Candidate odorant binding proteins and chemosensory proteins in the larval chemosensory tissues of two closely related noctuidae moths, *Helicoverpa armigera* and *H. assulta*

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**Abstract**

In order to acquire enough nutrients and energy for further development, larvae need to invest a large portion of their sensory equipments to identify food sources. Yet, the molecular basis of odor-driven behavior in larvae has been poorly investigated. Information on olfactory genes, particularly odorant binding proteins (OBPs) and chemosensory proteins (CSPs) which are involved in the initial steps of olfaction is very scarce. In this study, we have identified 26 OBP and 21 CSP genes from the transcriptomes of *Helicoverpa armigera* larval antennae and mouthparts. A comparison with the 34 OBP and 18 CSP genes of the adult antenna, revealed four novel OBPs and seven novel CSPs. Similarly, 27 OBPs (six novel OBPs) and 20 CSPs (6 novel CSPs) were identified in the transcriptomes of *Helicoverpa assulta* larval antennae and mouthparts. Tissue-specific profiles of these soluble proteins in *H. armigera* showed that 6 OBP and 4 CSP genes are larval tissue-specific, 15 OBPs and 13 CSPs are expressed in both larvae and adult, while the rest are adult-specific. Our data provide useful information for functional studies of genes involved in larval foraging.

**Introduction**

For survival, insects need a specialised sensory system to monitor environmental odors. Olfactory stimuli in Lepidoptera can be divided into intra-specific pheromones, mainly mediating communication between sexes, and plant volatiles used as cues for larval foraging and oviposition [1–3]. Odor detection is achieved by ten thousand chemosensilla on the two main sensory organs, antenna and mouthparts, housing olfactory sensory neurons (OSNs) that respond to volatiles and send electrical impulses to antennal lobes. From these organs cognate project
neurons (PN) convey electric signals to the mushroom bodies and lateral horn of the protocerebrum, triggering behavioral responses [4–6]. At the periphery, several protein families are involved in odor detection: odorant binding proteins (OBPs), chemosensory proteins (CSP), odorant receptors (OR) and ionotropic receptors (IR) [7–9]. Of these genes, the receptor families including ORs and IRs are the key elements which determine both sensitivity and specificity of chemical recognition. ORs are seven trans-membrane domain receptors expressed in the dendrite membrane of olfactory sensory neurons. OR perform their function as heterodimer with a specific ligand-binding ORx and a highly conserved co-receptor named Orco [10–12]. IRs belong to the ionotropic glutamate receptor (iGluR)-like protein family and can be activated by small molecules like acetates and amine-like volatile compounds [13–15].

Apart from receptors, two families of soluble proteins, OBPs and CSPs, also play essential roles in the first step of olfactory detection. OBPs are small soluble proteins generally with 135–220 amino acids. To maintain a compact and conserved structure, six conserved cysteines are paired in three interlocked disulphide bridges. Six $\alpha$-helices envelop a hydrophobic binding pocket [16–18]. OBPs are present at high concentrations (up to 10mM) in the lymph between the dendritic membrane and the cuticular wall [7, 19]. More interesting, there are some evidences that OBPs contribute to odorant recognition, rather than being passive odorant shuttles [20, 21]. Some studies have shown that OBPs perform the first filtering function in olfactory discrimination [19,20], besides a more general role in ferrying ligands through the sensillum lymph to the membrane of OSN dendrites. OBPs have also been shown to influence the response of ORx/ORco complexes to specific odors [21, 22]. CSPs represent another class of small soluble proteins abundant in the lymph of chemosensilla [23]. They are different from OBPs in amino acid sequence and structure, but appear to be similar in functions, although better evidence is needed to clarify their role in olfaction.

In Lepidoptera, both larvae and adults use their olfactory system to detect volatile chemicals, but their olfactory organs are completely different in morphology. In adults, a pair of antennae bear tens of thousands of sensilla, each of them housing two or more OSNs [24, 25]. Larvae are equipped with two different olfactory organs, antennae and mouthparts [26,27]. Unlike adult antennae, larval antennae and mouthparts contain few sensilla, but each of them houses a cluster of OSNs [28, 29]. With the rapid development of next generation sequence techniques, a large number of olfactory genes including IRs, ORs, OBPs and CSPs have been recently identified in the antennae of several moths, such as Manduca sexta [15, 30–32], Helicoverpa armigera [33–35], Helicoverpa assulta [34, 36], Cydia pomonella [37], Spodoptera littoralis [38, 39] and Chilo suppressalis [40], and many others. However, limited information is available for larval antennae and mouthparts.

Two Helicoverpa species, H. armigera and H. assulta are worldwide agricultural pests [41]. The behaviors of larvae and adults are largely triggered by olfactory stimuli. Previously, we performed a transcriptome analysis on adult antennae in both species. A total of 131 putative chemosensory unigenes were identified in H. armigera including 60 ORs, 19 IRs, 34 OBPs and18 CSPs. Similarly, in H. assulta we found 129 putative chemosensory unigenes, including 64 ORs, 19 IRs, 29 OBPs and 17 CSPs [34]. Skiri et al. (2005) have identified 65 glomeruli in each sex of H. armigera and 66 glomeruli in females of H. assulta [6], later supplemented by 15 new glomeruli in H. armigera [42]. Assuming that the number of glomeruli is equal to the number of ORs and IRs [43, 44], almost all olfactory receptors were identified in the two species. These data also agree with another study focused on both adults and larvae of H. armigera chemosensory tissues [35]. However, the repertoires of OBPs and CSPs in these two species may be incomplete by comparison with the numbers of OBPs and CSPs identified in the genome of B. mori (46 OBPs and 24 CSPs) [45]. This suggests that some OBP and CSP sources may occur in other chemosensory tissues, such as larval antennae and mouthparts. In this study, we
performed a transcriptome analysis to identify OBP and CSP genes in larval chemosensory organs of *H. armigera* and *H. assulta*. Moreover, we conducted RT-PCR assays on *H. armigera* adult and larval olfactory organs to find OBP and CSP genes with specific expression in larval antennae or mouthparts.

**Methods**

**Insect rearing**

*H. armigera* were reared at the Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. The *H. assulta* larvae were collected from the tobacco fields with the permission of the Experiment Station of Henan University of Science and Technology in Xuchang, Henan Province, China. Larvae were reared on an artificial diet and placed on a 16:8 h (light: dark) photoperiod at 27 ± 1˚C, 55–65% RH. Pupae were sexed and male and female individuals were placed in separate cages for eclosion. The adults were fed on 10% honey solution. In expression profile studies, all adult tissues were collected from 3-day-old male and female moths, all larval tissues were collected from fifth instar larvae.

**RNA extraction**

Fresh larval antennae and mouthparts were grinded in a liquid nitrogen cooled homogenizer, later adding 1mL of TriZol reagent (Invitrogen, Carlsbad, CA, USA) and the total RNA extraction were performed following the manufacturer’s instructions. The RNA sediment was dissolved in 20μL RNase-free water, RNA integrity was verified by gel electrophoresis. RNA quantity were measured on a Nanodrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, DE, USA) and purity was verified by gel electrophoresis.

**cDNA library construction and sequencing**

Five micrograms total RNA of each samples (*H. armigera* larval antennae, *H. armigera* larval mouthparts, *H. assulta* larval antennae, and *H. assulta* larval mouthparts,) was used to construct the cDNA library respectively. cDNA library construction and Illumina HiSeq 2000 (Illumina, San Diego, CA, USA) sequencing of the samples were performed at Beijing Genomics Institute (BGI, Shenzhen, China). The length of insert sequence was around 200 bp. The libraries were paired-end sequenced using PE90 strategy. The detailed procedures have been described in previous work from our laboratory [33, 34].

**Assembly and functional annotation**

After removing low quality reads, trimming low quality nucleotides of both ends, trimming 3 adaptors and poly-A/T tails, the remainder raw-reads were considered as clean-reads. De novo assembly in each sample was conducted using Trinity (version 20120608). Then the unigenes derived from the Trinity outputs were clustered by TGICL [46,47]. The consensus cluster sequences and singletons make up the unigenes dataset. The annotation of unigenes were performed via a NCBI blastx against non-redundant (nr) and SwissProt database. Candidate unigenes encoding putative OBPs and CSPs, were identified according to nr and SwissProt annotation results.

**Sequence and phylogenetic analysis**

The open reading frames (ORFs) of the putative chemosensory genes were predicted by using ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Putative N-terminal signal peptides of OBPs and CSPs were predicted by Signal IP 4.0 (http://www.cbs.dtu.dk/services/SignalP/).
Alignments of amino acid sequences (without signal peptides) were performed by ClustalX 2.0. The phylogenetic trees of OBPs and CSPs were constructed using MEGA5 software by the neighbor-joining method with Jones-Taylor-Thornton (JTT) model and the node support was assessed using a bootstrap procedure of 1000 replicates. The OBP data set contained OBP sequences identified in Lepidoptera (37 from *H. armigera*, 35 from *H. assulta*, 14 from *H. virescens*, 47 from *M. sexta* and 35 from *B. mori*). The CSP data set contained 25 sequences from *H. armigera*, 23 from *H. assulta*, 9 from *H. virescens*, 13 from *C. suppressalis*, and 16 from *B. mori*. The protein name and accession number of the genes used for phylogenetic tree building are listed in S1 Material.

Expression analysis by semi-quantitative reverse transcription PCR

Semi-quantitative reverse transcription PCR was performed to compare the expression levels of candidate chemosensory genes in larval antennae, larval mouthparts, adult antennae and adult abdomen in *H. armigera*. Total RNA was extracted from each sample as mentioned above. Before cDNA synthesis, total RNA was treated with DNase I (Fermentas, Vilnius, Lithuania) to remove residual genomic DNA. The cDNA was synthesized from total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Gene specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (S2 Material) and synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Taq MasterMix (CWBio, Beijing, China) was used for PCR reactions under general 3-step amplification of 94˚C for 30s, 60˚C for 30s, 72˚C for 30s. For most chemosensory genes, the PCR cycle-numbers were 28. PCR products were run on a 2% agarose gel and verified by DNA sequencing. The experiment was repeated using two independently prepared cDNA templates.

Results

Illumina sequencing and functional annotation

In this study, the transcriptomes of larval antennae and mouthparts in *H. armigera* and *H. assulta* were sequenced by Illumina HiSeq 2000 platform. After filtering, 51.1 million and 45.5 million clean-reads of 4.6 and 4.1 gigabases were generated for larval antennae and mouthparts of *H. armigera*, respectively. Meanwhile, 50.2 million and 52.9 million clean-reads of 4.5 and 4.8 gigabases were generated for larval antennae and mouthparts of *H. assulta*. These clean reads were assembled into 47,331, 41,705, 57,789 and 47,423 unigenes in *H. armigera* larval antennae and mouthparts, and in *H. assulta* larval antennae and mouthparts, respectively. After clustering and merging, 39,371 unigenes consisting of 12,724 distinct clusters and 26,647 distinct singletons were obtained for *H. armigera* and 44,352 unigenes consisting of 11,179 distinct clusters and 33,173 distinct singletons were obtained for *H. assulta* (Table 1).

A blastx homology search against the NCBI nr protein database revealed that 22,628 (57.5%) and 22,724 (51.2%) unigenes from *H. armigera* and *H. assulta*, respectively, showed sequence similarities to known proteins, with a cut-off E-value of $10^{-5}$. In the nr homologous species distribution, 46.78% (*H. armigera*) and 48.42% (*H. assulta*) annotated sequences closely matched the sequences of *B. mori*. The next most similar species was *D. plexippus* whose sequences matched 26.25% of those of *H. armigera* and 27.13% of *H. assulta*. Only a low percentage (<5%) of *H. armigera* and *H. assulta* sequences had orthologues in other species (S3 Material).

Identification of putative odorant-binding proteins

Based on the blastx sequence homology searching, a total of 26 and 27 OBP genes were obtained from *H. armigera* and *H. assulta* larval transcriptome respectively. Of these genes, 22
HarmOBPs and HassOBPs presented intact ORFs encoding for proteins of 135 to 195 aa, all exhibiting signal peptides at their N-termini (Table 2). A comparison with known OBPs of \textit{H. armigera} adult antenna revealed four novel OBPs in larvae, that we named as HarmOBP31, HarmOBP33, HarmOBP35 and HarmOBP36, and six new OBPs in \textit{H. assulta}, named as HassOBP33 to HassOBP38. All these novel genes were deposited in the GeneBank databases with the following accession numbers: HarmOBP31: KY810175, HarmOBP33: KY810179, HarmOBP35: KY810176, HarmOBP36: KY810177, HassOBP33: KY810180, HassOBP34: KY810178, HassOBP35: KY810181, HassOBP36: KY810182, HassOBP37: KY810183, HassOBP38: KY815028.

Insect OBPs are generally grouped into three main subfamilies: “Classic” OBPs with six conserved cysteines, “Minus-C” with only four cysteines, and “Plus-C” with more cysteines in addition to those of the conserved motif [45, 48, 49]. Among the larval OBPs, 14 of \textit{H. armigera} and 17 of \textit{H. assulta} were assigned to the Classic OBP group, while 3 can be classified as Minus-C OBPs in both species. 7 OBPs in both species belong to the Plus-C group, while others could not be assigned due to incomplete sequences (Fig 1).

A phylogenetic tree was constructed using OBP sequences from \textit{H. armigera}, \textit{H. assulta}, \textit{H. virescens}, \textit{M sexta} and \textit{B. mori} (Fig 2). Accordingly, the OBPs can be grouped into ABPI (antennal binding protein I), ABPII (antennal binding protein II), CRLBP (classic OBP), Minus-C, Plus-C, and PBP/GOBP (general odorant binding protein/pheromone binding protein) clusters based on the classification of OBPs from \textit{B. mori} [45]. At the same time, most OBPs of \textit{H. armigera} and \textit{H. assulta} defined as Minus-C and Plus-C clustered with \textit{B. mori} proteins of the same groups. However, among “classic” OBPs, only two sequences were found in the CRLBP branch, the others in the ABPX branches. Based on the bootstrap values on the tree, for all novel HarmOBPs we could find orthologous genes in \textit{H. assulta} with more than 90% sequence identity. Only for HassOBP38 we could not identify an orthologue in \textit{H. armigera}.

### Identification of candidate chemosensory proteins

In our transcription sets, a total of 21 sequences in \textit{H. armigera} and 20 sequences in \textit{H. assulta} can be matched with sequences of known CSPs in other Lepidoptera species. Of these, 17 HarmCSPs and HassCSPs had full-length ORFs and predicted signal peptides. Their lengths range from 107 to 292 amino acids (Table 3). A comparison with CSPs previously reported for

| Sample | Total Number | Total Length (nt) | Mean Length (nt) | N50 Total Consensus Sequences | Distinct Clusters | Distinct Singletons |
|--------|--------------|------------------|-----------------|-------------------------------|------------------|-------------------|
| \textit{H. armigera} | | | | | | |
| Contig | Harm-L-A | 83,523 | 37,101,992 | 444 | 1247 | - | - |
| | Harm-L-MP | 71,965 | 32,582,057 | 453 | 1244 | - | - |
| Unigene | Harm-L-A | 47,331 | 43,705,425 | 907 | 1953 | 11,955 | 29,750 |
| | Harm-L-MP | 41,705 | 46,761,853 | 1188 | 2298 | 39,371 | 12,724 |
| All | | 93,038 | 83,864,349 | 1188 | 2298 | 39,371 | 12,724 |
| \textit{H. assulta} | | | | | | |
| Contig | Hass-L-A | 103,673 | 38,454,494 | 371 | 831 | - | - |
| | Hass-L-MP | 78,235 | 39,088,209 | 461 | 1241 | - | - |
| Unigene | Hass-L-A | 57,789 | 40,537,882 | 701 | 1554 | 57,789 | 8,354 |
| | Hass-L-MP | 47,423 | 42,770,981 | 902 | 2028 | 47,423 | 9,677 |
| All | | 100,212 | 83,106,863 | 1058 | 2104 | 44,352 | 11,179 |

[Table 1. Summary of data used for transcriptome assembly.](https://doi.org/10.1371/journal.pone.0179243.t001)
Table 2. Unigenes of candidate odorant binding proteins in larval chemosensory tissues of *H. armigera* and *H. assulta*.

| Unigene reference | Gene name | Length (bp) | ORF (aa) | Blastx best hit (Reference/Name/Species) | E value | Identity | Signal peptide | Full length |
|-------------------|-----------|-------------|----------|------------------------------------------|---------|-----------|----------------|-------------|
| **Unigene 16494** | HarmGOBP2 | 634         | 162      | emb|CAC08211.1[general odorant-binding protein 2 precursor (GOBP2)] [Helicoverpa armigera] | 6E-115  | 100%      | Yes            | Yes         |
| **Unigene 15587** | HarmOBP1  | 617         | 147      | gb|AEX07272.1[odorant-binding protein [Helicoverpa assulta]] | 1E-80   | 94%       | Yes            | Yes         |
| **Unigene 25962** | HarmOBP2  | 276         | 51       | gb|AGH70103.1[odorant-binding protein 7 [Spodoptera exigua]] | 4E-22   | 84%       | Yes            | No          |
| **Unigene 8467**  | HarmOBP3  | 662         | 147      | gb|AGC92788.1[odorant-binding protein 3 [Helicoverpa assulta]] | 2E-97   | 96%       | Yes            | Yes         |
| **Unigene 3463**  | HarmOBP4  | 611         | 147      | gb|AEX07276.1[odorant-binding protein [Helicoverpa assulta]] | 2E-89   | 91%       | Yes            | Yes         |
| **CL5000.Contig1**| HarmOBP5  | 641         | 147      | gb|AEX07271.1[odorant-binding protein [Helicoverpa assulta]] | 1E-101  | 99%       | Yes            | Yes         |
| **CL1168.Contig2**| HarmOBP6  | 641         | 147      | gb|AEX07270.1[odorant-binding protein [Helicoverpa assulta]] | 2E-98   | 97%       | Yes            | Yes         |
| **Unigene 9848**  | HarmOBP9  | 703         | 148      | gb|AGC92789.1[odorant-binding protein 9 [Helicoverpa assulta]] | 7E-106  | 99%       | Yes            | Yes         |
| **CL3933.Contig2**| HarmOBP14 | 769         | 137      | gb|AFI57167.1[odorant-binding protein 18 [Helicoverpa armigera]] | 1E-94   | 100%      | Yes            | Yes         |
| **CL3679.Contig2**| HarmOBP15 | 631         | 168      | gb|ADY17882.1[odorant-binding protein [Spodoptera exigua]] | 6E-82   | 75%       | Yes            | Yes         |
| **Unigene 12555** | HarmOBP16 | 714         | 186      | gb|AEX07273.1[odorant-binding protein [Helicoverpa assulta]] | 3E-78   | 62%       | Yes            | Yes         |
| **Unigene 9920**  | HarmOBP17 | 473         | 137      | gb|AGM38607.1[odorant binding protein [Chilo suppressalis]] | 1E-58   | 69%       | Yes            | Yes         |
| **Unigene 371**   | HarmOBP19 | 533         | 148      | ref|NP_001141088.1[odorant-binding protein 4 [Bombyx mori]] | 3E-37   | 46%       | Yes            | Yes         |
| **Unigene 16501** | HarmOBP21 | 550         | 142      | gb|AFD34178.1[odorant-binding protein 2 [Argyresthia conjugella]] | 3E-45   | 54%       | Yes            | Yes         |
| **Unigene 24118** | HarmOBP22 | 576         | 140      | gb|AFG72998.1[odorant-binding protein 1 [Spodoptera exigua]] | 3E-55   | 57%       | Yes            | No          |
| **Unigene 3491**  | HarmOBP23 | 811         | 241      | gb|AGH70107.1[odorant binding protein 11 [Spodoptera exigua]] | 3E-95   | 80%       | Yes            | No          |
| **Unigene 10971** | HarmOBP25 | 658         | 195      | gb|AEX07273.1[odorant-binding protein [Helicoverpa assulta]] | 1E-136  | 98%       | Yes            | Yes         |
| **Unigene 14030** | HarmOBP26 | 669         | 154      | gb|EHJ67765.1[odorant binding protein [Danaus plexippus]] | 1E-60   | 69%       | Yes            | Yes         |
| **CL376.Contig1** | HarmOBP27 | 577         | 147      | gb|AEX07279.1[odorant-binding protein [Helicoverpa armigera]] | 7E-97   | 96%       | Yes            | Yes         |
| **Unigene 15643** | HarmOBP28 | 643         | 147      | gb|BAI44700.1[odorant binding protein [Bombyx mori]] | 6E-52   | 56%       | Yes            | Yes         |
| **Unigene 6228**  | HarmOBP29 | 608         | 142      | gb|AAR28763.1[odorant-binding protein-2 precursor [Spodoptera frugiperda]] | 1E-46   | 62%       | Yes            | Yes         |
| **Unigene 8997**  | HarmOBP30 | 794         | 135      | gb|AFI57166.1[odorant-binding protein 17 [Helicoverpa armigera]] | 1E-93   | 99%       | Yes            | Yes         |
| **Unigene 9430**  | HarmOBP31 | 755         | 150      | gb|AEX07271.1[odorant-binding protein [Helicoverpa assulta]] | 2E-59   | 61%       | Yes            | Yes         |
| **Unigene 5227**  | HarmOBP33 | 351         | 99       | ref|XP_004928233.1[general odorant-binding protein 9a-like [Bombyx mori]] | 1E-41   | 65%       | No             | No          |
| **Unigene 6209**  | HarmOBP35 | 656         | 146      | gb|AFI57165.1[odorant-binding protein 16 [Helicoverpa armigera]] | 5E-108  | 99%       | Yes            | Yes         |
| **Unigene 7375**  | HarmOBP36 | 537         | 149      | ref|NP_001141088.1[odorant-binding protein 4 [Bombyx mori]] | 4E-40   | 45%       | Yes            | Yes         |

(Continued)
Table 2. (Continued)

| Unigene reference | Gene name | Length (bp) | ORF (aa) | Blastx best hit (Reference/Name/Species) | E value | Identity | Signal peptide | Full length |
|--------------------|-----------|-------------|----------|------------------------------------------|---------|----------|----------------|-------------|
| **H. assulta**     |           |             |          |                                          |         |          |                |             |
| Unigene23306       | HassGOBP1  | 328         | 109      | sp|Q27226.1|general odorant binding protein 1 [Heliothis virescens] | 9E-73   | 96%      | Yes            | No          |
| Unigene21063       | HassGOBP2  | 624         | 162      | gb|AAQS4909.1|general odorant binding protein 2 [Helicoverpa assulta] | 8E-115  | 100%     | Yes            | Yes         |
| CL3828.Contig1     | HassOBP1   | 1042        | 147      | gb|AE007272.1|odorant-binding protein [Helicoverpa assulta] | 1E-81   | 98%      | Yes            | Yes         |
| CL2155.Contig1     | HassOBP2   | 752         | 143      | gb|AGH70103.1|odorant binding protein 7 [Spodoptera exigua] | 7E-85   | 82%      | Yes            | Yes         |
| Unigene16541       | HassOBP3   | 618         | 147      | gb|AGC92788.1|odorant-binding protein 3 [Helicoverpa assulta] | 2E-100  | 100%     | Yes            | Yes         |
| Unigene8150        | HassOBP4   | 581         | 147      | gb|AE007276.1|odorant-binding protein [Helicoverpa assulta] | 2E-96   | 97%      | Yes            | Yes         |
| Unigene6153        | HassOBP5   | 637         | 147      | gb|AE007271.1|odorant-binding protein [Helicoverpa assulta] | 1E-101  | 99%      | Yes            | Yes         |
| Unigene5533        | HassOBP6   | 626         | 147      | gb|AE007270.1|odorant-binding protein [Helicoverpa assulta] | 2E-101  | 99%      | Yes            | Yes         |
| Unigene8860        | HassOBP9.2 | 698         | 148      | gb|AGC92789.1|odorant-binding protein 9 [Helicoverpa assulta] | 5E-105  | 99%      | Yes            | Yes         |
| Unigene18089       | HassOBP14  | 1747        | 137      | gb|AFI57167.1|odorant-binding protein 18 [Helicoverpa armigera] | 1E-89   | 99%      | Yes            | Yes         |
| Unigene18604       | HassOBP15  | 579         | 166      | gb|ADY17882.1|odorant binding protein [Spodoptera exigua] | 1E-82   | 76%      | Yes            | No          |
| Unigene4097        | HassOBP19  | 516         | 148      | ref|NP_001140188.1|odorant-binding protein 4 [Bombyx mori] | 1E-37   | 47%      | Yes            | Yes         |
| Unigene5122        | HassOBP22  | 379         | 125      | gb|AGM38613.1|odorant binding protein [Chilo suppressalis] | 2E-54   | 58%      | No             | No          |
| Unigene1471        | HassOBP23  | 863         | 241      | gb|AGH70107.1|odorant binding protein 11 [Spodoptera exigua] | 8E-96   | 81%      | Yes            | No          |
| Unigene12884       | HassOBP25  | 621         | 194      | gb|AE007273.1|odorant-binding protein [Helicoverpa assulta] | 8E-115  | 100%     | Yes            | Yes         |
| Unigene8198        | HassOBP26  | 669         | 181      | gb|EHJ67765.1|odorant binding protein [Danaus plexippus] | 9E-73   | 96%      | Yes            | No          |
| CL3623.Contig1     | HassOBP27  | 573         | 147      | gb|AE007279.1|odorant-binding protein [Helicoverpa armigera] | 1E-22   | 48%      | No             | No          |
| Unigene6098        | HassOBP28  | 585         | 147      | db|BAA44700.1|odorant binding protein [Bombyx mori] | 1E-37   | 47%      | Yes            | Yes         |
| Unigene6100        | HassOBP29  | 602         | 142      | gb|AAR28763.1|odorant-binding protein 2 precursor [Spodoptera frugiperda] | 2E-54   | 58%      | No             | No          |
| Unigene11827       | HassOBP30  | 811         | 135      | gb|AFI57166.1|odorant-binding protein 17 [Helicoverpa armigera] | 8E-96   | 81%      | Yes            | No          |
| Unigene6144        | HassOBP31  | 789         | 150      | gb|AE007271.1|odorant-binding protein [Helicoverpa assulta] | 5E-134  | 97%      | Yes            | Yes         |
| Unigene10157       | HassOBP33  | 609         | 152      | ref|XP_00492833.1|general odorant-binding protein 99a-like [Bombyx mori] | 1E-60   | 69%      | Yes            | Yes         |
| Unigene13394       | HassOBP34  | 476         | 137      | gb|AGM38607.1|odorant binding protein [Chilo suppressalis] | 8E-98   | 97%      | Yes            | Yes         |
| Unigene16490       | HassOBP35  | 636         | 146      | gb|AGC92791.1|odorant-binding protein 16 [Helicoverpa assulta] | 3E-52   | 56%      | Yes            | Yes         |
| Unigene2048        | HassOBP36  | 527         | 149      | ref|NP_001140188.1|odorant-binding protein 4 [Bombyx mori] | 8E-45   | 60%      | Yes            | Yes         |
| Unigene20923       | HassOBP37  | 634         | 142      | gb|AFD34178.1|odorant binding protein 2 [Argyresthia conjugella] | 1E-93   | 99%      | Yes            | Yes         |
| Unigene31389       | HassOBP38  | 356         | 118      | gb|AGC92793.1|odorant-binding protein 19 [Helicoverpa assulta] | 4E-59   | 62%      | Yes            | Yes         |

https://doi.org/10.1371/journal.pone.0179243.t002
Candidate OBPs and CSPs in the larval chemosensory tissues of *Helicoverpa armigera* and *H. assulta*

*H. armigera* and *H. assulta* adult antenna revealed seven new sequences in *H. armigera* (HarmCSP20 to HarmCSP26) and six in *H. assulta* (HassCSP20 to HassCSP25). All candidate CSPs exhibit the four conserved cysteine pattern characteristic of this family (Fig 3). These sequences were used to build a neighbor-joining tree with the CSPs of *C. suppressalis*, *B. mori* and *H. virescens*. In the tree we could recognize four groups of genes clustered together with a 99% bootstrap value, while the remaining sequences could not be grouped. Based on this homology analysis, we named the novel CSPs as HarmCSP20/HassCSP20, HarmCSP21/HassCSP21, HarmCSP22/HassOBP22, HarmCSP23/HassCSP23, HarmCSP24, HarmCSP25, HarmCSP26, HassCSP24 and HassCSP25 following the numbers assigned to previously reported CSPs (Fig 4). All these novel genes were deposited in the GeneBank: HarmCSP20-26 (GeneBank accession numbers: KY810184, KY810185, KY810186, KY810187, KY810188, KY815026, KY815027), HassCSP20-25 (GeneBank accession numbers: KY810189, KY810190, KY810191, KY810192, KY810193, KY810194).

**Expression of the OBPs and CSPs in larva and adult *H. armigera***

To better understand the functional role of OBPs and CSPs in larval olfactory systems, we investigated the expression patterns of all candidate HarmOBPs and HarmCSPs via semi-quantitative reverse transcription PCR. The tissues used were larval antenna, larval mouthpart, adult antenna and adult abdomen. The results reported in Fig 5 show that all OBPs except HarmOBP16 were successfully detected in target tissues. Six OBPs were exclusive to larval tissues including HarmOBP36, HarmOBP27 and HarmOBP19 specific for larval mouthparts, while HarmOBP26, HarmOBP31 and HarmOBP35 were expressed in both larval antennae and mouthparts. On the other hand, we found that five OBPs (HarmOBP2, HarmOBP15 and HarmOBP21, HarmOBP22 and HarmOBP23) are exclusively expressed in adult antenna. The remaining 13 OBPs showed expression in both larval and adult tissues. Of this latter group HarmGOBP2, HarmOBP4, HarmOBP9, HarmOBP17 and HarmOBP25 where were preferentially expressed in adult antenna, while the others did not show significant differences between...
Candidate OBPs and CSPs in the larval chemosensory tissues of *Helicoverpa armigera* and *H. assulta*. Compared to OBPs, CSPs were more expressed in non-olfactory tissues suggesting diverse functions. Eight of them showed similar expression levels in all tissues, while the others were specifically detected in olfactory organs. In particular, four genes (HarmCSP20, 22, 23 and 24) were specific of larval olfactory tissues, one (HarmCSP14) was detected only in adult antenna, and three (HarmCSP7, HarmCSP15 and HarmCSP25) were found in both larval and adult olfactory organs with no significant differences.

![Phylogenetic tree of OBPs from *H. armigera*, *H. assulta* and other Lepidoptera insects](https://doi.org/10.1371/journal.pone.0179243.g002)
| Unigene reference | Gene name | ORF (aa) | Blastx best hit (Reference/Name/Species) | E value | Identity | Signal peptide | Full length |
|-------------------|-----------|----------|------------------------------------------|---------|----------|----------------|-------------|
| **H. armigera**    |           |          |                                          |         |          |                |             |
| Unigene12801      | HarmCS1   | 745      | gb|ABB91378.1|chemosensory protein [Helicoverpa assulta] | 1.00E-67 | 99% Yes Yes |
| Unigene12890      | HarmCS2   | 523      | gb|AGR939574.1|chemosensory protein 4 [Agrotis ipsilon] | 1.00E-73 | 86% Yes Yes |
| Unigene2914       | HarmCS4   | 381      | gb|AFR92093.1|chemosensory protein 9 [Helicoverpa armigera] | 2.00E-65 | 98% No No  |
| Unigene4249       | HarmCS5   | 730      | gb|AGY49267.1|putative chemosensory protein [Sesamia inferens] | 4.00E-65 | 71% Yes Yes |
| Unigene4261       | HarmCS7   | 684      | gb|AGY49261.1|putative chemosensory protein [Sesamia inferens] | 1.00E-47 | 61% Yes Yes |
| CL750.Contig1     | HarmCS8   | 546      | gb|AFR92095.1|chemosensory protein 11 [Helicoverpa armigera] | 9.00E-91 | 99% Yes Yes |
| Unigene8213       | HarmCS9   | 389      | gb|AGH20055.1|chemosensory protein 17, partial [Helicoverpa armigera] | 2.00E-68 | 100% No No  |
| Unigene2863       | HarmCS10  | 1000     | gb|AGY49264.1|putative chemosensory protein [Sesamia inferens] | 2.00E-64 | 92% Yes Yes |
| Unigene6030       | HarmCS12  | 377      | gb|AFR92092.1|chemosensory protein 8 [Helicoverpa armigera] | 1.00E-68 | 100% Yes No  |
| CL1562.Contig1    | HarmCS14  | 2323     | ref|NP_001037069.1|chemosensory protein 9 precursor [Bombyx mori] | 1.00E-81 | 70% Yes Yes |
| Unigene8181       | HarmCS15  | 516      | gb|AGH20053.1|chemosensory protein 15, partial [Helicoverpa armigera] | 1.00E-75 | 99% Yes Yes |
| CL5163.Contig1    | HarmCS16  | 544      | gb|AGR939578.1|chemosensory protein 8 [Agrotis ipsilon] | 1.00E-66 | 76% Yes Yes |
| CL513.Contig1     | HarmCS18  | 864      | gb|AGY49260.1|putative chemosensory protein, partial [Sesamia inferens] | 1.00E-45 | 99% Yes Yes |
| Unigene2882       | HarmCS19  | 1216     | gb|AFR92094.1|chemosensory protein 10 [Helicoverpa armigera] | 3.00E-25 | 42% Yes Yes |
| CL1091.Contig1    | HarmCS20  | 1056     | gb|AGH20054.1|chemosensory protein 16, partial [Helicoverpa armigera] | 8.00E-75 | 93% Yes Yes |
| CL750.Contig3     | HarmCS21  | 567      | gb|AFR92098.1|chemosensory protein 14 [Helicoverpa armigera] | 4.00E-85 | 93% Yes Yes |
| Unigene14000      | HarmCS22  | 545      | gb|BAG71920.1|chemosensory protein 12 [Papilio xuthus] | 2.00E-43 | 58% Yes Yes |
| Unigene7878       | HarmCS23  | 507      | ref|NP_001037066.1|chemosensory protein precursor [Bombyx mori] | 2.00E-49 | 60% Yes Yes |
| CL750.Contig2     | HarmCS24  | 404      | gb|AFR92098.1|chemosensory protein 14 [Helicoverpa armigera] | 3.00E-64 | 91% No No |
| Unigene12750      | HarmCS25  | 577      | gb|AFR92093.1|chemosensory protein 9 [Helicoverpa armigera] | 5.00E-74 | 82% Yes Yes |
| Unigene4081       | HarmCS26  | 458      | gb|AIW65100.1|chemosensory protein [Helicoverpa armigera] | 5E-85 | 99% Yes Yes |
| **H. assulta**    |           |          |                                          |         |          |                |             |
| Unigene15996      | HassCS1   | 746      | gb|ABB91378.1|chemosensory protein [Helicoverpa assulta] | 1.00E-86 | 98% Yes Yes |
| Unigene20555      | HassCS2   | 530      | gb|AGR939574.1|chemosensory protein 4 [Agrotis ipsilon] | 1.00E-73 | 94% Yes Yes |
| CL2298.Contig1    | HassCS4   | 374      | gb|AFR92093.1|chemosensory protein 9 [Helicoverpa armigera] | 6.00E-65 | 99% No No |
| CL332.Contig1     | HassCS5   | 693      | gb|AGY49267.1|putative chemosensory protein [Sesamia inferens] | 9.00E-66 | 88% Yes Yes |

(Continued)
Discussion

In Lepidoptera, the main tasks of adults are reproduction and species dispersal. To accomplish them they use a sophisticated olfactory system for correct mating and oviposition on the suitable host plant [50, 51]. Compared to adults, larvae show limited activity, their major tasks being feeding, growing and accumulating energy [52,53]. Therefore larvae are expected to harbor a simpler olfactory system than adults. One of the characteristics of monophagous insects is the strict specificity to their host, a typical example being the specialization of M. sexta for Solanaceous plants [32]. In this case, the mother choses the host plant while ovipositing and larvae may not need to move away through their life [54, 55]. In contrast, larvae of polyphagous species often ignore their mother’s choices, disperse actively, and often move between different host plants for feeding [56, 57]. For example, sometimes larvae need to abandon their prior host and select another one, because the plant resources are exhausted, or because of competition with other herbivores, or else because the plant has become infected [58–61]. Such differences in foraging behaviors are genetically determined [56].

H. armigera and H. assulta are two closely related species both representing serious pests in China and other countries. H. armigera is a polyphagous insect which attacks about 180 species...
of plants [62], while *H. assulta* is oligophagous, mainly feeding on tobacco [63]. In both species antennae and mouthparts are the main chemosensory structures guiding the larvae to their host plants. Thus, a study of larval antennae and mouthparts at the molecular level can provide useful information for larva-based pest control.

In this work, we focused on two families of soluble protein OBPs and CSPs which play some roles in the interactions between odorant molecules and olfactory receptors. We identified a total of 26 OBPs and 21 CSPs in the larval chemosensory tissues of *H. armigera* as well as 27 OBPs and 20 CSPs in *H. assulta*. Combined with the data available for in adult antennae, the total number of OBP genes identified in *H. armigera* and *H. assulta* are 38 and 35 respectively. These numbers are lower, although in the same order, than those reported for other species (46 in *B. mori*) [45]. The total number of CSP genes identified in *H. armigera* (25) and *H. assulta* (23) are also in the same order of magnitude as in other species such as *B. mori* (21), and *S. littoralis* (23) [39, 45].

For most of HarmOBPs and HarmCSPs we could find homologue genes in *H. assulta*. The high similarities in sequence between pairs of orthologous genes suggest that *H. armigera* and *H. assulta* larvae detect similar volatile substances. This idea is supported by the observation that often mixed populations of the two species are present on tobacco and some solanaceous plants [63]. However, for some genes we could not find orthologs in the sister species. This fact, if confirmed, could suggest that during evolution, the two species can have developed some unique characteristics in their chemosensory systems to become adapted to different ecosystems. For nearly half of the HarmCSPs, we detected expression in non-olfactory organ, such as adult abdomen, suggesting roles different from chemosensing. Similarly, in other species, some CSPs were found to be expressed in non-olfactory tissues, such as the pheromone glands, where they likely assist delivery of semiochemicals in the environment [64–67], or in reproductive organs, with putative roles in egg and embryo development [68, 69].

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**Fig 3.** Sequences alignment of candidate HarmCSPs and HassCSPs. All these CSPs were characteristic of four conserved cysteine residues marked with "$\ast$". 

![Fig 3. Sequences alignment of candidate HarmCSPs and HassCSPs.](https://doi.org/10.1371/journal.pone.0179243.g003)
OBPs and CSPs are expressed both in adults and in larvae chemo-sensory organs, suggesting some common olfactory related behaviors. In particular, the gene encoding GOBP2 is expressed in larval antenna, where it might bind pheromone cues. Such hypothesis was originated from what was observed in *Plutella xylostella* [70]. However, for all PBP genes we could not find their expression in *H. armigera* larval tissues. This case, although being inconsistent with what was observed in *S. littoralis* [53], was common in other species. We also found three OBPs and six CSPs presenting larva-specific expression, suggesting that they may be involved in larval-foraging behaviors. Three OBPs and ten CSPs were found to be expressed more in larval antennae than in mouthparts, whereas the other proteins were only detected in larval mouthparts, suggesting that these genes may be involved in taste.

![Phylogenetic tree of CSPs](https://doi.org/10.1371/journal.pone.0179243.g004)

**Fig 4.** Phylogenetic tree of CSPs *H. armigera, H. assulta and other Lepidoptera insects.* Harm: *H. armigera* (red), Hass: *H. assulta* (blue), Hvirs: *H. virescens* (black), Bm: *B. mori* (aquamarine), Csups: *C. suppressalis* (cyan). The red and blue pentastars represented newly identified HarmCSPs and HassCSPs respectively.

https://doi.org/10.1371/journal.pone.0179243.g004
Our results contribute to a better understanding of the chemoreception mechanisms of larvae at the molecular level and might help the development of larva-targeted strategies for population control in these two important agricultural pests.

Supporting information

S1 Material. Accession numbers for amino acid sequences of OBPs and CSPs used in phylogenetic analyses.

(SDOCX)

S2 Material. Primers for RT-PCR expression analyses of *H. armigera* OBPs and CSPs.

(SDOCX)
S3 Material. Species distribution of unigenes’ best-hit annotation term in nr database.
(A) H. armigera unigenes. (B) H. assulta unigenes.

(TIF)

Acknowledgments
We thank Dr. Junfeng Dong (Henan University of Science and Technology) for providing the H. assulta and Miss Liyan Yang for insect rearing. This work was supported by National Natural Science Foundation of China (31372264 to SD, 31230062 to GW, 31321004 to GW&YL, and 31471833 to YL).

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