810192.1 |
| HassCSP24 | chemosensory protein 24 | Helicoverpa assulta | KY810193.1 |
| HassCSP25 | chemosensory protein 25 | Helicoverpa assulta | KY810194.1 |
| HviCSP2 | chemosensory protein 2 | Heliotis virescens | YA101511.1 |
| HviCSP1 | chemosensory protein 1 | Heliotis virescens | YA101512.1 |
| LbotCSP10 | chemosensory protein 10 | Lobesia botrana | MG788191.1 |
| LbotCSP13 | chemosensory protein 13 | Lobesia botrana | MG788194.1 |
| LbotCSP14 | chemosensory protein 14 | Lobesia botrana | MG788195.1 |
| LbotCSP18 | chemosensory protein 18 | Lobesia botrana | MG788196.1 |
| LbotCSP19 | chemosensory protein 19 | Lobesia botrana | MG788197.1 |
| LbotCSP2 | chemosensory protein 20 | Lobesia botrana | MG788198.1 |
| LbotCSP20 | chemosensory protein 20 | Lobesia botrana | MG788199.1 |
| LbotCSP21 | chemosensory protein 21 | Lobesia botrana | MG788200.1 |
| LbotCSP23 | chemosensory protein 23 | Lobesia botrana | MG788201.1 |
| LbotCSP3 | chemosensory protein 3 | Lobesia botrana | MG788193.1 |
| LbotCSP4 | chemosensory protein 4 | Lobesia botrana | MG788196.1 |
| LbotCSP5 | chemosensory protein 5 | Lobesia botrana | MG788197.1 |
| LbotCSP6 | chemosensory protein 6 | Lobesia botrana | MG788189.1 |
| OfurCSP1 | chemosensory protein 10 | Ostrinia furnacalis | LC027712.1 |
| OfurCSP12 | chemosensory protein 12 | Ostrinia furnacalis | LC027713.1 |
| OfurCSP13 | chemosensory protein 13 | Ostrinia furnacalis | LC027714.1 |
Table S3 (continued).

| Gene     | Name                  | Species          | Accession number |
|----------|-----------------------|------------------|------------------|
| OfurCSP16| chemosensory protein 16 | Ostrinia furnacalis | LC027717.1       |
| OfurCSP2 | chemosensory protein 2 | Ostrinia furnacalis | LC027703.1       |
| OfurCSP4 | chemosensory protein 4 | Ostrinia furnacalis | LC027705.1       |
| OfurCSP5 | chemosensory protein 5 | Ostrinia furnacalis | LC027706.1       |
| OfurCSP6 | chemosensory protein 6 | Ostrinia furnacalis | LC027707.1       |
| OfurCSP7 | chemosensory protein 7 | Ostrinia furnacalis | LC027708.1       |
| OfurCSP8 | chemosensory protein 8 | Ostrinia furnacalis | LC027709.1       |
| PxutCSP1 | chemosensory protein 1 | Papilio xuthus    | AB260116.1       |
| PxutCSP10| chemosensory protein10 | Papilio xuthus    | AB260126.1       |
| PxutCSP11a| chemosensory protein11a | Papilio xuthus    | AB430775.1       |
| PxutCSP11b| chemosensory protein11b | Papilio xuthus    | AB430776.1       |
| PxutCSP12| chemosensory protein12 | Papilio xuthus    | AB430777.1       |
| PxutCSP13| chemosensory protein13 | Papilio xuthus    | AB430778.1       |
| PxutCSP2 | chemosensory protein2  | Papilio xuthus    | AB260117.1       |
| PxutCSP3 | chemosensory protein3  | Papilio xuthus    | AB260118.1       |
| PxutCSP4 | chemosensory protein4  | Papilio xuthus    | AB260119.1       |
| PxutCSP4a| chemosensory protein4a | Papilio xuthus    | AB430771.1       |
| PxutCSP4b| chemosensory protein4b | Papilio xuthus    | AB430772.1       |
| PxutCSP4c| chemosensory protein4c | Papilio xuthus    | AB430773.1       |
| PxutCSP5 | chemosensory protein5  | Papilio xuthus    | AB260120.1       |
| PxutCSP6 | chemosensory protein6  | Papilio xuthus    | AB260121.1       |
| PxutCSP7 | chemosensory protein7  | Papilio xuthus    | AB260122.1       |
| PxutCSP8 | chemosensory protein8  | Papilio xuthus    | AB260123.1       |
| PxutCSP8a| chemosensory protein8a | Papilio xuthus    | AB260124.1       |
| PxutCSP8b| chemosensory protein8b | Papilio xuthus    | AB430774.1       |
| PxylCSP1 | chemosensory protein CSP1 | Plutella xylostella | EF186791.1       |
| PxylCSP2 | chemosensory protein CSP2 | Plutella xylostella | EF186792.1       |
| PxylCSP3 | chemosensory protein CSP3 | Plutella xylostella | EF202828.1       |
| PxylCSP4 | chemosensory protein CSP4 | Plutella xylostella | EF202829.1       |
| PxylCSP5 | chemosensory protein5  | Plutella xylostella | EF202830.1       |
| SexiCSP1-1| chemosensory protein 1 | Spodoptera exigua | KM275345.1       |
| SexiCSP1 | chemosensory protein1  | Spodoptera exigua | EF186793.1       |
| SexiCSP2-1| chemosensory protein2 | Spodoptera exigua | KM275346.1       |
| SexiCSP2 | chemosensory protein2  | Spodoptera exigua | EF186794.1       |
| SexiCSP3-1| chemosensory protein 3 | Spodoptera exigua | KM275347.1       |
| SexiCSP3 | chemosensory protein3  | Spodoptera exigua | EF186795.1       |
| SexiCSP6 | chemosensory protein6  | Spodoptera exigua | KM275350.1       |
| SltCSP   | chemosensory protein   | Spodoptera litura | DQ007458.1       |

Fig. S1. Alignment of mature CresCSPs and CSPs of other Lepidoptera. Amino acids conserved in all CSPs are shown with a black background. The sequences used in the construction of this phylogenetic tree are listed in Table S3.
