Antennal transcriptome analysis of the piercing moth *Oraesia emarginata* (Lepidoptera: Noctuidae)

Bo Feng¹, Qianshuang Guo¹, Kaidi Zheng¹, Yuanxia Qin², Yongjun Du¹*  

¹ Institute of Health and Environmental Ecology, Wenzhou Medical University, University Town, Wenzhou, Zhejiang, China, ² Department of Research and Development, Newcon Inc., Ningbo, Zhejiang, China  

* Current address: Institute of Pesticide and Environmental Toxicology, Zhejiang University, Hangzhou, Zhejiang, China  

* yongjundu@zju.edu.cn

Abstract

The piercing fruit moth *Oraesia emarginata* is an economically significant pest; however, our understanding of its olfactory mechanisms in infestation is limited. The present study conducted antennal transcriptome analysis of olfactory genes using real-time quantitative reverse transcription PCR analysis (RT-qPCR). We identified a total of 104 candidate chemosensory genes from several gene families, including 35 olfactory receptors (ORs), 41 odorant-binding proteins, 20 chemosensory proteins, 6 ionotropic receptors, and 2 sensory neuron membrane proteins. Seven candidate pheromone receptors (PRs) and 3 candidate pheromone-binding proteins (PBPs) for sex pheromone recognition were found. *OemaOR29* and *OemaPBP1* had the highest fragments per kb per million fragments (FPKM) values in all ORs and OBPs, respectively. Eighteen olfactory genes were upregulated in females, including 5 candidate PRs, and 20 olfactory genes were upregulated in males, including 2 candidate PRs (*OemaOR29* and 4) and 2 PBPs (*OemaPBP1* and 3). These genes may have roles in mediating sex-specific behaviors. Most candidate olfactory genes of sex pheromone recognition (except *OemaOR29* and *OemaPBP3*) in *O. emarginata* were not clustered with those of studied noctuid species (type I pheromone). In addition, *OemaOR29* was belonged to cluster PRIII, which comprise proteins that recognize type II pheromones instead of type I pheromones. The structure and function of olfactory genes that encode sex pheromones in *O. emarginata* might thus differ from those of other studied noctuids. The findings of the present study may help explain the molecular mechanism underlying olfaction and the evolution of olfactory genes encoding sex pheromones in *O. emarginata*.

Introduction

Olfaction plays a key role in foraging [1–3], mating [4,5], and oviposition behaviors [6–8] of insects. Insect olfaction studies have provided fundamental insights into chemosensory
biology and chemical ecology and have provided valuable opportunities for pest management [9–14]. Lepidopterans are often used for olfaction studies, as these have extensive and sensitive olfactory repertoires. However, molecular studies on olfaction in Lepidopterans lag behind those of other insect models such as fruit fly and mosquitoes [15].

Lepidoptera sex pheromones are divided into two main types based on their chemistry [16]. Type I pheromone components have 10- to 18-carbon, even numbered straight chain acetates, aldehydes, and alcohols. Type II pheromones consist of polyunsaturated C_{17}-C_{23} straight chains, skipped conjugated polyenic hydrocarbons and the corresponding epoxide derivatives [17]. Type I pheromones occur in about 75% of all studied moth species, whereas type II pheromones occur in about 15% of identified Lepidopteran pheromones [17]. These two major types of sex pheromones are produced through distinct pathways that involve different biosynthetic sites, substrates, and enzymes, as well as respectively employ specific endocrine regulatory mechanisms. However, both types of pheromones have the same function in mate recognition and attraction in moths [16,18].

Genes encoding Lepidopteran olfactory proteins have been identified in *Bombyx mori* [19], and also in the pest species *Manduca sexta* [20], *Heliothis virescens* [21], *Spodoptera litura* [22], *S. littoralis* [23,24], *Agrotis ipsilon* [25], and *Dendrolimus spp*. [26]. Sex pheromones of above species are type I. However, studies on the olfactory genes that encode type II pheromones are limited.

The piercing fruit moth *Oraesia emarginata* Fabricius (Lepidoptera: Noctuidae) is an important pest of fruits such as citrus, pear, peach, and plum. The larvae feed on plants belonging to the Menispermaceae. Adult moths obtain nutrition from ripe fruits. Mated females lay eggs on Menispermaceae plants (Fig 1) [27]. The electroantennographic and behavioral responses of *O. emarginata* to volatiles from ripe fruits [28] and the repellency of a volatile compound, sec-butyl β-styryl ketone have been studied [29]. However, little is known about the olfactory mechanism of *O. emarginata*. Type II pheromones were identified as female sex pheromones in *Oraesia* species. The major and minor sex pheromone components of the related *O. excavate* were identified as cis-9,10-epoxy-(Z)-6 –heneicosene and cis-9,10-epoxy-(Z,Z)-3,6- heneicosadiene [30]. Although the sex pheromone of female *O. emarginata* was not published, it was similar to epoxide components from a preliminary identification (Du et al., unpublished data). In the present study, we achieved significant coverage of olfactory genes with *de novo* transcriptome and measured gene expression using real-time quantitative reverse transcription PCR analysis (RT-qPCR) for comparison between the sexes. We also discuss the diversification of olfactory genes for the recognition of type I and type II pheromones.

### Materials and methods

**Insects**

*O. emarginata* larvae were collected from fields in Gannan City of Jiangxi Province, China and reared in the laboratory at 25 ± 1˚C and 75 ± 5% relative humidity with a 14-h light/10-h dark photoperiod. Our field collection activities did not impact endangered or protected species. Larvae were fed fresh leaves of *Cocculus orbiculatus* until pupation. Emergence of males and females was checked every morning, and adults were separately maintained in ventilated wooden cages (35 cm × 35 cm × 50 cm). Emerging adult moths were fed with 10% glucose water soaked into cotton.

**Extraction of total RNA from tissues**

Antennae of 4-d-old adults were used. A total of 25 adults (males and females separately) were collected after 3.5 h of the dark cycle. Antennae samples from each group were immediately
homogenized in TRNzol-A+ (TIANGEN Biotech, Beijing, China) on ice, and total RNA was extracted according to the manufacturer’s instructions. The concentration and purity of the total RNA were determined by using a NanoDrop2000 spectrophotometer (ThermoFisher, Waltham, MA, USA). RNA with an A260/A280 ratio between 1.75–2.05, an A260/A230 ratio > 1, and a concentration > 400 ng/μL was used for the experiments. Total RNA was treated with DNase I (Takara, Kusatsu, Shiga, Japan) to remove any genomic DNA. RNA extractions were performed in triplicate.

De novo transcriptome analysis
The same amount of RNA collected from male and female antennae was pooled for transcriptome analysis. The cDNA library for transcriptome analysis was prepared using a TruSeq SBS Kit v3-HS (Illumina, San Diego, CA, USA), following the manufacturer’s recommendations. The library was sequenced using Illumina HiSeq™ 2000 (Illumina, San Diego, CA, USA) with a
90-bp read length for the paired-end reads by BGI (Shenzhen, Guangdong, China). Dirty reads containing adapters and unknown or low-quality bases were discarded from the raw reads to obtain clean reads for analysis. *De novo* transcriptome assembly was conducted with the short reads assembly program, Trinity (r20140413p1, min_kmer_cov:2) [31]. BLASTx (v2.2.28+) alignment (E value < 0.00001) between unigenes and protein databases (NCBI non-redundant protein database, Swiss-Prot, Kyoto Encyclopedia of Genes and Genome (KEGG), and Clusters of Orthologous Groups (COG)) was successively performed. Gene ontology (GO) annotations of the unigenes were determined using Blast2go (http://www.blast2go.org/) [32].

**Olfactory gene analysis**

The candidate olfactory gene was manually obtained from gene annotation. In addition, a 50% ORF length cutoff was used in identifying putative genes to prevent a gene from being counted twice. The candidate OBPs and CSPs were searched for the presence of N-terminal signal peptides using SignalP4.0 (http://www.cbs.dtu.dk/services/SignalP/) using default parameters [33]. The signal peptides likely contained significant phylogenetic information and were included in the phylogenetic analyses of OBPs and CSPs [34]. Amino acid sequence alignment was performed using CLUSTALX2.1 using default parameters [35]. For phylogenetic analysis, known amino acid sequences of olfactory genes from other insects were downloaded (S1 File). Phylogenetic analyses were conducted using the maximum likelihood method of MEGA 6.0, which was based on the Jones-Taylor-Thornton (JTT) substitution model, partial deletion cutoff, a nearest neighbor interchanges (NNI) heuristic search, and other default parameters [36]. Node support for the phylogenetic tree was assessed using the bootstrap method with 1,000 bootstrap replicates.

**Profiling analysis of gene expression based on the antennal transcriptome**

Gene expression levels were calculated using the fragments per kb per million fragments (FPKM) method based on the results of antennal transcriptome analysis. The number of fragments that uniquely aligned to a gene was divided by the total number of fragments that uniquely aligned to all genes and by the base number in the CDS of that gene [37]. The FPKM method can eliminate the influence of different gene lengths and sequencing levels on the calculation of gene expression.

**RT-qPCR analysis of olfactory gene expression in the antennae**

Single-stranded cDNAs were synthesized from 1 μg of total RNA using the ReverTra Ace qPCR RT Kit (Toyobo, Kita-ku, Osaka, Japan) following the manufacturer’s recommendations. RT-qPCR was performed with SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), following the manufacturer’s protocols, in a CFX-96™ PCR Detection System (Bio-Rad). The cycling conditions were an initial cycle at 95°C for 30 s, followed by 39 cycles of 95°C for 5 s and 60°C for 5 s. Dissociation curves with 0.3°C/s melt rates were used to check for the presence of non-specific dsDNA SYBR Green hybrids. Only primers with a single PCR amplification product were used in the subsequent analyses. The amplification efficiency of each primer was calculated from the slope of the standard curve [38]. The PCR primers used are listed in S1 Table. Ubiquinol-cytochrome c reductase (*UCCR*) and arginine kinase (*AK*) were used as reference genes. The difference in gene expression was measured by using the $2^{-\Delta\Delta C_{t}}$ algorithm [39]. Differential gene expression between females and males was measured, with the female antennae used as reference. Expression levels of target genes were normalized independent of
each-reference-gene-with-the-algorithm-and-then-averaged. When-the-gene-expression-of-the-female-antennae-was-very-low-the-gene-expression-of-the-male-antennae-was-used-as-control. RNA-extraction-was-repeated-three-times-for-each-sample-and-two-or-more-RT-qPCR-replicates-were-prepared-for-each-sample.

Data analysis

Data analysis was conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The significance of the difference between means was determined using the student’s \( t \)-test. The critical \( P \) value for each test was set at 0.05.

Results

De novo antennal transcriptome assembly

Using the Illumina HiSeq™ 2000 sequencing system, 117,410,034 raw reads were obtained from the antennal samples. After removing low-quality (< Q20) adaptor and contaminating sequence reads, 103,301,292 (a total of 9,297,116,280 bp) clean reads were generated from antennae, and 42,992 unigenes were assembled (N50 = 1,098), with a mean length of 713 bp. More than 58% (24,954) of the unigenes were aligned to sequences in various protein databases. GO annotation was performed to obtain information on their molecular function, biological process, and cellular location (S1 Fig). The raw sequence of the transcriptome has been deposited to the National Center for Biotechnology Information (NCBI) (GenBank Accession Number PRJNA358570; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA358570).

Analysis of olfactory genes

The 35 candidate OR genes encoding an olfactory receptor co-receptor (OemaORco), OemaOR18, 7 candidate pheromone receptors (PRs, OemaOR3, 4, 21, 26, 28, 29, and 30) and 26 general OR genes were identified from O. emarginata antennae (Table 1, Fig 2). Candidate PRs of O. emarginata were clustered together with previously reported PRs in the phylogenetic tree. Eight general ORs (OemaOR11, 14, 17, 19, 20, 25, 27, and 32) were clustered with OfurOR34, MsexOR42, and AdisOR9 into a specific group, with a bootstrap support value of 87 (Fig 2). Two general OR genes (OemaOR24 and 35) were not clustered with any reported ORs from Lepidopteran species with sufficient bootstrap values (bootstrap values < 50). Full open reading frame (ORF) of 8 OR genes (OemaOR5, 9, 19, 22, 26, 29, 35 and ORco) were obtained, with the mean length of 435 aa.

The 41 candidate odorant-binding protein (OBP) genes were identified from O. emarginata antennae. and these encoded 34 OBPs, 2 general odorant-binding proteins (GOBPs), 3 pheromone-binding proteins (PBPs), an antennal-binding protein (OemaABPX), and OemaOBP25 (DmelOBP73a analogue) (Table 2, Fig 3). All OemaOBPs were clustered with those of Lepidopteran species with sufficient bootstrap values (bootstrap values > 60). Seven OemaOBP genes (OemaOBP4, 11, 13, 18, 23, 27, and 35) were clustered with AipsOBP4, SlitABP1, SlitOBP12, SexiABP1, HvirABP2, HarmOBP7, and HarmOBP7.2 with a bootstrap support value of 61, and the latter 7 OBPs were clustered into a subgroup with a bootstrap support value of 99 (Fig 3). The mean length of the OBPs was 166 aa, and the full ORF of the 37 OBP genes were obtained. Thirty-three OBPs were a classic group with six conserved cysteines, 3 OBPs (OemaOBP9, 28, and 30) were of the minus-C group with C2 and C5 missing, and 5 OBPs (OemaOBP3, 12, 20, 29 and 33) were of the plus-C OBP group with more than six conserved cysteines (Fig 4).

A total of 20 candidate chemosensory protein (CSP) genes were identified in O. emarginata, with a mean length of 128 aa. The full ORF of the 16 CSP genes were obtained (Table 3, Fig 5).
Table 1. BLASTp results of candidate olfactory receptors of *O. emarginata*.

| Gene name | Full ORF | Group | FPKM | Gene length (aa) | Reference gene ID | Reference gene name | E_value | Similarity (%) |
|-----------|---------|-------|------|----------------|------------------|---------------------|---------|----------------|
| *OemaOR1* | No | General | 6.1 | 271 | All01102.1 | Odorant receptor [*Dendrolimus kikuchi*] | 4.54E-129 | 70.1 |
| *OemaOR3* | No | Pheromone | 10.0 | 269 | AGS41448.1 | Olfactory receptor 9 [*A. segetum*] | 2.25E-32 | 24.9 |
| *OemaOR4* | No | Pheromone | 7.0 | 299 | AGY14585.2 | Putative odorant receptor [*Sesamia inferens*] | 2.98E-81 | 45.5 |
| *OemaOR5* | Yes | General | 6.6 | 402 | AGG08877.1 | Putative olfactory receptor 44 [*S. litura*] | 0 | 83.8 |
| *OemaOR6* | Yes | General | 6.7 | 392 | BAR43469.1 | Putative olfactory receptor 27 [*Ostrinia fumacalis*] | 0 | 78.1 |
| *OemaOR7* | No | General | 9.6 | 329 | CAD31950.1 | Putative chemosensory receptor 9 [*H. virescens*] | 4.02E-95 | 47.4 |
| *OemaOR8* | No | General | 3.6 | 207 | AIG51892.1 | Odorant receptor [*Helicoverpa armigera*] | 3.38E-121 | 82.6 |
| *OemaOR9* | Yes | General | 13.9 | 437 | AIG51891.1 | Odorant receptor, partial [*H. armigera*] | 0 | 65.9 |
| *OemaOR10* | No | General | 4.1 | 249 | AIG51890.1 | Odorant receptor [*H. armigera*] | 6.71E-117 | 63.5 |
| *OemaOR11* | No | General | 7.5 | 194 | AJD81541.1 | Olfactory receptor 1, partial [*H. assulta*] | 4.75E-77 | 56.7 |
| *OemaOR12* | No | General | 13.5 | 277 | All01072.1 | Odorant receptor [*D. houli*] | 4.55E-130 | 65.0 |
| *OemaOR13* | No | General | 9.9 | 358 | AGK90004.1 | Olfactory receptor 12 [*H. armigera*] | 1.70E-137 | 53.2 |
| *OemaOR14* | No | General | 13.2 | 274 | AGG08878.1 | Putative olfactory receptor 12 [*S. littoralis*] | 3.28E-115 | 62.8 |
| *OemaOR15* | No | General | 1.7 | 289 | AIG51902.1 | Odorant receptor, partial [*H. armigera*] | 2.38E-108 | 54.7 |
| *OemaOR16* | No | General | 9.2 | 251 | AIG51898.1 | Odorant receptor [*H. armigera*] | 1.19E-75 | 49.8 |
| *OemaOR17* | No | General | 9.0 | 369 | ABQ84982.1 | Chemosensory receptor 12 [*S. littoralis*] | 3.46E-129 | 50.1 |
| *OemaOR18* | No | General | 10.6 | 353 | ACL81186.1 | Partial olfactory receptor 18 [*H. zea*] | 1.17E-175 | 69.4 |
| *OemaOR19* | Yes | General | 3.5 | 463 | AGG08878.1 | Putative olfactory receptor 12 [*S. littoralis*] | 3.47E-148 | 45.4 |
| *OemaOR20* | No | General | 5.6 | 248 | ABQ84982.1 | Chemosensory receptor 12 [*S. littoralis*] | 1.23E-72 | 47.6 |
| *OemaOR21* | No | Pheromone | 4.5 | 266 | AGI96751.1 | Olfactory receptor 16 [*S. littoralis*] | 9.95E-80 | 46.2 |
| *OemaOR22* | Yes | General | 10.9 | 424 | AFL70813.1 | Odorant receptor 50, partial [*M. sexta*] | 1.05E-123 | 44.6 |
| *OemaOR23* | No | General | 5.9 | 237 | All01083.1 | Odorant receptor [*D. kikuchi*] | 7.66E-99 | 59.9 |
| *OemaOR24* | No | General | 6.7 | 308 | AIG51858.1 | Odorant receptor, partial [*H. armigera*] | 3.39E-90 | 43.5 |
| *OemaOR25* | No | General | 17.1 | 339 | ABQ84982.1 | Chemosensory receptor 12 [*S. littoralis*] | 1.49E-131 | 62.6 |
| *OemaOR26* | Yes | Pheromone | 8.4 | 447 | AGK90019.1 | Olfactory receptor 14b [*H. assulta*] | 2.51E-131 | 46.3 |
| *OemaOR27* | No | General | 19.1 | 392 | AGG08878.1 | Putative olfactory receptor 12 [*S. littoralis*] | 5.13E-142 | 50.8 |
| *OemaOR28* | No | Pheromone | 6.5 | 276 | ACL81180.1 | Putative olfactory receptor 11 [*S. littoralis*] | 5.16E-54 | 37.3 |
| *OemaOR29* | Yes | Pheromone | 39.1 | 467 | AGH58120.1 | Odorant receptor 11 [*S. exigua*] | 1.04E-180 | 53.5 |
| *OemaOR30* | No | General | 6.7 | 259 | AIG51856.1 | Odorant receptor [*H. armigera*] | 7.40E-49 | 32.8 |
| *OemaOR31* | No | General | 4.5 | 197 | AIG51896.1 | Odorant receptor, partial [*H. armigera*] | 3.70E-39 | 36.5 |
| *OemaOR32* | No | General | 15.1 | 390 | AGG08878.1 | Putative olfactory receptor 12 [*S. littoralis*] | 1.72E-129 | 47.4 |

(Continued)
In the phylogenetic tree, OemaCSP9 and OemaCSP16 were clustered the homologous genes of other insect species into two conserved groups (Fig 5). The bootstrap values of 5 CSPs (OemaCSP1, 2, 7, 8, and 10) were < smaller than 50%, although these were clustered with studied CSPs of the Lepidopteran species. OemaCSP16 differed from the other CSPs in terms of the number of amino acids (Fig 6).

Six candidate ionotropic receptor (IR) genes and 2 sensory neuron membrane protein (SNMP) genes were identified in O. emarginata, and their mean lengths were 535 aa and 522 aa, respectively (Tables 4 and 5). All O. emarginata IRs and SNMPs were clustered with Lepidopteran IRs and SNMPs, respectively, with the bootstrap values > 80% (Figs 7 and 8). The full ORF of 2 SNMP genes was obtained.

**Expression of olfactory genes with RNA sequences**

The FPKM values of the chemosensory receptors were < 60, and OemaORco showed the highest FPKM value (Tables 1 and 4). The FPKM value of OemaOR29 was higher, but those of the other candidate PRs were lower than the general ORs, including OemaOR14, 25, 27, and 32 (Table 1). The FPKM values of OemaIR75p and OemaIR21a were larger than those of the co-receptors OemaIR25a and OemaIR8a (Table 4). In contrast to chemosensory receptors, 39.0% of the OBP and 52.4% of the CSP genes showed FPKM values > 300, including 3 candidate PBPs (Tables 2 and 3). OemaPBP1 showed the highest FPKM value among all OBPs, and OemaCSP19 had the highest FPKM value among all chemosensory genes. The FPKM value of OemaSNMP1 was < 20, but that of OemaSNMP2 was > 500 (Table 5).

**Expression of all olfactory genes between male and female antennae**

Five candidate PRs (OemaOR3, 21, 26, 28, and 30), OemaOR13, OemaOR16, OemaOR30, OemaORco, 2 GOBPs, 7 OBPs (OemaOBP4, 9–11, 26, 27, and 29), and OemaSNMP1 were expressed at significantly higher levels in females, and OemaOR26, OemaOR28, OemaOR13, and OemaOBP10 were specifically expressed in females (Fig 9). Two candidate PRs (OemaOR29 and 4), OemaOR18, 4 general ORs (OemaOR8, 15, 20, and 25), 2 PBPs (OemaPBP1 and 3), 3 OBPs (OemaOBP6, 13, and 21), 6 CSPs (OemaCSP1, 5, 6, 9, 10, and 19), OemaIR21a, and OemaSNMP2 were expressed at significantly higher levels in males compared to that in females, and OemaOR29, OemaOR4, OemaOR18, OemaOR15, OemaPBP1, and OemaPBP3 were specifically expressed in males (Fig 9).

**Phylogeny of pheromone recognition genes of types I and II pheromones**

In the phylogenetic tree, 4 orthologous PRs clusters for type I pheromones were obtained (Cluster PRI–PRIV), and candidate PRs of the noctuid species (excluding O. emarginata) formed subclusters of these 4 clusters, with high bootstrap support (≥ 89, Fig 10). OemaOR29
Fig 2. Phylogenetic analysis of putative OR gene sequences of *O. emarginata* (black circle). The tree was rooted with Orco lineage (pink color). Bootstrap values < 50% are not shown. Color legend: Orange = PR group, yellow = OR18 group, green = OemaORs group, and blue = other general OR groups. Adis, *Athetis dissimilis*, Aips, *A. ipsilon*, Bmor, *B. mori*, Hvir, *H. virescens*, Msex, *M. sexta*, Oema, *O. emarginata*, Ofur, *O. furmacalis*, Slitu, *S. litura*.

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## Table 2. BLASTp results of candidate odorant-binding proteins of *O. emarginata.*

| Gene name | Full ORF | Group | FPKM | ORF length (aa) | Reference gene ID | Reference gene name | E_value | Similarity (%) |
|-----------|----------|-------|------|----------------|------------------|---------------------|---------|----------------|
| OemaOBP1  | Yes      | Classic | 2833 | 148            | AEB54581         | OBP5 [H. armigera]  | 1.78E-58 | 64.2           |
| OemaOBP2  | Yes      | Classic | 24   | 210            | EHJ64212         | Odorant-binding protein 2 [Danaus plexippus] | 3.99E-80 | 72.9           |
| OemaOBP3  | Yes      | Plus   | 33   | 155            | AGK24580         | Odorant-binding protein 4 [Chilo suppressalis] | 2.82E-65 | 60.6           |
| OemaOBP4  | Yes      | Classic | 7    | 161            | AEB54591         | OBP7 [H. armigera]  | 3.09E-17 | 33.5           |
| OemaOBP5  | Yes      | Classic | 1436 | 178            | AGS36751         | OBP10, partial [S. inferens] | 2.31E-57 | 49.4           |
| OemaOBP6  | Yes      | Classic | 196  | 142            | AGC92789         | Odorant-binding protein 9 [H. assulta] | 1.45E-19 | 28.9           |
| OemaOBP7  | Yes      | Classic | 16   | 145            | ADY17886         | Odorant binding protein [S. exigua] | 2.98E-69 | 67.6           |
| OemaOBP8  | Yes      | Classic | 11   | 147            | AFM77984         | Odorant binding protein 6 [S. exigua] | 8.21E-53 | 61.9           |
| OemaOBP9  | Yes      | Minus  | 113  | 146            | AAL60425         | Antennal binding protein 7 [M. sexta] | 3.45E-44 | 56.8           |
| OemaOBP10 | Yes      | Classic | 1796 | 153            | AGP03457         | SexiOBP11 [S. exigua] | 7.60E-79 | 71.9           |
| OemaOBP11 | Yes      | Classic | 28   | 139            | AEB54591         | OBP7 [H. armigera]  | 7.76E-22 | 38.8           |
| OemaOBP12 | Yes      | Plus   | 60   | 200            | AGC92793         | Odorant-binding protein 19 [H. assulta] | 1.04E-30 | 36.0           |
| OemaOBP13 | Yes      | Classic | 917  | 149            | CAC33574         | Antennal binding protein [H. virescens] | 1.33E-29 | 37.3           |
| OemaOBP14 | Yes      | Classic | 312  | 147            | AEB54586         | OBP2 [H. armigera]  | 6.72E-72 | 69.4           |
| OemaOBP15 | Yes      | Classic | 119  | 146            | Alli00997        | Odorant binding protein [D. kikuchi] | 2.51E-66 | 62.3           |
| OemaOBP16 | Yes      | Classic | 1497 | 155            | AGP03456         | SexiOBP10 [S. exigua] | 1.35E-64 | 68.6           |
| OemaOBP17 | Yes      | Classic | 1796 | 153            | AFG73000         | Odorant-binding protein 2 [Cnaphalocrocis medinalis] | 4.76E-78 | 76.5           |
| OemaOBP18 | Yes      | Classic | 11   | 149            | CAC33574         | Antennal binding protein [H. virescens] | 5.11E-31 | 40.3           |
| OemaOBP19 | Yes      | Classic | 15   | 334            | XP_011559551     | General odorant-binding protein 71-like [Plutella xylostella] | 2.06E-80 | 73.7           |
| OemaOBP20 | Yes      | Plus   | 37   | 189            | AGR39564         | Odorant binding protein 1, partial [A. ipsilon] | 2.49E-55 | 46.6           |
| OemaOBP21 | Yes      | Classic | 9327 | 153            | AGH70104         | Odorant binding protein 8 [S. exigua] | 1.32E-77 | 83.3           |
| OemaOBP22 | Yes      | Classic | 161  | 146            | AAL60415         | Antennal binding protein 4 [M. sexta] | 1.50E-72 | 78.1           |
| OemaOBP23 | Yes      | Classic | 11   | 158            | CAC33574         | Antennal binding protein [H. virescens] | 1.94E-14 | 36.1           |
| OemaOBP24 | Yes      | Classic | 81   | 248            | Alli00994        | Odorant binding protein [D. kikuchi] | 7.81E-88 | 59.0           |
| OemaOBP25 | Yes      | Classic | 3    | 184            | Alli00978        | Odorant binding protein [D. hou] | 2.22E-124 | 96.7       |
| OemaOBP26 | No       | Classic | 4    | 208            | NP_001140186     | Odorant-binding protein 2 precursor [B. mori] | 1.04E-101 | 67.8           |
| OemaOBP27 | Yes      | Classic | 9    | 146            | AEX07271         | Odorant-binding protein [H. assulta] | 2.25E-11 | 35.9           |
| OemaOBP28 | Yes      | Minus  | 551  | 133            | AGH70105         | Odorant binding protein 9 [S. exigua] | 8.22E-83 | 91.7           |
| OemaOBP29 | Yes      | Plus   | 19   | 157            | AGK24578         | Odorant-binding protein 2 [C. suppressalis] | 1.75E-16 | 74.4           |
| OemaOBP30 | Yes      | Minus  | 4    | 141            | AGK24581         | Odorant-binding protein 5 [C. suppressalis] | 2.49E-24 | 38.3           |
| OemaOBP31 | No       | Classic | 96   | 130            | AGC92798         | Odorant-binding protein 9 [H. assulta] | 4.65E-09 | 26.2           |
| OemaOBP32 | No       | Classic | 4    | 127            | Alli00989        | Odorant binding protein [D. hou] | 6.62E-38 | 46.5           |
| OemaOBP33 | Yes      | Classic | 323  | 172            | NP_001159621     | Odorant binding protein LOC100307012 [B. mori] | 4.88E-07 | 38.8           |
| OemaOBP34 | Yes      | Classic | 4    | 182            | EHU4351          | Odorant-binding protein 2 [D. plexippus] | 2.06E-102 | 79.7           |
| OemaOBP35 | No       | Classic | 5    | 123            | AEX07270         | Odorant-binding protein [H. assulta] | 9.52E-16 | 34.1           |
| OemaABPX | Yes      | Classic | 890  | 136            | AGS36754         | OBPABPX, partial [S. inferens] | 2.62E-62 | 69.1           |
| OemaOBP1 | Yes      | Classic | 1796 | 164            | AAW50670         | General odorant binding protein 1 [H. assulta] | 1.16E-89 | 75.0           |
| OemaOBP2 | Yes      | Classic | 1796 | 161            | AII72932         | General odorant-binding protein 2 [S. litura] | 4.06E-99 | 87.6           |
| OemaOBP1 | Yes      | Classic | 10342| 166            | AAC36315         | Pheromone binding protein [H. zeal] | 6.90E-76 | 66.0           |
| OemaOBP2 | Yes      | Classic | 1796 | 168            | AAF16710         | Pheromone binding protein 2 [M. sexta] | 5.17E-79 | 63.1           |
| OemaOBP3 | Yes      | Classic | 2245 | 163            | AFM36758         | Pheromone-binding protein 3 [A. ipsilon] | 3.97E-78 | 66.3           |

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Fig 3. Phylogenetic analysis of putative OBP gene sequences of O. emarginata (black circle), other moth species (black lines), and Diptera species (green lines). The tree was rooted with the Lepidopteran GOBP-PBP group (green color). Bootstrap values < 50% are not shown. Color legend: Orange = conserved OBP groups, pink = expanded OemaOBPs group, green = Lepidoptera GOBP-PBP group, and blue = other general OBP groups.

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Fig 4. Aligned putative full ORF of OBP gene sequences of *O. emarginata*. Six conserved cysteines are highlighted in blue.

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and ObruOR1 (the only identified pheromone receptor for type II sex pheromones from the geometrid O. brumata) belonged to cluster PRIII (Fig 10). Other candidate PRs of O. emarginata were not grouped with any of these 4 clusters, but 5 (OemaOR3, 4, 21, 26, and 28) were clustered, with a bootstrap support of 78 (Fig 10).

The PBPs and GOBPs of all test species were clustered into 3 (Cluster PBPI-PBPIII) and 2 (Cluster GOBPI-II) apparent clusters, with good bootstrap support (≥ 52), respectively (Fig 11). OemaPBP3 and OemaGOBP1 were clustered with orthologous PBPs and GOBP1s of the other noctuids for type I pheromones, respectively (bootstrap support 56) (Fig 11). However, OemaPBP1, OemaPBP2, and OemaGOBP2 were not clustered within PBPs and GOBP2s from other noctuid species for type I pheromones. OemaPBP2 was clustered with MsexPBP2, with a bootstrap value of 74 (Fig 11).

**Discussion**

The unique life history of *O. emarginata* might have driven the increase in the number of chemosensory genes

*O. emarginata* has a unique life history. The larvae feed on Menispermaceae plants, but adults suck on the juices of ripe fruits. Mating behavior is mediated by female sex pheromones. Mated females oviposit on Menispermaceae plants. Odorant classes from different species might thus be different [52]. Moths of *O. emarginata* must recognize a range of different odors with diverse chemical structures emitted from conspecifics, fruits, or orchard background and larval host plants. The olfactory acuity and discriminatory power in *O. emarginata* may have evolved to fulfill its ecological needs. We found 104 candidate olfactory genes in the antennae of *O. emarginata*, including 35 ORs, 41 OBPs, 20 CSPs, 6 IRs, and 2 SNMPs. In these 104
olfactory genes, 2 ORs (OemaOR24 and 35) and 5 CSPs (OemaCSP1, 2, 7, 8, and 10) were not effectively clustered with those of other Lepidopterans (bootstrap values < 50) in the phylogenetic analysis. In addition, 8 OemaORs (OemaOR11, 14, 17, 19, 20, 25, 27, and 32) were clustered into the clade of OfurOR34, MsexOR42, and AdisOR9 (bootstrap value = 87) (Fig 2), and 7 OemaOBPs (OemaOBP4, 11, 13, 18, 23, 27, and 35) were clustered with AipsOBP4, SlitABP1,
Fig 6. Aligned putative full ORF of CSP gene sequences of *O. emarginata*. Four conserved cysteines are highlighted in blue.

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SlitOBP12, SexiABP1, HvirABP2, HarmOBP7, and HarmOBP7.2 (bootstrap value = 61) in the phylogenetic trees (Fig 3). Some of those genes might be species-specific to *O. emarginata* and used to recognize the odors produced by the Menispermaceae and fruits.

The number of chemosensory binding proteins (including OBPs and CSPs) was slightly smaller than in *B. mori*, which included the whole genome, but larger than in other moth species studied using the same protocol (antennal transcriptome). These other species included polyphagous insects such as *S. litura* (Table 6). The larger number of chemosensory binding proteins might be due to the life history of *O. emarginata* and the larger database in our study.

We found a total of 103,301,292 reads that were assembled into 2,202,660 contigs, and compared to 55,288,304 reads assembled into 105,971 contigs in *S. litura* [51]. However, the number of chemosensory receptors was lower than in most other moths (Table 6). The low expression level of chemosensory receptor genes (FPKM < 60) and short read length (250 bp) of the transcriptome analysis might have resulted in short sequences for many chemosensory receptor genes. However, the long sequence of gustatory receptor genes (about 400 aa and 800 aa for OR and IR, respectively) [53,54] and the criterion of 50% ORF length cutoff might have excluded some gustatory receptors with short sequences. No gustatory receptor gene was identified in the antennae, which suggests that the antennae of *O. emarginata* are not major taste organs. The proboscis, which harbors considerably fewer sensilla than antennae, are believed to specialize in taste reception in some moths [37,55]. In addition, the long sequence of gustatory receptor genes (about 400 aa) and the criterion of 50% ORF length cutoff might have excluded some gustatory receptors with short sequences.

**Olfactory genes with sex-specific expression**

We identified 2 candidate PRs (*OemaOR29* and 4) and 2 candidate PBPs (*OemaPBP1* and 3) that showed male-biased expression and might be involved with female sex pheromone recognition in *O. emarginata*. Our results were consistent with the study on the sex pheromone recognition in a sibling species *O. excavata*, which produces two sex pheromone compounds at the ratio of 86:14 [30]. *OemaOR29* was clustered with *ObruOR1* and AsegOR3 in the phylogenetic tree, which recognized the pheromonal tetraene of *O. brumata*, 3Z,6Z,9Z-19:H and the

**Table 4. BLASTp results of candidate ionotropic receptors of *O. emarginata***

| Gene name     | Full ORF | FPKM | ORF length (aa) | Reference Gene ID | Reference gene name                                      | E_value | Similarity (%) |
|---------------|----------|------|-----------------|-------------------|----------------------------------------------------------|---------|----------------|
| OemaIR21a     | No       | 15.8 | 514             | ADR64678.1        | Chemosensory ionotropic receptor IR21a [S. littoralis]    | 5.06E-180 | 51.9           |
| OemaIR25a     | No       | 9.5  | 910             | AJD81628.1        | Ionotropic receptor 25a, partial [H. assulta]             | 0       | 95.7           |
| OemaIR75p     | No       | 17.5 | 534             | ADR64684.1        | Chemosensory ionotropic receptor IR75p [S. littoralis]    | 6.11E-145 | 40.6           |
| OemaIR76b     | No       | 6.2  | 557             | AGY49253.1        | Putative ionotropic receptor [S. inferens]               | 0       | 73.8           |
| OemaIR87a     | No       | 4.6  | 277             | ADR64689.1        | Chemosensory ionotropic receptor IR87a [S. littoralis]    | 3.03E-125 | 69.0           |
| OemaIR8a      | No       | 14.8 | 575             | AFC91764.1        | Putative ionotropic receptor IR8a, partial [Cydia pomonella] | 0       | 87.5           |

**Table 5. BLASTp results of candidate SNMP genes of *O. emarginata***

| Gene name     | Full ORF | FPKM | ORF length (aa) | Reference gene ID | Reference gene name                                      | E_value | Similarity (%) |
|---------------|----------|------|-----------------|-------------------|----------------------------------------------------------|---------|----------------|
| OemaSNMP1     | Yes      | 19   | 525             | AF462067.1        | Sensory neuron membrane protein [H. armigera]            | 0       | 79.0           |
| OemaSNMP2     | Yes      | 505  | 518             | AGN48099          | Sensory neuron membrane protein 2 [S. littoralis]        | 0       | 73.0           |
Fig 7. Phylogenetic analysis of putative IR gene sequences of *O. emarginata* (black circles). The tree is rooted with IR25a and IR8a lineages. Bootstrap values < 50% are not shown. Bmor, *B. mori*; Dmel, *D. melanogaster*; Harm, *H. armigera*; Msx, *M. sexta*; Oema, *O. emarginata*; Slit, *S. litura*.

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triene 3Z,6Z,9Z-21:H separately [56]. OemaPBP1 and OemaPBP3 were ranked in the clusters PBPI and PBPIII in the phylogenetic analysis, respectively, which showed an equally consistent association with male-specific pheromone sensitive sensilla [57]. Orthologous genes in the clusters PBPI and PBPIII play critical and minor roles in female sex pheromone perception, respectively [58–61]. OemaOR29 and OemaPBP1 showed the highest FPKM values in all ORs and OBPs, respectively, and might be used to recognize the main sex pheromone component. OemaOR4 and OemaPBP3 might be involved in the recognition of the minor sex pheromone component. Further studies are needed to verify the function of these genes.

Five candidate pheromone receptor genes (OemaOR3, 21, 26, 28, and 30) showed female-biased expression, and OemaOR26, and OemaOR28 were specifically expressed in females. The function of these genes is unknown, but these might be used by females to recognize male

![Phylogenetic analysis of putative SNMP gene sequences of O. emarginata (black circles), D. melanogaster (black lines), other moth species (purple lines), and Hymenopteran species (green lines).](https://doi.org/10.1371/journal.pone.0179433.g008)
pheromones. Production of short-range pheromones has been reported in male butterflies [62]; these function in female mate selection, act as an aphrodisiac, and arrest female departure [63,64].

Besides the candidate PR genes, some genes with sex-specific expression were detected; for example, OemaOR13 was female-specific. These genes might also be correlated with sex specific behaviors such as the recognition of oviposition cues by females [65–67].

Diversification of olfactory recognition to sex pheromones

Type II pheromones have mainly been found in the moth superfamilies Geometroidea and Noctuoidea [17], but olfactory genes for type II pheromones were only identified in the geometrids A. selenaia cretacea [68,69] and O. brumata [56] and the erebids L. dispar [70–72] and Hyphantria cunea [73]. The sex pheromone of female O. emarginata was not published, but it...
Fig 10. The phylogeny of Lepidopteran PRs. The tree was rooted with Orco lineage (yellow color). Bootstrap values < 50% are not shown. Genes of *O. emarginata*, *O. brumata*, and other noctuid species are indicated by black circles, black triangles, and diamonds, respectively. Clusters PRI—PRIV for type I pheromones are indicated in red, green, purple, and blue, respectively. Aseg, *A. segetum*, Atra, *Amyelois transitella*, Bmor, *B. mori*, Harm, *H. armigera*, Hvir, *H. virescens*, Obru, *O. brumata*, Oema, *O. emarginata*, Onub, *O. nubilalis*, Pxyl, *P. xylostella*, Sexi, *S. exigua*, Slit, *S. litura*.

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Fig 11. The phylogeny of Lepidopteran PBPs. The tree was rooted with GOBP lineage. Bootstrap values < 50% are not shown. Genes of *O. emarginata*, other species with type II pheromones, and the other noctuid species are indicated by black circles, black triangles, and diamonds, respectively. Clusters PBPI—PBPIII are indicated by orange, purple, and blue colors, respectively. Acon, *Argyresthia conjugella*, Aips, *A. ipsilon*, Apol, *A. polyphemus*, Asel, *Ascosis selenaria cretacea*, Bmor, *B. mori*, Cpun, *C. punctiferalis*, Csup, *C. suppressalis*, Ehip, *Eogystia hippophaecolus*, Harm, *H. armigera*, Hass, *H. assulta*, Gmol, *G. molesta*, Ldis, *Lymandria dispar*, Msex, *M. sexta*, Obru, *O. brumata*, Oema, *O. emarginata*, Ofur, *O. furnacalis*, Onub, *O. nubilalis*, Pxy, *P. xylostella*, Sexi, *S. exigua*, Sinf, *S. inferens*, Slit, *S. litura*.

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was similar to the epoxide components of a preliminary identification (Du et al., unpublished data). In addition, cis-9,10-epoxy-(Z)-6-heneicosene and cis-9,10-epoxy-(Z,Z)-3,6-heneicosadiene were identified as the major and minor sex pheromone components from a sibling species, *O. excavate* [30]. In the present study, 7 candidate PRs and 3 candidate PBPs were obtained from the noctuid *O. emarginata* using antennal transcriptome analysis.

The diversification of olfactory recognition to sex pheromones has been verified for type I pheromones in noctuids such as *A. segetum*, *H. armigera*, and *S. litura*, and the phylogeny of moth PRs and PBPs for type I pheromone identified several apparent orthologous clusters (cluster PRI—PRIV for PRs and cluster PBPI—PBPIII for PBPs). PRs and PBPs from different clusters specifically respond to different type I sex pheromone components [59,74]. Although the functions of PRs for type II pheromone recognition were not identified, phylogenetic analysis clustered 3 candidate PRs of *H. cunea* [73] and 7 candidate PRs of *O. emarginata* into three groups. These findings are indicative of the diversification in olfactory recognition to type II pheromones.

Phylogenetic analysis did not separate the PRs and PBPs for types I and II pheromones, thereby suggesting that PRs and PBPs for types I and II pheromones evolved from a common ancestor. However, type I pheromones differed from type II pheromones in its chemical characteristics. *OemaOR29* and *ObruOR1* belonged to cluster PRIII of type I pheromone recognition, which is under strong purifying selection (a very small dN/dS values), and did not respond to any type I sex pheromone components [75]. On the contrary, *ObruOR1* was verified to specifically recognize the pheromonal tetraene of *O. brumata*, 3Z,6Z,9Z-19:H, and the orthologous receptor *AsegOR3* responded strongly to the triene 3Z,6Z,9Z-21:H instead of any female sex pheromone of *A. segetum* [56]. Cluster III might be specialized in the recognition type II sex pheromone components. In addition, 6 other candidate PRs of *O. emarginata* were not grouped within any of the four PR clusters of type I sex pheromones, but 5 of these were grouped into a specific cluster, with a bootstrap support value of 78. The candidate main sex pheromone-binding protein *OemaPBP1* was not clustered into the subgroup of PBPI genes from other noctuid species in the phylogenetic tree. These results indicate that the olfactory genes for sex pheromones in *O. emarginata* might differ from those of other noctuid species.

### Table 6. Chemosensory genes in insects.

| Species          | GR | OR | IR | OBP | CSP | SNMP | Reference |
|------------------|----|----|----|-----|-----|------|-----------|
| *A. ipsilon*     | 1  | 42 | 24 | 33  | 12  | 2    | [25]      |
| *B. mori*        | 65 | 66 | 18 | 46  | 22  | 1    | [40,41]   |
| *C. suppressalis*| /  | 47 | 20 | 26  | 21  | 2    | [42]      |
| *C. pomonella*   | 20 | 58 | 21 | /   | /   | /    | [43,44]   |
| *D. houi*        | /  | 33 | 10 | 23  | 17  | 2    | [45]      |
| *D. kikuchii*    | /  | 33 | 9  | 27  | 17  | 2    | [45]      |
| *H. armigera*    | /  | 60 | 19 | 34  | 18  | 2    | [46]      |
| *H. assulta*     | /  | 64 | 19 | 29  | 17  | 2    | [46]      |
| *M. sexta*       | 1  | 47 | 6  | 18  | 19  | 2    | [20]      |
| *O. fumacalis*   | 5  | 56 | 21 | 23  | 10  | 2    | [47,48]   |
| *O. emarginata*  | 0  | 35 | 6  | 41  | 20  | 2    | The study |
| *S. inferens*    | /  | 39 | 3  | 24  | 24  | 2    | [49]      |
| *S. littoralis*   | 6  | 47 | 17 | 37  | 21  | /    | [50]      |
| *S. litura*      | /  | 26 | 9  | 21  | 18  | /    | [51]      |

/ means the number of genes in the family was not reported.

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and the diversification of pheromone recognition genes for types I and II sex pheromones might exist in noctuid species.

Conclusions

A total of 104 candidate olfactory genes, including 7 candidate PRs and 3 candidate PBPs were identified from the noctuid *O. emarginata*. Seven olfactory genes of *O. emarginata* were not effectively clustered with those of other Lepidoptera, and OemaORs and OemaOBPs in 2 clusters were strongly expanded. These changes in olfactory genes in *O. emarginata* might correlate with its unique life history. Most candidate PRs and PBPs (except for OemaOR29 and OemaPBP3) of *O. emarginata* were not clustered with other noctuid species. OemaOR29 was grouped into cluster PRIII of type I pheromones, which recognized type II pheromones instead of type I pheromones. Noctuid species might thus have undergone diversification of the pheromone recognition gene for types I and II sex pheromones. Our results increase our understanding of the molecular mechanism of *O. emarginata* olfaction and the evolution of olfactory genes associated with sex pheromones.

Supporting information

S1 Fig. GO annotation.
(TIF)

S1 Table. Primers used in this study.
(DOC)

S1 File. Amino acid sequences of the olfactory genes used in the phylogenetic analysis.
(TXT)

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Author Contributions

Conceptualization: YD BF.

Data curation: BF QG.

Formal analysis: BF KZ YD.

Funding acquisition: YD YQ.

Investigation: BF QG YQ.

Methodology: BF KZ.

Project administration: YD.

Resources: BF KZ.

Software: BF.

Supervision: YD.
Validation: BF YD.
Visualization: BF YD.
Writing – original draft: BF YD.
Writing – review & editing: YD.

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