Identification of chemosensory genes in the greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Olfaction, one of the most significant sensations influencing insect behaviour, has been an efficient target for pest management. In this study, we analysed the antennal transcriptome of the greater wax moth, *Galleria mellonella* L. which is a predominant honeybee pest and is now becoming a potential threat to the global honeybee industry. A *de novo* antennal RNA-sequence assembly resulted in 24,683 unigenes and identified 24 odorant-binding proteins, 62 odorant receptors, 4 ionotropic receptors and 2 sensory neuron membrane proteins. Additionally, seven antennal-binding proteins, six pheromone-binding proteins and seven general odorant-binding proteins were identified from *G. mellonella*. Phylogenetic analysis suggested majority of the genes be closely associated with orthologs from other lepidopteran species. The identification of candidate genes and functional annotation of the olfactory genes will facilitate future functional studies on chemoreception processes in this species and other lepidopterans. This study lays the groundwork for future research that might lead to cutting-edge approaches in pest management.

Keywords: Chemosensory genes, *Galleria mellonella*, lepidopterans, olfaction, pest management.

OLFACTION in insects is critical for foraging, host selection, mate recognition, oviposition site identification and predation[1]. Understanding and analysing the functions of the insect olfactory system has advanced over time[2]. Antennae are important sensory appendages in insect olfaction, which perceive chemical stimuli in two steps. Odour molecules from the environment initially enter the sensillae through the cuticular pores into the sensillar lymph, where they bind to water-soluble odorant-binding proteins (OBPs)[3,4]. The sensillar lymph, an integral membrane protein found on the dendrites of olfactory receptor neurons (ORNs) which are triggered to produce signals that induce insect response to the stimulus, is another pathway by which the OBPs are disseminated to odorant receptors (ORs)[5–7]. Ionotropic receptors (IRs) are chemoreceptors in the olfactory receptor family[8,9], which are involved in a wide range of activities, including the formation of social clusters and hearing[10,11], chemosensation, thermosensation and hygrosensation[12]. Further, sensory neuron membrane proteins (SNMPs), which are situated on the dendrites of the olfactory sensory neurons (OSNs), are considered to be linked with pheromone reception[13–15]; SNMPs are crucial for the detection of pheromones in fruit flies and other moth species[5,16]. Tissue- and sex-specific expression profiling and functional assessment of putative chemosensory genes are two crucial areas of focus in understanding the mechanism of olfaction in insects. Knockdown of certain OBPs, ORs, IRs and SNMPs using CRISPR and RNA interference techniques can effectively prevent the communication between insect pests and their hosts[17–20]. Identification of chemosensory genes and phylogenetic analyses may provide potential information and insights about the functions of vital chemosensory genes.

The greater wax moth (GWM), *Galleria mellonella* Linnaeus (Lepidoptera: Pyralidae), is a ubiquitous and destructive pest of honeybee colonies. *G. mellonella* larvae devour wax, bee larvae, pollen and honey, and gradually destroy the honeycomb, especially in stressed colonies. This pest is becoming an inherent menace to the global honeybee industry[21]. The existing management of GWM involves heat treatment, chemical fumigation, biological control and sterile insect technique (SIT)[22]. These management tools are less promising in several ways and are not cost-effective[21]. *G. mellonella* has an unusual olfactory system; the male produces a mixture of sex pheromones, such as nonanal, decanal, hexanal, heptanal, undecanal, 6,10,14-trimethylpentacanol-2, and 5,11-dimethylpentacosane to attract virgin females to initiate mating[23–27]. Moreover, adult *G. mellonella* uses olfactory modifications to find host volatiles and sex pheromones[28,29]. Therefore, a deeper understanding of the biological pathways involved in olfactory communication can lead to developing behaviour-based novel pest management strategies.

Zhao *et al.*[9] have identified 22 OBPs, 20 chemosensory proteins (CSPs), 46 ORs, 17 IRs and 2 SNMPs while Jiang *et al.*[30] reported 21 OBPs, 18 CSPs, 43 ORs, 18 IRs and 2 SNMPs from *G. mellonella*. Interestingly, the chemosensory genes identified are fewer than those reported in many other lepidopteran species, suggesting the presence of additional undiscovered genes. The transcriptome data generated in present study helped identify a total of 112 olfactory-related genes. Additionally, the evolutionary links between the discovered unigenes and olfaction genes from different lepidopterans were analysed. These findings will aid in developing a better resource and facilitate the functional characterization of the olfactory genes of *G. mellonella* and other important agricultural insect pests.

A total of 100 antennae of male and female moths of *G. mellonella* were excised one day after eclosion, immediately frozen in liquid nitrogen, and stored at –80°C until the RNA was extracted. According to the protocol and steps outlined by Vyas *et al.*[31] and Krishnarao *et al.*[32], RNA isolation, cDNA library construction, RNA sequencing, RNA analysis, Illumina sequencing, *de novo* transcriptome assembly,

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functional annotation, data collection of olfactory and chemosensory-related sequences of other lepidopteran species, identification of olfactory genes and phylogenetic analysis of antennal transcriptomics were performed. In brief, RNA was extracted using Direct-zolTM RNA Mini Prep (Zymo Research), and its quantity and quality were determined using Qubit RNA BR test (Thermo Fisher Scientific), and Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) and RNA Nano kit (Agilent Technologies, CA, USA) respectively. The NEB Next Poly (A) mRNA magnetic isolation module (Catalog: E7490, New England Biolabs) was then employed to enrich the mRNA using 500 ng of total RNA. Further, library quality assessment was done using an Agilent D5000 screen tape system (Catalog: 5067-5588, Agilent Technologies) in a 4150-tape station system (Catalog: G2992AA, Agilent Technologies).

Illumina HiSeq was used to generate the sequence data. The raw sequence reads have been deposited under SRA accession PRJNA691708. *De novo* transcriptome was assembled using Trinity v.2.5.1 (ref. 33). The transcripts from the *de novo* transcriptome assembly were used to predict the protein-coding sequences with the help of TransDecoder. Employing the BLASTp module of DIAMOND[34], the protein sequences were annotated against the NCBI nr, UniProt/Swiss-Prot, and UniRef100 protein databases. The sequences of olfactory and chemosensory-related genes of other lepidopteran species were downloaded using the NCBI protein sequence tool and the sequences obtained after cleaning were aligned with MUSCLE using the MEGA11 program[35].

Sequence homology analysis was conducted for the designation and arbitrarily numbering of all the olfactory genes identified in the *G. mellonella* antennal transcriptome. Evolutionary analyses were conducted in MEGA11 (ref. 35). To assess the branch strength of each tree, a bootstrap analysis with 500 iterations was used. The analysis comprised 238 amino acid sequences with a total of 729 positions in the final dataset for the GmelOBP tree, which included ABP, GOBP and PBP sequences, 211 amino acid sequences with a total of 646 positions in the GmelOR tree, 31 amino acid sequences with a total of 474 positions in the GmelIR tree and 21 amino acid sequences with a total of 503 positions in the GmelSNMP tree. The Newick tree generated was then transferred to Fig Tree v1.4.4 using the bootstrap values for further analysis and figure preparation.

Totally 64,110,108 reads were obtained from both female and male (pooled) antennae samples with a length of 151 bp (Q20 content at 99.51%, Q30 content at 91.99% and GC content was 50.00%). The reads were assembled into 127,504 transcripts. The median and average length of the contigs was 344 and 679.06 nt respectively, with the total assembled bases of 86,582,440. The GC content was 36.57% and the number of N10, N20, N30, N40 and N50 contigs was 4636, 3283, 2395, 1750 and 1193 respectively (Table 1).

Table 1. Summary of *de novo* antennal transcriptome of *Galleria mellonella* L.

RAW sequence data and quality	
Total number of reads	64,110,108
Read length (bp)	151
GC per cent of transcripts	50.00
Q20 per cent of transcripts	99.51
Q30 per cent of transcripts	91.99
Assembly	
Total genes	108,165
Assembled transcripts	127,504
Per cent GC	36.57
Median contig length	344
Average contig	679.06
Total assembled bases	86,582,440
N10 of contig	4636
N20 of contig	3283
N30 of contig	2395
N40 of contig	1750
N50 of contig	1193
Annotation and functional classification	
Transcript annotations against NCBI nr	23,076
Transcript annotations against UniProt	14,879
Transcript annotations against UniRef100	23,204
Transcript annotations against Pfam	20,726
Transcript annotations against KO	4464
Transcript annotations against GO	45,889
Olfactory genes	
Odorant-binding proteins	24
Antennal-binding proteins	07
General odorant-binding proteins	07
Pheromone-binding proteins	06
Odorant receptors	62
Ionotropic receptors	04
Sensory neuron membrane proteins	02
Total number of olfactory genes	112

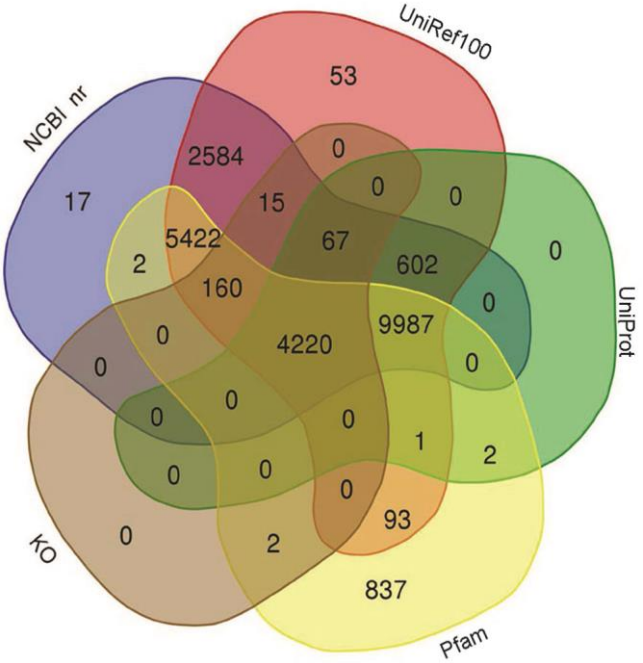

Figure 1. Venn diagram showing the annotation of *Galleria mellonella* transcripts against the NCBI nr, UniProt, UniRef100 and Pfam databases.

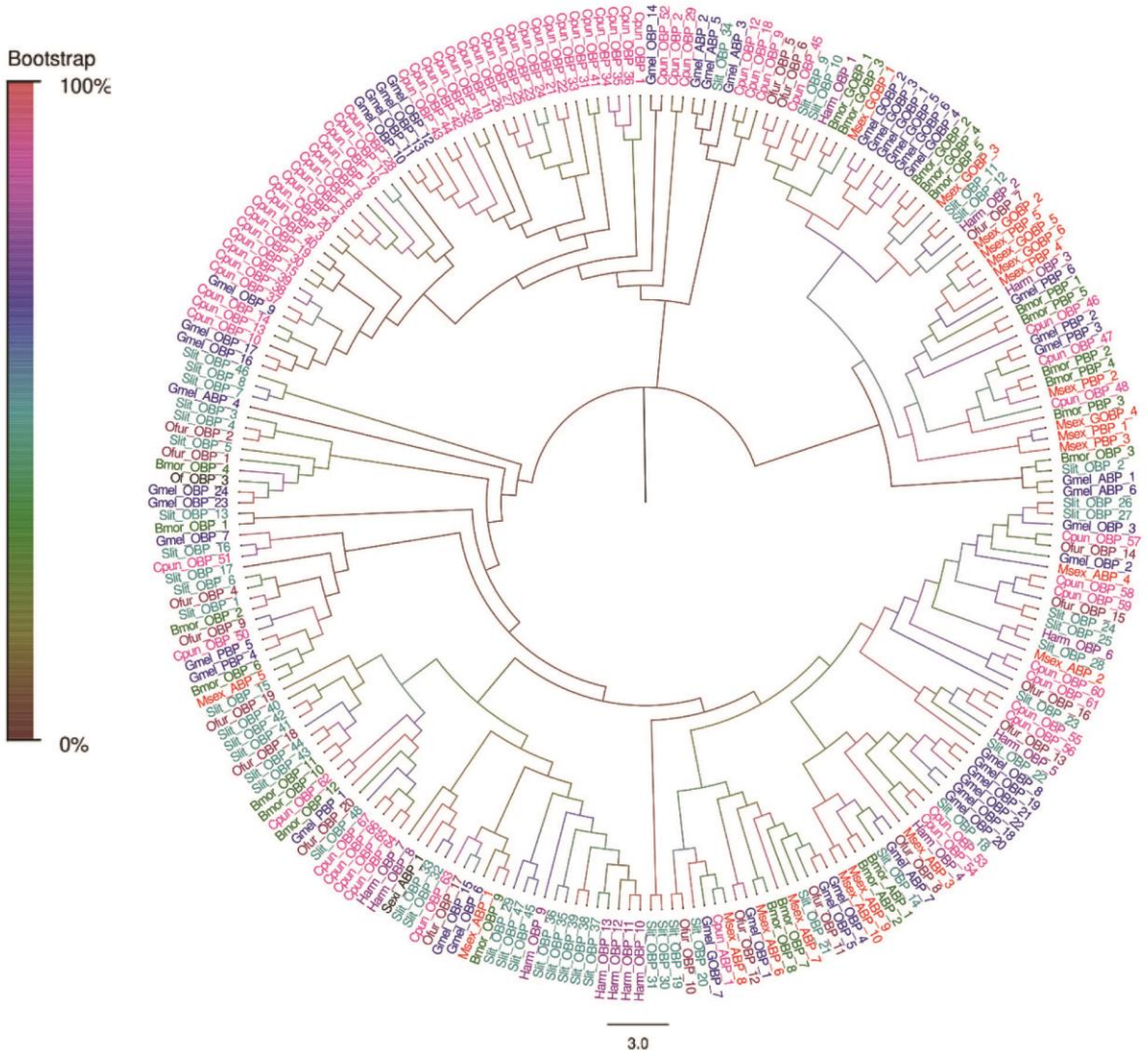

Figure 2. Maximum likelihood dendrogram based on protein sequences of candidate odorant-binding proteins in *G. mellonella* antennal transcriptome.

Among the 127,504 assembled *G. mellonella* transcripts, a total of 23,076 (18.10%), 14,879 (11.67%), 23,204 (18.20%) and 20,726 (16.25%) unigenes were annotated from the pooled antennal transcriptome of *G. mellonella* against NCBI nr, UniProt, UniRef100 and Pfam respectively (Table 1, Figure 1 and Supplementary Datasheets 1 and 3). In terms of species distribution, the *G. mellonella* unigenes were most closely related to those from other lepidopteran species, particularly *Amyelois transtiella* W. (Pyralidae), *Bombyx mori* L. (Bombycidae), and *Helicoverpa armigera* H. (Noctuidae) against NCBI nr and UniRef100 hits (Supplementary Figures 1 and 2), whereas in UniProt hits, best matches were *Drosophila melanogaster* M. and *Homo sapiens* L. (Supplementary Figure 3).

Global standardization of the classification of the functional properties of genes is known as gene ontology (GO). Molecular functions (10,976), cellular components (13,362) and biological processes (21,551) were the three main GO categories used to classify the functional groups of the 45,889 annotated unigenes (Supplementary Datasheet 2 and Figure 4). The biological complexity of the genes was examined using KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis. KEGG is a high-level database repository for comprehending molecular-level information, particularly using high-throughput experimental technologies, and high-level functions and utilities of biological systems (such as cells, animals and ecosystems). A total of 4464 unigenes could be assigned to different KEGG pathways using KO (KEGG Orthology) (Supplementary Datasheet 1 and Figure 1). Additionally, the KO classification of unigenes was matched to five categories, i.e. cellular processes, processing of genetic and environmental information, processing of information related to human disorders, metabolism, and organismal systems. Signal transduction was the most

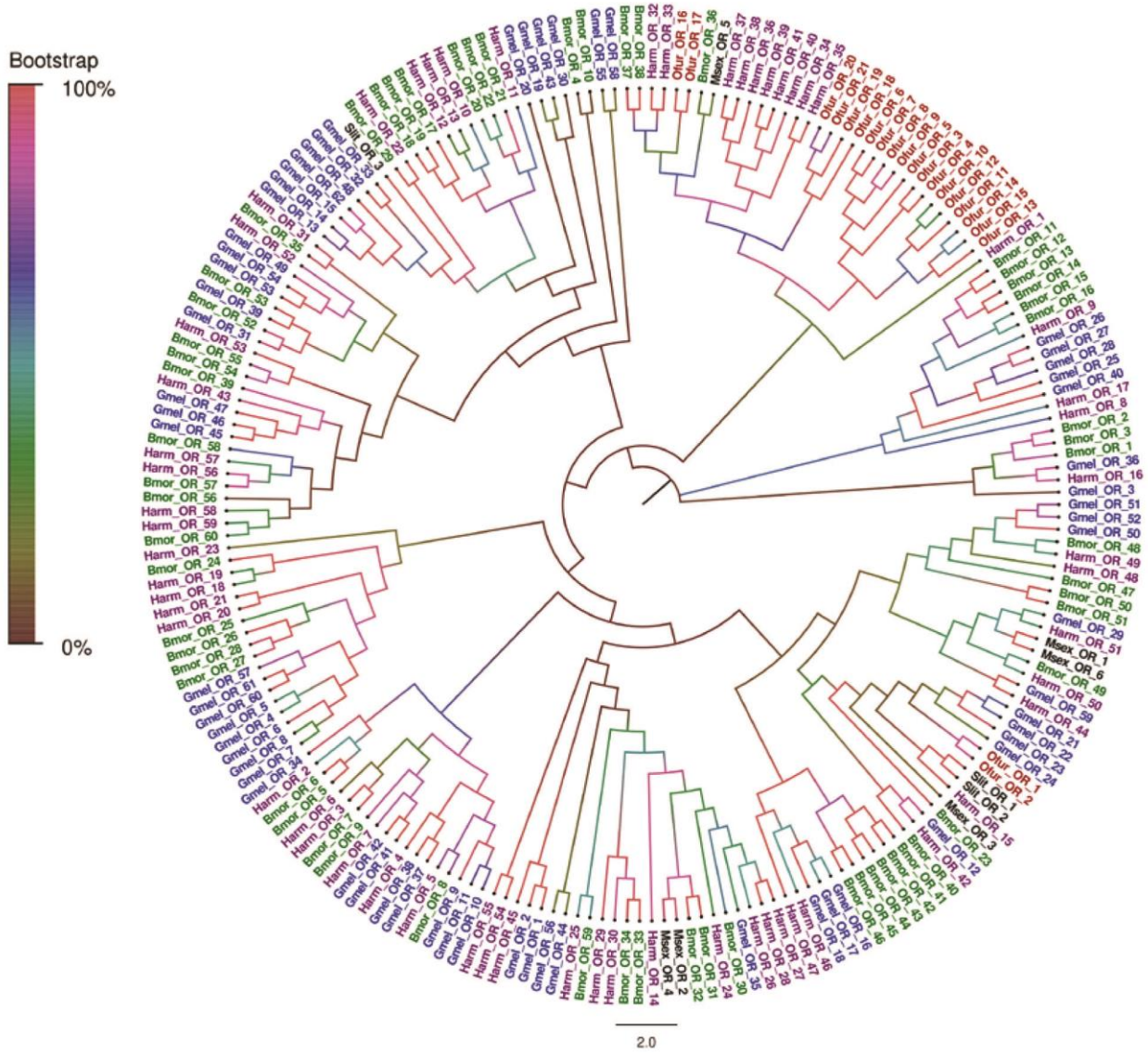

Figure 3. Maximum likelihood dendrogram based on protein sequences of candidate odorant receptors in *G. mellonella* antennal transcriptome.

abundant category among the KO classes, while cellular community-prokaryotes were the least abundant group (Supplementary Figure 5).

A total of 112 olfactory-related genes, including 24 OBPs, a few specialized OBPs like seven general odorant-binding proteins (GOBPs), six pheromone binding proteins (PBPs), seven antennal binding proteins (ABPs), 62 ORs, four IRs and two SNMPs were identified from the antennal transcriptome of *G. mellonella*.

OBPs are the prime responders in the insect olfactory system that deliver semiochemicals to the ORs. Among the assembled unigenes, 44 were annotated encrypting OBPs, including GOBPs, PBPs and ABPs, which were in the range 308–3794 bp (Supplementary Table 1). An evolutionary (phylogenetic) analysis was done with the 44 *G. mellonella* OBPs and 194 OBPs, including GOBPs, PBPs and ABPs from other lepidopterans species like *Conogethes punctiferalis* (Guenee), *Spodoptera litura* (Fab.), *B. mori*, *Manduca sexta* (Linn.), *H. armigera*, *Ostrinia furnacalis* (Guenee) and *Spodoptera exigua* (Hubner). All the identified putative OBP proteins grouped with at least one lepidopteran orthologue, either in the same clade or a distinct clade (Figure 2). Information regarding the unigene sequences, lengths, reads, best NCBI nr, UniProt, UniRef100 and Pfam hits, and GO of the 44 OBPs is available in Supplementary Table 1.

Sex pheromone sensing and general odorant detection processes both include insect ORs. There were 62 ORs in the antennal transcriptome of *G. mellonella*. They were nearly full sequences with lengths ranging from 347 to 3649 bp. Phylogenetic analysis of 62 *G. mellonella* ORs and 149 ORs from other lepidopterans species like *S. litura*, *B. mori*, *M. sexta*, *H. armigera* and *O. furnacalis* revealed that *G. mellonella* is likely associated with *B. mori* and *H. armigera* (Figure 3). Moreover, several species-specific branches

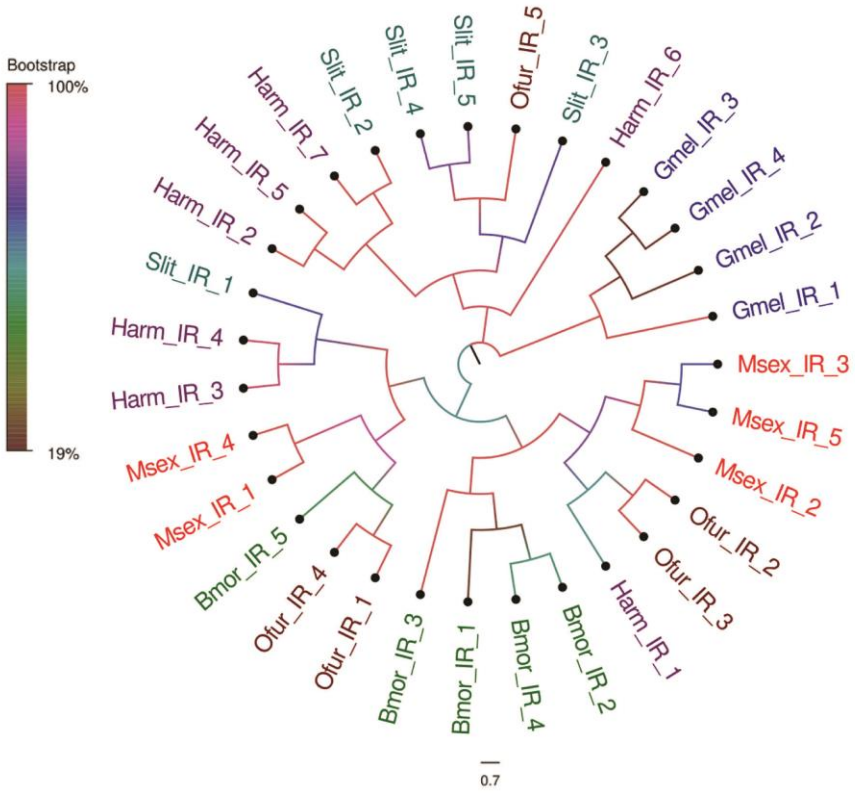

Figure 4. Maximum likelihood dendrogram based on protein sequences of candidate ionotropic receptors in *G. mellonella* antennal transcriptome.

were detected, including *G. mellonella*. Information on the unigene sequences, lengths, reads, best NCBI nr, UniProt, UniRef100 and Pfam hits, and GO of the 62 ORs is provided in Supplementary Table 2.

IRs are the chief receptors for various odorants and different sensations. The antennal transcriptome of *G. mellonella* contained four IRs that ranged in size from 1740 to 2346 bp. When these IRs were phylogenetically analysed with those of other lepidopteran species like *S. litura*, *B. mori*, *M. sexta*, *H. armigera* and *O. furnacalis*, species-specific clades were detected among all tested lepidopterans, indicating the unique mating behaviour (Figure 4). Information on the unigene sequences, lengths, reads, best NCBI nr, UniProt, UniRef100 and Pfam hits, and GO is provided in Supplementary Table 3.

The chemosensory system of insects depends significantly on the proteins found in the SNMPs. Two SNMPs from the antennal transcriptome of *G. mellonella*, measuring 3318 and 3190 bp respectively, were identified in the present study. Phylogenetic analysis with *S. litura*, *B. mori*, *M. sexta*, *Agrotis ipsilon* (Hufnagel), *Heliothis virescens* (Fabricius), *Cnaphalocrocis medinalis* Guenee and *Chilo suppressalis* (Walker) revealed that *G. mellonella* SNMPs were divergent from each other and most of them were species-specific clades (Figure 5). Information on the unigene sequences, lengths, reads, best NCBI nr, UniProt, UniRef100 and Pfam hits, and GO of the two SNMPs is available in Supplementary Table 4.

The ability of insects to recognize molecules in their surroundings is essential to their survival. Generally, chemoperception plays a prominent role in insects while finding suitable ecological niches and mating partners[36]. Antennae are the chief olfactory sensory organs that help insects locate habitats, oviposition sites and recognize mating partners[36,37]. Olfaction is an essential sense of smell for all insects, including the GWM to survive and reproduce in nature[9].

The transcriptome profile of pooled antennae from male and female *G. mellonella* was generated in the present study to examine the olfactory mechanism of this globally distributed species. In total, 112 OR genes were identified, including 24 OBPs and specialized ones like seven GOBPs, six PBPs, seven ABPs, 62 ORs, four IRs and two SNMPs. Our findings, along with those of Zhao *et al.*[9] and Jiang *et al.*[30], provide a comprehensive data repository for the olfactory genes of *G. mellonella*. Zhao *et al.*[9] reported 22 OBPs, 20 CSPs, 46 ORs, 25 IRs and two SNMPs in *G. mellonella*, while Jiang *et al.*[30] identified 22 OBPs, 46 ORs, 25 IRs, two SNMPs and 20 CSPs.

We found numerous unigenes linked to the KEGG pathways and GO categories, demonstrating that the antenna has many active genes. The most strongly represented KEGG pathways in environmental information processing and, more specifically, signal transduction suggest that the antenna is actively involved in responding to environmental signals and aiding the exceptional olfaction system of

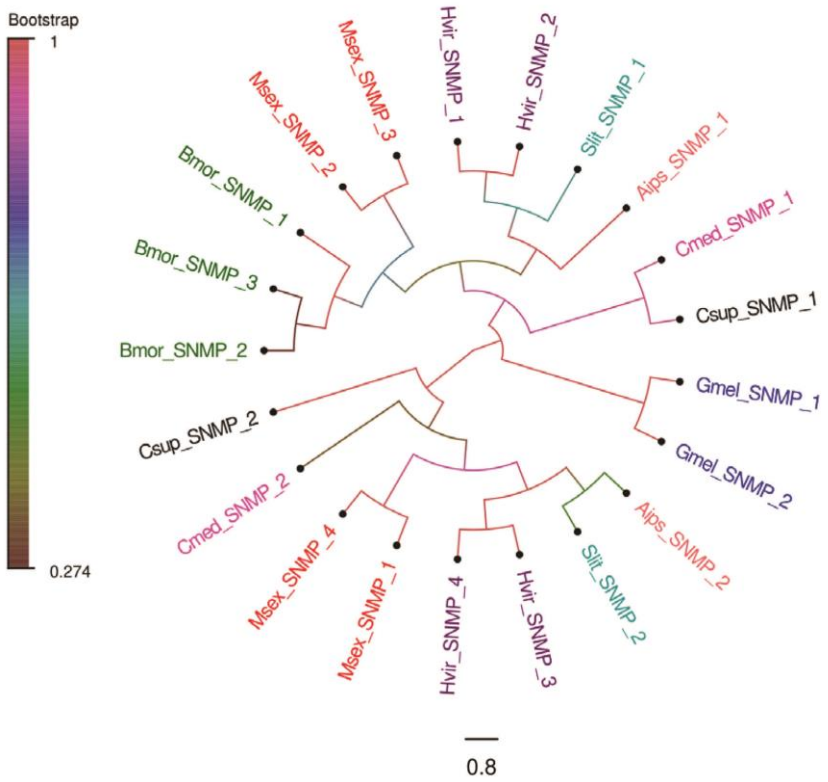

Figure 5. Maximum likelihood dendrogram based on protein sequences of sensory neuron membrane proteins in *G. mellonella* antennal transcriptome.

G. mellonella while detecting various odours (Supplementary Figure 4).

Over the past few decades, lepidopteran insect chemosensory gene families have been extensively studied, leading to a significant understanding of the molecular mechanisms underlying pheromone perception in many insects[9,30,36,38]. The small, water-soluble proteins, i.e. OBPs are abundant in the sensillar lymph of insect antennae[39]. They transport semiochemicals to ORs, which in turn send electrical impulses to the insect brain[40–42]. In addition, OBPs protect odorant molecules from odorant-degrading enzymes[40,43] and the amount of OBPs in the sensillum lymph varies with insect species[44–46].

The findings of Zhao *et al.*[9] and Jiang *et al.*[30] along with those reported in the present study offer a comprehensive data resource for the olfactory genes in *G. mellonella*. We found 44 OBPs in *G. mellonella* antennae, including seven GOBPs, six PBPs, and seven ABPs. This is comparable to those of previously identified lepidopteran species, such as *O. furnacalis* (23 genes), *S. exigua* (24 genes), *P. xylostella* (24 genes), *Spodoptera frugiperda* (J. E. Smith) (25 genes) and *C. suppressalis* (26 genes)[47–50]. Some studies have reported a greater number of OBPs in *Spodoptera littoralis* (Boisduval) (49 genes), *D. melanogaster* (52 genes), *M. sexta* (49 genes) and *B. mori* (46 genes)[51–54].

OSNs of insects are equipped with ORs on the dendritic membrane to detect odorants released by the chemosensory proteins and OBPs, and convert them into electrical impulses[55,56]. In the present study, 62 ORs were identified from the antennal transcriptome of *G. mellonella*. The number of *G. mellonella* ORs identified in this study was more than that found in other lepidopteran insects, including *Sesamia inferens* W. (39), *C. punctiferalis* (46 and 59) and *H. armigera* (47)[38,57–59]. However, the genomes of two model lepidopteran insect species, viz. *B. mori* and *S. litura*, contained 66 and 72 genes respectively[51,60].

Odorant-specific IRs have been associated with a diversity of behaviours, including general attraction/aversion, courtship and oviposition[61]. IRs are also important receptors for odorants such phenyl acetaldehyde, amines and acids[9,62]. We found four IRs from the *G. mellonella* antennae in the present study, which is less than that reported by Zhao *et al.*[9] and Jiang *et al.*[30]. Given that the GWM is more active during the first three days of its life cycle and that its activity decreases with advancing age, it is possible that the variation in numbers is the result of various sampling periods and stages of the *G. mellonella* life cycle[29].

The membrane-bound SNMPs, first found in the olfactory receptor neurons of Lepidoptera[63], are considered to be involved in detecting odour pheromones[16,64]. Similar to the findings of Zhao *et al.*[9] and Jiang *et al.*[30], we identified two SNMPS in the antennae of *G. mellonella*. They share a high degree of homology with orthologs found in other insect species, proving that their functions have not changed over time. Previous studies have reported that SNMPs play an important role in pheromone signalling in *D. melanogaster*,

H. virescens and *B. mori*[16,64,65], supporting their role in detecting of pheromones.

Phylogenetic analysis using genes from these species was done to determine the differences in sexual behaviour between *G. mellonella* and other moths, including *C. punctiferalis*, *S. litura*, *B. mori*, *M. sexta*, *H. armigera*, *O. furnacalis*, *S. exigua*, *A. ipsilon*, *H. virescens*, *C. medinalis* and *C. suppressalis* (Figures 2–5). The olfactory genes of *G. mellonella* were placed in various clades with other recognized lepidopteran genes, as expected. However, in evolutionary studies across these species, multiple species-specific branches were found, demonstrating the distinct olfactory mechanism of this pest in contrast to other species. Zhao *et al.*[9] and Jiang *et al.*[30] also reported the same phenomenon in *G. mellonella*, highlighting the further functional assessments of putative chemosensory genes to knock down the particular OBPs, ORs, IRs and SNMPs by RNA interference or CRISPR techniques, which can improve the chances of blocking communication between the insect and host as well as the insect and insect interactions[17–20]. In addition, numerous studies have demonstrated that various olfactory genes are expressed in non-olfactory tissues such as legs, abdomen and larval stages of other insect species[55,66]. We may have overlooked several olfactory genes in the present study because we have only sequenced the antennal transcriptome of *G. mellonella*. The identification of other chemosensory genes will require the study of additional tissues and developmental stages. Studies have shown that non-olfactory tissues such as the abdomen, legs and larval stages of other insect species express several olfactory genes[55,66]. Thus, some of the olfactory genes may have been missed in the present study because we exclusively sequenced the antennal transcriptome of *G. mellonella*. More research into new tissues and developmental stages is required to identify other chemosensory genes.

In conclusion, we have developed a transcriptome dataset for *G. mellonella* antennae in this study. The antennal transcriptome of *G. mellonella* revealed a total of 112 OR genes, with 24 OBPs and a few specialized ones as well. Future analysis of these genes will help identify the molecular mechanisms underlying *G. mellonella* olfaction.

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