

The Antennal Sensilla and Expression Patterns of Olfactory Genes in the Lower Termite *Reticulitermes aculabialis* (Isoptera: Rhinotermitidae)

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Abstract

The insect olfactory system plays pivotal roles in insect survival and reproduction through odor detection. Morphological and physiological adaptations are caste-specific and evolved independently in workers, soldiers, and reproductives in termites. However, it is unclear whether the olfactory system is involved in the division of labor in termite colonies. In the present study, the antennal sensilla of alates, workers, soldiers, nymphs, and larvae of the termite *Reticulitermes aculabialis* Tsai et Hwang (Isoptera: Rhinotermitidae) were investigated. Transcriptomes were used to detect olfactory genes, and differential expression levels of olfactory genes were confirmed in various castes by qRT-PCR analysis. Nine types of sensilla were identified on the antennae of *R. aculabialis*, and soldiers possessed all 9 types. In 89,475 assembled unigenes, we found 16 olfactory genes, including 6 chemosensory protein (CSP) and 10 odorant-binding protein (OBP) genes. These OBP genes included 8 general odorant-binding protein genes (GOBPs) and 2 pheromone-binding protein-related protein (PBP) genes. Five CSP genes were more highly expressed in alates than in workers, soldiers, larvae, and nymphs, and the expression levels of *CSP6* were significantly higher in nymphs. Seven GOBP and two PBP genes exhibited significantly higher expression levels in alates, and there were no significant differences in the expression levels of *GOBP2* among workers, soldiers, alates, and larvae. These results suggest that alates, as primary reproductives, have unique expression patterns of olfactory genes, which play key roles in nuptial flight, mate seeking, and new colony foundation.

Key words: termite, antennal sensilla, olfactory gene, odorant-binding protein, chemosensory protein

Caste differentiation in termites is one of the most conspicuous examples of facultative polyphenism in animals (Korb and Hartfelder 2008). Colony efficiency is based on the division of labor, leading to allocation of specific tasks and behavioral specializations between different castes. Termites contain three castes in their colonies: workers, soldiers, and reproductives. Lower termites have flagellates in their guts including all termites except Termitidae (higher termites). In *Reticulitermes*, newly hatched larvae develop into workers or nymphs. The nymphs follow the reproductive pathway to develop into alates (alate adults) that fly off at the same time in great swarms, mate, and create new colonies as primary reproductives. Soldiers develop from workers and specialize in colony defence (Su et al. 2017). As workers and soldiers are blind, they obtain environmental information using antennal sensilla (Yanagawa et al. 2009). Antennae are the major sensory and olfactory organs that process a wide range of sensilla types for the treatment of chemical signals, which allow them to survive in different environments (Bawin et al. 2017,

Li et al. 2018, Silva et al. 2019). The antennal sensilla may play a role in nestmate recognition in workers and soldiers (Huang et al. 2012). To perform specialized social interactions, every individual needs chemical communication, which requires a special chemosensory system in the division of labor. The diversification of antennal sensilla and chemosensory genes is crucial for the complex social organization and ecological dominance of social insects (Hojo et al. 2015, Balbuena and Farina 2020). It is reasonably anticipated that individuals performing different tasks have different sensitivities to various chemicals and may have a distinct set of chemoreceptors (Slessor et al. 2005).

The antenna has numerous hair-like organs called sensilla that contain receptors for olfactory, chemosensory, hygrosensory, and gustatory perception. These sensilla perform distinct functions depending on their structure, which markedly affects insect behavior and plays important roles in the survival of many insect species (Rocha et al. 2007, Sakurai et al. 2014, Ma et al. 2017). Olfactory

sensilla are identified by the pores along the cuticular surface of the antenna and detect plant volatiles and pheromones (Steinbrecht 1997, Hallem et al. 2006, Yuvaraj et al. 2013, Ruschioni et al. 2015, Rani et al. 2021). Although morphological features of the antennal sensilla of termites have been reported (Yanagawa et al. 2009, Chu et al. 2019, Fu et al. 2020), little is known about the types of antennal sensilla in various castes of termites.

The process of sensing outside volatiles is called olfaction, which is very important in nestmate recognition, nest defence, and foraging (Xu et al. 2009, Gu et al. 2014, Sun et al. 2019). The olfactory coreceptors participate in termite magnetic orientation under both light and darkness and are essential for termites to perceive their trail pheromones (Gao et al. 2020, Gao et al. 2021). In the insect olfactory system, chemosensory proteins mainly include odorant-binding proteins (OBPs), chemosensory proteins (CSPs), odorant receptors (ORs), sensory neuron membrane proteins (SNMPs), and ionotropic receptors (IRs) (Liu et al. 2012, Leal 2013, Gu et al. 2014, Liu et al. 2015a, b). OBPs have been found in the damp wood termite *Zootermopsis nevadensis* Hagen (Isoptera: Termopsidae) (Ishida et al. 2002). OBPs are divided into pheromone-binding proteins, general odorant-binding proteins, and antennal-binding proteins, all of which function as carrier proteins (Lartigue et al. 2002, Gu et al. 2019). Ultimately, the olfactory sensilla recognize active components capable of triggering a response in the insect brain that results in a behavioral change.

In this study, the morphology of antennal sensilla in workers, soldiers, alates, nymphs, and larvae of *Reticulitermes aculabialis* was investigated by scanning electron microscopy. We conducted RNA sequencing of workers, soldiers, and alates of *R. aculabialis* and identified OBP and CSP genes. Finally, the differential expression levels of the OBP and CSP genes were confirmed in workers, soldiers, alates, larvae, and nymphs using quantitative real-time PCR (qRT-PCR). Our findings might help to understand how the olfactory system of termites is involved in the adaptability of social organizations.

Materials and Methods

Termites

Three colonies of *R. aculabialis* were collected from Northwest University in Xi'an, Shaanxi Province, China. The last instar nymphs appeared in colonies from September to April next year. Lower termites were characterized based on their unique flexibility in development. There were morphological differences among workers, nymphs, alates, and soldiers (Fig. 1). Late instar workers, soldiers, and last instar nymphs were collected in March and alates in May 2019 when the alates were swarming. Late instar workers (sixth and seventh instar workers) were identified by the presence of 16 or more antennal segments (Su et al. 2015). Larvae were collected in July 2019. The larvae were small white. The workers had light brown bodies. The nymphs had white bodies and wing buds. The soldiers had large, highly sclerotized heads and powerful mandibles. The alates were characterized by darkened pigmentation, a hard cuticle, and black wings. Their heads were stored in liquid nitrogen for RNA extraction.

Light Microscopy and Scanning Electron Microscopy (SEM)

The heads of workers, soldiers, alates, nymphs, and larvae were cut with a blade, and then antennae were observed using a digital microscope ($n = 10$). The number of flagellum subsegments was counted,

and the length of the antenna was measured. For observation of antennal sensilla using SEM, samples were cleaned using a Skymen JP-38005 ultrasonic cleaning apparatus (Skymen Co., China) for 3 min ($n = 5$). Gradient dehydration was performed with 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol. Ethanol and tert-butanol were prepared to fix specimens at ratios of 2:1, 1:1, and 1:2, and the treatment time of each stage was 30 min. Then, the specimens were stored temporarily in tert-butanol. Their heads were sprayed with gold in a JEC-3000FC autofine coater (JEOL Ltd., Japan) for 100 s. The antennal sensilla were observed and photographed under a Hitachi TM3030Plus SEM (Hitachi Ltd., Japan). Sensilla types were classified based on external morphology as described by Yanagawa et al. (2009), Wang et al. (2018), and Fu et al. (2020).

RNA Extraction, cDNA Library Construction, and Sequencing

Total RNA was extracted from the heads of 20 individuals of workers, soldiers, and alates of *R. aculabialis* using RNAiso Plus reagent (TaKaRa Bio. Inc., Japan), respectively (three biological replications). After the extraction of RNA, RNA quality was verified using an A2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Next, poly(A) mRNA was isolated using oligo-dT magnetic beads (Qiagen Co., Ltd., Shanghai, China). Fragmented buffer was added to produce short mRNA fragments. Taking these short fragments as a template, first-strand cDNA was synthesized using random hexamer primers. Using a buffer, dNTPs, RNAaseH, and DNA polymerase I, second-strand cDNA was generated. The cDNA was checked using agarose gel electrophoresis and was verified to have a fragment length of 200 bp. After refinement, PCR was used to build the final cDNA library. Following agarose gel electrophoresis and extraction of cDNA from the gels, the cDNA fragments were purified and enriched by PCR to construct the final cDNA library. The cDNA library was built using an Illumina sequencing platform (Illumina HiSeq 2500) with paired-end Gene Denovo Co. (Guangzhou, China). Perl software was used to filter clean reads by removing low-quality sequences (those with greater than 50% of bases with fewer than 20 and greater than 5% N bases and those with an unknown base in one sequence) and reads containing adaptor sequences.

De Novo Assembly, Read Mapping, and Bioinformatic Analysis

Trinity is a secluded strategy and programming bundle with three constituents: inchworm, chrysalis, and butterfly. Linear contigs are collected by inchworm in Trinity (version 2.0.6), which assembles reads produced using a greedy k-mer-based approach. Next, chrysalis bunch-related contigs were compared to segments of joined transcripts or generally novel fragments of paralogous genes, after which a de Bruijn graph for each group of related contigs is assembled. Finally, butterfly breaks down the read pairings with regard to the comparison de Bruijn graph, yielding one straight succession for each, and joined isoforms, and transcripts are obtained from paralogous genes. Sequencing reads were mapped to reference arrangements utilizing SOAP aligner/soap2, an instrument intended for short sequence alignment. The gene functions and classification were analysed based on searches against the following databases: the National Center for Biotechnology Information (NCBI) non-redundant nucleotide (Nr), the Swiss-Prot database, EuKaryotic Orthologous Groups (KOG), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Expression levels of genes were calculated by the total number



Fig. 1. Morphology of the larvae, workers, nymphs, alates, and soldiers of *R. aculabialis*. (A) The larvae were small white. The larvae developed into workers and nymphs. (B) The workers had light brown bodies. (C) The nymphs had white bodies and wing buds (WB). (D) The soldiers developed from workers and had large, highly sclerotized heads, and powerful mandibles. (E) The alates developed from nymphs. They were characterized by darkened pigmentation, a hard cuticle, and black wings.

of reads covered by that gene. Using the same method, we calculated the expression quantities of all mapped genes. The expression levels of these genes were computed only for genes whose reads were uniquely mapped. Uniquely mapped reads per kilobase per million (RPKM) were used to compute the expression quantities of the genes. RPKM was calculated using the formula $RPKM = \text{number of reads} / (\text{gene length} / 1,000 \times \text{total number of reads} / 1,000,000)$. R software was used to calculate expression and data statistics (<http://www.r-project.org/>). Differentially expressed genes (DEGs) among the workers, soldiers, and alates were conducted using edgeR. The false discovery rate (FDR) was used to determine the threshold for the p value following multiple tests, and the $FDR \leq 0.05$ threshold and an absolute value of the

$\log_2 \text{Ratio} \geq 1$ were used to judge the significance of the gene expression differences in the analysis.

Quantitative Real-time PCR

The heads of 20 individuals of larvae, workers, soldiers, nymphs, and alates were used for RNA extraction, respectively (three biological replications). Total RNA was obtained from their heads using RNAiso Plus reagent (TaKaRa Bio. Inc., Japan). From 50 ng mRNA, cDNA was synthesized. cDNA for qPCR was synthesized using Primer Script RTase (Takara Bio. Inc., Japan). The quantitative reaction was performed on a Light Cycler 480 with software version 1.2.0.0625 (Roche Diagnostics, Switzerland). The

gene-specific primers were designed by Primer 5.0 (Supp Table 1 [online only]). The beta-actin gene and the primer sequences of beta-actin used in this study were well validated in termite *R. aculabialis* and *R. labralis* Hsia et Fan (Isoptera: Rhinotermitidae) (Rasheed et al. 2019, Ye et al. 2019). RT-qPCR was performed in 20- μ l reactions containing 10 μ l of SYBR Premix Ex Taq™ II (TaKaRa Bio. Inc., Japan), 6 μ l of nuclease-free water, 0.8 μ l of forward primer, 0.8 μ l of reverse primer, 0.4 μ l of ROX reference dye, and 2 μ l of cDNA. All reactions in the qPCR framework were standardized utilizing Ct values relating to beta-actin (a reference gene) levels as indicated by an investigation of dependable reference qualities for articulation based on *R. aculabialis*. Relative quality articulations were determined by utilizing the $2^{-\Delta\Delta C_t}$ method. For each sample, three technical replicates were performed. Differences in the expression levels of these genes among workers, soldiers, alates, nymphs, and larvae were tested for significance by one-way ANOVA, with means separated using the least significant difference (LSD) using R statistical software. The values are presented as the mean \pm SD.

Results

Antennal Morphology of Workers, Soldiers, Alates, Nymphs, and Larvae

Termites have a pair of antennae that serve as major chemosensory organs, containing many sensilla on the flagellar regions of the antenna. Each antenna consists of three basic parts: a scape, a pedicel, and a flagellum. The flagellum was composed of a number of subsegments (Fig. 3A). The numbers of antennal segments in alates, nymphs, soldiers, workers, and larvae of *R. aculabialis* were 17–18, 15–17, 13–17, 13–16, and 10–13, respectively ($n = 10$). The lengths of antennae in alates ($1889.5 \pm 143.4 \mu\text{m}$) and nymphs ($1732.5 \pm 170.5 \mu\text{m}$) were significantly longer than those in workers ($1344.9 \pm 150.5 \mu\text{m}$), soldiers ($1389.6 \pm 213.3 \mu\text{m}$), and larvae ($552.9 \pm 55.6 \mu\text{m}$) ($F = 90.72$; $df = 4,45$; $p < 0.0001$) (Fig. 2).

Types of Antennal Sensilla in Workers, Soldiers, Alates, Nymphs, and Larvae

In total, nine different morphological types of sensilla were identified on the antennae of *R. aculabialis*: sensilla chaetica, sensilla trichodea, spherical sensilla, sensilla furcella, Böhm sensilla, dome-shaped sensilla, sensilla basiconica, sensilla coeloconica, and sensilla campaniformia. Soldiers possessed all 9 types. Workers, nymphs, and alates had no sensilla campaniformia, and larvae had no sensilla

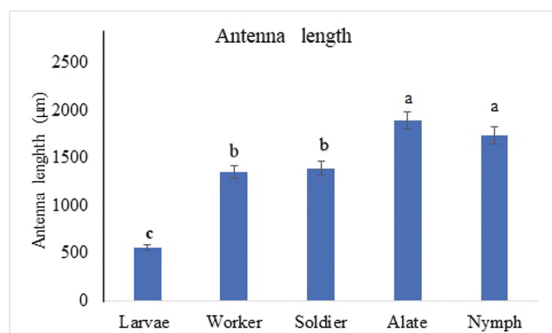


Fig. 2. Antenna lengths of larvae, workers, soldiers, nymphs, and alates of *R. aculabialis*. The x-axis indicates different individuals, and the y-axis represents antenna length. The columns represent the means; bars represent the standard deviation of the mean ($n = 10$). Different letters above each bar indicate significantly different groups. $p < 0.05$ was considered significant.

campaniformia or dome-shaped sensilla (Table 1). Sensilla chaetica formed the longest antenna bristles, which tapered towards the tip. They were inserted in a cuticular socket at the base and were movable (Fig. 3A and B). Sensilla trichodea were long and tapered gradually from base to tip, bent towards the antenna, and had no cuticular socket at the base (Fig. 3B). Spherical sensilla were round like a ball (Fig. 3C). Sensilla furcella had one or two short furcella with a bulbous base (Fig. 3D). Böhm sensilla were short, smooth, and sharp-tipped, with thorn-like bristles (Fig. 3E). Sensilla basiconica were elongated pegs with blunt apical parts (Fig. 3F). The sensillum featured a smooth dome in the middle of a round cuticular collar (Fig. 3G). Sensilla coeloconica had comparatively deep pits (Fig. 3H). Sensilla campaniformia was sunken and had a round cuticular collar in the antennal tip of soldiers (Fig. 3I).

Transcriptome Sequence and De Novo Assembly

Using mRNA extracted from workers, soldiers, and alates of *R. aculabialis*, we built an RNA sequencing library. Ultimately, 43,531,652 clean reads were generated, with an average length of 831 bp via HiSeq 2,000 paired-end sequencing of *R. aculabialis*. Minimum and maximum lengths, extending from 201 bp to 44,333 bp for a total of 89,475 unigenes, were gathered using the Trinity program. The N50 length was 1319 bp, and 45.36% GC content was observed, which remained constant. Average quality values ≥ 20 were acquired for over 98.32% of cycles. The total assembled unigene lengths ranged from 273 bp to 21,363 bp. There were 21,363 unigenes (23.88%) with lengths ranging from 300 to 400 bp, 15,859 unigenes (17.72%) with lengths ranging from 401 to 500 bp, 32,754 unigenes (36.61%) with lengths ranging from 501 to 1,000 bp, 11,533 unigenes (12.89%) with lengths ranging from 1,001 to 2,000 bp, and 7,966 unigenes (8.90%) with lengths ranging from 2,001 to $>3,000$ bp. These outcomes indicated that sequencing yield and quality were adequate for further examination. In total, 40,972 unigenes were successfully annotated to Nr, Swiss-Prot, KEGG, or KOG databases. A large number of *R. aculabialis* unigenes closely matched the insect genomes, especially termite *Z. nevadensis*. We identified 10,210 DEGs (upregulated and downregulated genes) among workers, soldiers, and alates. There were 3,494 DEGs in ‘workers vs alates’, 4,123 DEGs in ‘soldiers vs alates’, and 2,593 DEGs in ‘soldiers vs workers’ (Fig. 4). All raw sequence reads have been deposited in the NCBI SRA database and are accessible through SRA accession number SRP199695.

Olfactory Genes of *R. aculabialis*

Our analysis of the RNA-seq data of *R. aculabialis* identified 16 olfactory genes. Of these 16 olfactory genes, 8 genes were similar to the olfactory genes of the termite *Z. nevadensis*, 6 genes were similar to the olfactory genes of *Blattella germanica* (L.) (Blattaria: Blattellidae), and two genes were similar to the olfactory genes of *Bemisia tabaci* Gennadius (Homoptera: Aleyrodidae). These olfactory genes included 6 chemosensory protein (CSP) genes and 10 odorant-binding protein (OBP) genes, including 8 general odorant-binding protein genes (GOBPs), and two pheromone-binding protein-related protein (PBP) genes (Table 2). CSPs and OBPs are responsible for olfaction in insects, which is important for both insect survival and reproduction.

Expression Levels of CSP Genes in Workers, Soldiers, Larvae, Nymphs, and Alates

We performed qRT-PCR analysis on the relative expression levels of 16 olfactory genes. Five CSP genes were more highly expressed

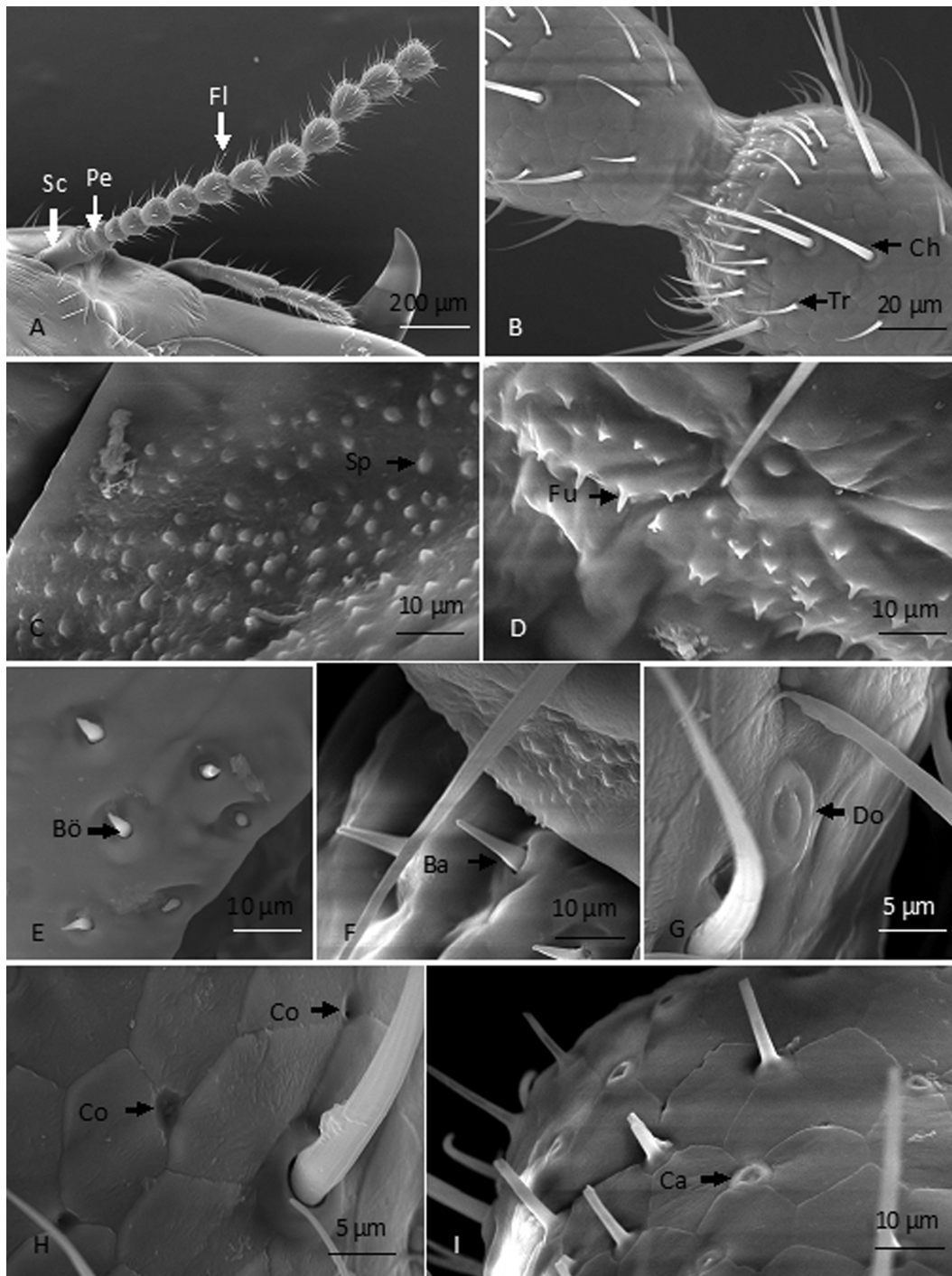


Fig. 3. Morphology of antennal sensilla in *R. aculabialis*. (A) The antenna had three basic parts: a scape, a pedicel, and a flagellum. The flagellum was composed of a number of subsegments. (B) Sensilla chaetica and sensilla trichodea. (C) Spherical sensilla. (D) Sensilla furcella. (E) Böhm Sensilla. (F) Sensilla basiconica. (G) Dome-shaped sensilla. (H) Coeloconic sensilla. (I) Sensilla campaniformia. Sc: scape; Pe: pedicel; Fl: flagellum; Ch: sensilla chaetica; Tr: sensilla trichodea; Sp: spherical sensilla; Fu: sensilla furcella (furcella-shaped sensilla); Bö: Böhm sensilla; Ba: sensilla basiconica; Do: dome-shaped sensilla; Co: coeloconic sensilla; Ca: sensilla campaniformia.

in alates than in workers, soldiers, larvae, and nymphs. The expression levels of *CSP1* were approximately 3-, 1-, and 12-fold higher in alates than in workers, soldiers, and larvae, respectively, while the expression levels of *CSP1* in nymphs were nearly undetectable. The expression levels of *CSP2* were approximately 24-, 11-, 12- and 391-fold higher in alates than in workers, soldiers, larvae, and nymphs, respectively. The expression levels of *CSP3* exhibited significant

differences in alates, with 10-, 6-, 7-, and 52-fold higher expression levels compared to workers, soldiers, larvae, and nymphs, respectively ($F = 67.57$; $df = 4,10$; $p < 0.0001$). The expression levels of *CSP4* were approximately 6-, 7-, and 37-fold higher in alates than in soldiers, larvae, and nymphs, respectively. The expression levels of *CSP4* in workers were negligible. The expression levels of *CSP5* were 1-, 16-, 16-, and 628-fold higher in alates than in workers, soldiers,

Table 1. Types of antennal sensilla in *R. aculabialis*

Samples	Sensilla chaetica	Sensilla trichodea	Spherical sensilla	Sensilla furcella	Böhm Sensilla	Dome-shaped sensilla	Sensilla basiconica	Sensilla coeloconica	Sensilla campaniformia
Workers	+	+	+	+	+	+	+	+	-
Soldiers	+	+	+	+	+	+	+	+	+
Nymphs	+	+	+	+	+	+	+	+	-
Alates	+	+	+	+	+	+	+	+	-
Larvae	+	+	+	+	+	-	+	+	-

Presence (+) or absence (-) of sensillum types on antennae of different samples.

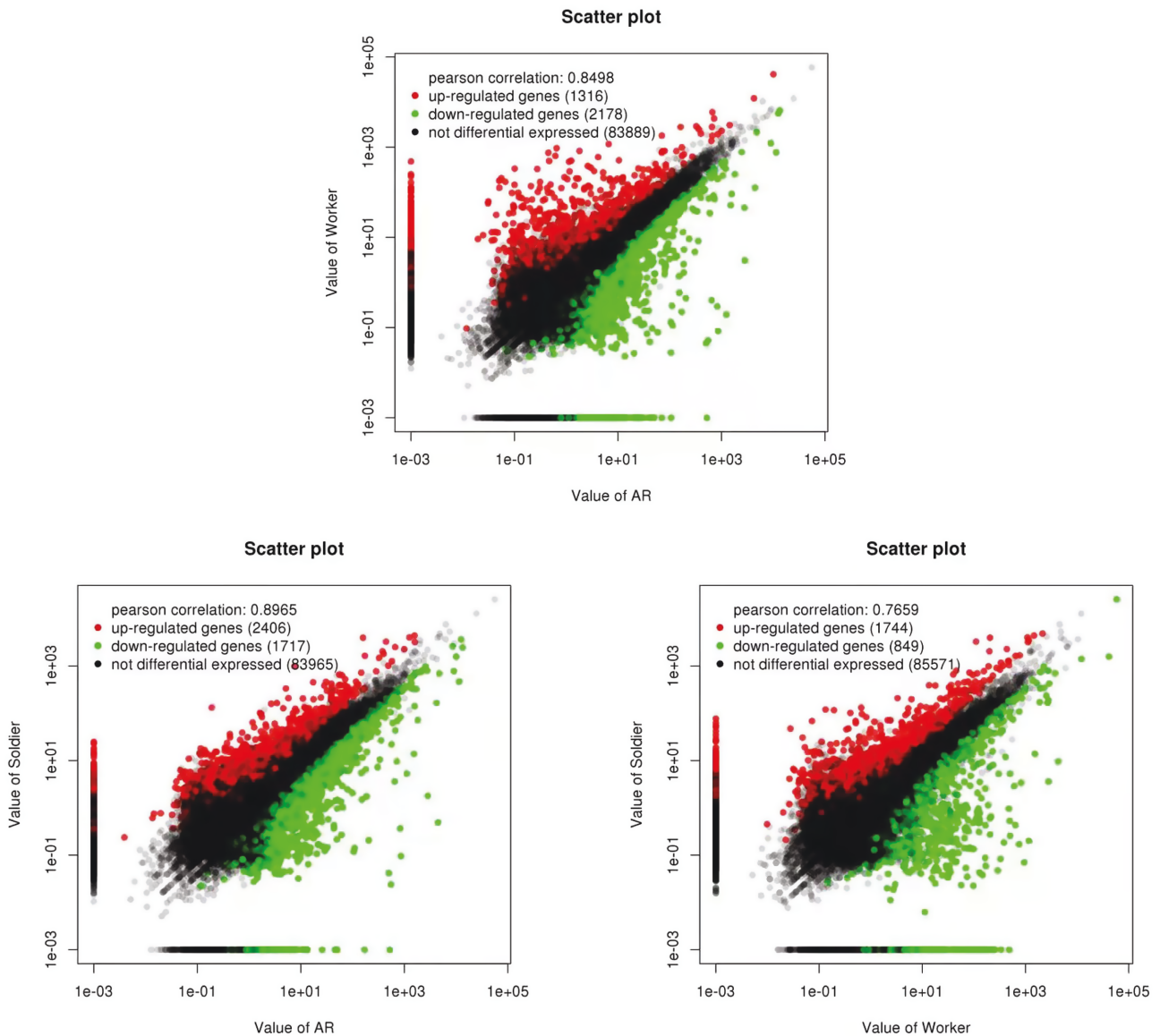


Fig. 4. The scatter plot of the differentially expressed genes in workers, soldiers, and alates. Red and green scatter plot represent upregulated and downregulated genes, respectively. Black scatter plot represents that the genes were not differential expressed. The parameters $FDR \leq 0.001$ and $\log_2\text{Ratio} \geq 1$ were used as the thresholds to judge the significance of gene expression differences.

larvae, and nymphs, respectively. The expression levels of *CSP6* were significantly higher in nymphs than in workers, soldiers, alates, and larvae ($F = 9.43$; $df = 4, 10$; $p = 0.002$), with no significant differences in expression levels among workers, soldiers, larvae, and alates ($p > 0.9486$) (Fig. 5).

Expression Levels of OBP Genes in Workers, Soldiers, Larvae, Nymphs, and Alates

We also performed qRT-PCR analysis of 10 OBP genes, including 8 GOBP and 2 PBP genes. Our results indicated that 7 GOBPs exhibited higher expression levels in alates than in workers,

Table 2. 16 olfactory protein genes of *R. aculabialis* were identified and annotated

Unigene ID	Annotation
Unigene 0017650	Chemosensory protein [<i>Blattella germanica</i>]
Unigene 0034266	Chemosensory protein [<i>Blattella germanica</i>]
Unigene 0065462	Chemosensory protein [<i>Blattella germanica</i>]
Unigene 0075670	Chemosensory protein [<i>Blattella germanica</i>]
Unigene 0012196	Chemosensory protein [<i>Blattella germanica</i>]
Unigene 0073794	Chemosensory protein [<i>Blattella germanica</i>]
Unigene 0003742	General odorant-binding protein 19a [<i>Zootermopsis nevadensis</i>]
Unigene 0007349	General odorant-binding protein 19a [<i>Zootermopsis nevadensis</i>]
Unigene 0011105	General odorant-binding protein 19a [<i>Zootermopsis nevadensis</i>]
Unigene 0011248	General odorant-binding protein 19a [<i>Zootermopsis nevadensis</i>]
Unigene 0085557	General odorant-binding protein 19a [<i>Zootermopsis nevadensis</i>]
Unigene 0045066	General odorant-binding protein 7 [<i>Bemisia tabaci</i>]
Unigene0045067	General odorant-binding protein 7 [<i>Bemisia tabaci</i>]
Unigene 0084274	General odorant-binding protein 1 [<i>Zootermopsis nevadensis</i>]
Unigene 0078046	Pheromone-binding protein-related protein 3 [<i>Zootermopsis nevadensis</i>]
Unigene 0079389	Pheromone-binding protein-related protein 3 [<i>Zootermopsis nevadensis</i>]

soldiers, larvae, and nymphs. There were no significant differences in the expression levels of *GOBP2* (Unigene 0007349) among workers, soldiers, alates, and larvae ($p > 0.6592$), but the expression levels in nymphs were significantly lower ($F = 7.20$; $df = 4,10$; $p = 0.0053$). The expression levels of *PBP1* and *PBP2* in alates were approximately 3-, 10-, 9-, and 21-fold and 7-, 5-, 8-, and 11-fold higher than those in workers, soldiers, larvae, and nymphs, respectively, and significant differences were present in alates compared to levels in workers, soldiers, larvae, and nymphs ($F = 13.82$, $df = 4,10$, $p = 0.0004$; $F = 191.50$, $df = 4,10$, $p < 0.0001$, respectively), with no significant difference in the expression levels among workers, soldiers, larvae, and nymphs ($p > 0.6062$, $p > 0.3145$, respectively) (Fig. 6).

Discussion

In this study, we observed that different castes of *R. aculabialis* exhibited distinct types of antennal sensilla, with soldiers having more types of sensilla compared to workers and alates. Interestingly, although the soldiers were developed from workers via two moults, the types of antennal sensilla significantly changed, and sensilla campaniformia was added to the soldiers. The antennal sensilla of insects can play roles in locating sexual counterparts and host plants (Rani et al. 2021). Antennal sensilla of termites have gustatory, chemosensory, mechanosensory, and hygrosensory receptors (Rocha et al. 2007, Yanagawa et al. 2009). Previous studies have shown that sensilla chaetica, sensilla trichodea, and sensilla basiconica have olfactory functions (Yanagawa et al. 2009, Wang et al. 2018). In this

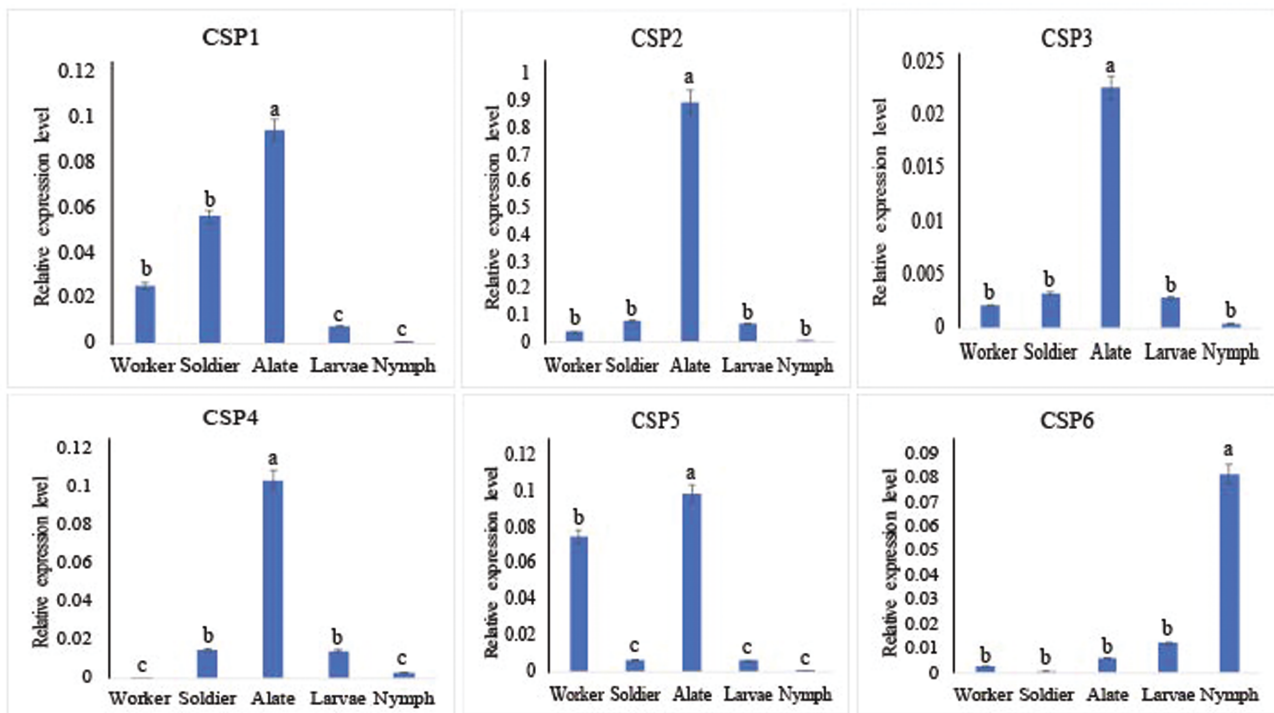


Fig. 5. The relative expression levels of CSP genes in workers, soldiers, larvae, nymphs, and alates of *R. aculabialis* by qRT-PCR analysis. The columns represent the means; bars represent the standard deviation. Different letters on each bar indicate a significant difference ($p < 0.05$). CSP, chemosensory protein; CSP1, Unigene 0017650; CSP2, Unigene 0034266; CSP3, Unigene 0065462; CSP4, Unigene 0075670; CSP5, Unigene 0012196; CSP6, Unigene 0073794.

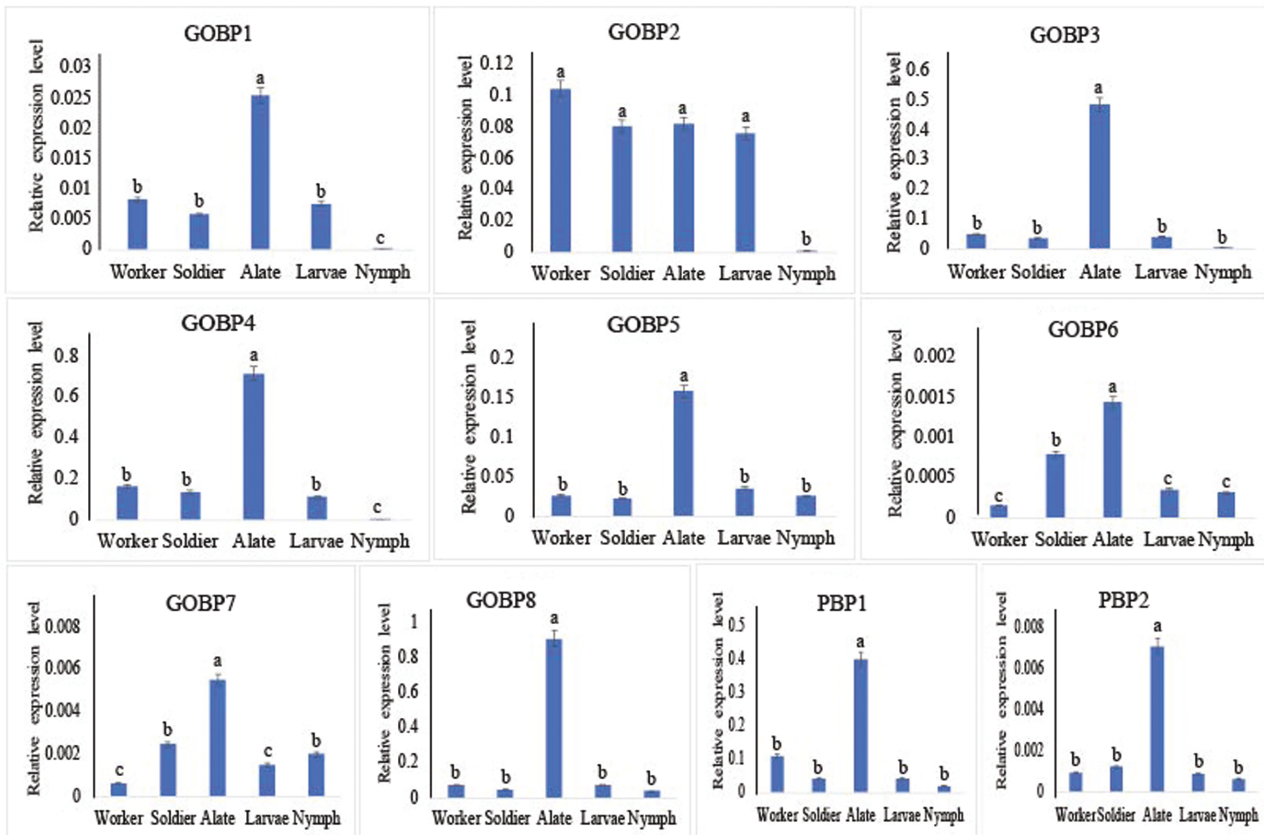


Fig. 6. The relative expression levels of OBP genes in workers, soldiers, larvae, nymphs, and alates of *R. aculabialis* by qRT-PCR analysis. The columns represent the means; bars represent the standard deviation. Different letters on each bar indicate a significant difference ($p < 0.05$). GOBP, general odorant-binding protein; GOBP1, Unigene 0003742; GOBP2, Unigene 0007349; GOBP3, Unigene 0011105; GOBP4, Unigene 0011248; GOBP5, Unigene 0085557; GOBP6, Unigene 0045066; GOBP7, Unigene 0045067; GOBP8, Unigene 0084274; PBP, pheromone-binding protein-related protein; PBP1, Unigene 0078046; PBP2, Unigene 0079389.

study, we found that termite castes exhibited different types of sensilla that might perform distinct functions. In alates and workers, 8 types of sensilla were observed. A previous study revealed that males detect sex pheromones produced by females with the help of sensilla basiconica in the cigarette beetle *Lasioderma serricorne* Fabricius (Coleoptera: Anobiidae) (Okada et al. 1992). In social insects, queen pheromones represent the queen's fecundity to workers and prevent them from becoming reproductively active (Oi et al. 2015). Furthermore, sensilla basiconica act as the site for the synthesis of chemicals that are involved in mate recognition (Palma et al. 2019). In *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae), sensilla chaetica of workers detects different fungal odors that affect the elicitation of grooming behaviors (Yanagawa et al. 2009). In termite colonies, soldiers have only one function – to defend their colony – for which they can use chemical defenses. Our findings suggest that soldiers have sensilla campaniformia in the antennal tip, indicating that sensilla campaniformia is related to defense.

By transcriptome analysis of *R. aculabialis*, we identified 6 CSP genes and 10 OBP genes. OBPs are divided into two subfamilies, GOBPs and PBPs. In the olfactory system of insects, OBPs and CSPs capture volatile compounds from the outside environment and are sent to the olfactory receptor. These olfactory systems are crucial for the reproduction and survival of insects (Leal 2013). In *Z. nevadensis*, 29 OBPs were identified from genomic data, and one of them was specifically expressed in alates (Terrappon et al. 2014). Based on the transcriptomic data, nine OBPs and three CSPs were identified in *R. speratus*, and the expression levels of six OBPs and two CSPs were

higher in workers and soldiers than in alates. However, in this study, the verification of gene expression levels was not performed by qRT-PCR (Mitaka et al. 2016). Generally, GOBPs sense plant volatiles, but they also act as a sex pheromone-binding component (Zhou et al. 2008). Research conducted by Gu et al. (2019) reported that in *Clostera restituta* Walker (Lepidoptera: Notodontidae), the GOBP2 gene exhibited 2-fold higher expression levels in the antennae of females than in the antennae of males, and two pheromone-binding proteins (PBP1, PBP2) and OBPs (OBP9, 10 and 16) were highly expressed in male antennae compared to females. Their results suggest that these proteins might be involved in host and mate recognition and foraging in *C. restituta*. The general odorant-binding protein genes *GOBP1* and *GOBP2* are highly expressed on the antennae of female *Amyelois transitella* Walker (Lepidoptera: Pyralidae) and may act as female attractants (Leal et al. 2005, Leal et al. 2009). In the transcriptomes of *R. speratus*, only six genes were differentially expressed between the female and male primary reproductives in heads. Therefore, the researchers focused on the genes that were differentially expressed among castes (reproductives, workers, and soldiers) (Shigenobu et al. 2022). In this study, the expression levels of primarily GOBPs were higher in alates than in workers, soldiers, nymphs, and larvae, indicating that the GOBPs are related to flying alates that leave their colonies and that these individuals use these proteins for pairing and the establishment of new colonies. Previous studies have provided evidence that GOBPs bind to female pheromones in the silk moths *Antberaea* (Lepidoptera: Saturniidae) and *Bombyx* (Lepidoptera: Bombycidae), which are present in both

male and female antennae, and GOBP sensing sensilla are sensilla basiconica (Steinbrecht et al. 1995, Ziegelberger 1995, Zhou et al. 2008). We suggest that GOBPs in the termite *R. aculabialis* are likely to be involved in sensing general odorants and plant volatiles, acting as attractants and binding to sex pheromones.

Our study reveals that the expression levels of the pheromone-binding genes *PBP1* and *PBP2* are significantly higher in alates, indicating that alates use PBPs to find suitable mates to perform their reproductive activities. PBPs have been found in long trichoid sensilla of the male antenna in *Bombyx mori* Linnaeus (Lepidoptera: Bombycidae) and *Antheraea polyphemus* Cramer (Lepidoptera: Saturniidae) (Steinbrecht et al. 1995). Sex pheromones are species-specific and consist of a hydrocarbon chain that contains an oxygenated functional group, such as an ester, aldehyde, alcohol, or epoxide, primarily involved in the recognition of insect sex pheromones (Tillman et al. 1999, Grobe-Wilde et al. 2006). A previous study reported that all pheromone-binding proteins and general odorant-binding protein genes in *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae) have conserved exon and intron splice sites and intron numbers. These are antennal genes, and these genes bind to sex pheromones, although their binding capacity is different. Despite the binding of these genes to sex pheromones, all five PBPs and GOBPs were found to bind some plant volatiles with significant affinities, and PBPs sense some plant volatiles (Liu et al. 2015a). Poivet et al. (2012) suggested that in *Spodoptera littoralis*, PBPs are used to detect sex pheromones absorbed or present on the egg, and when the female moth lays eggs on the leaves of the plant, this pheromone sensation is used by larvae in their search for food. The larvae of *Sesamia inferens* also use PBPs to search for food (Zhang et al. 2013).

In this study, we identified 6 CSP genes in *R. aculabialis* and found that most CSP genes (*CSP1*, 2, 3, 4, and 5) were expressed at higher levels in alates. In the ant *Camponotus japonicus* Mayr (Hymenoptera: Formicidae), transcriptome analysis revealed 12 CSP genes, and the CSP genes were differentially expressed among castes (Hojo et al. 2015). CSPs act as carrier proteins and aid in olfaction by transporting lymph (Pelosi et al. 2005, Foret et al. 2006, Maleszka et al. 2007). CSPs also take part in gustation and the olfactory system of insects, similar to OBPs, by carrying hydrophobic ligands (Pelosi et al. 2005, Foret and Maleszka 2006). Some CSPs are also expressed at particular developmental stages, such as eggs and embryos (Wanner et al. 2005, Foret et al. 2006). CSP5 in the honeybee *Apis mellifera* Linnaeus (Hymenoptera: Apidae) was shown to act as a carrier protein and was used for synthesis of the embryonic integument. If the expression of CSP5 was blocked in the embryo by double-stranded RNA, it resulted in many abnormalities in all regions of the body (Maleszka et al. 2007). CSPs are expressed in sensory and nonsensory tissue of insects, indicating that these proteins may be involved in functions other than chemosensation, including female survival, reproduction, regeneration of limbs, and embryonic development (Nomura et al. 1992, Maleszka et al. 2007, Guo et al. 2011, Gu et al. 2012, Zhang et al. 2012, Gong et al. 2012). CSPs exhibited differential expression patterns among different castes of *R. aculabialis*, indicating that CSPs are involved in the physiological functions of different castes.

Conclusions

In summary, nine types of sensilla were identified on the antennae of *R. aculabialis*. We found 16 olfactory genes, including 6 chemosensory protein (CSP) and 10 odorant-binding protein (OBP) genes by transcriptome analysis of *R. aculabialis*. The expression levels of

most of olfactory genes were significantly higher in alates than in workers, soldiers, larvae, and nymphs by qRT-PCR. Different castes of *R. aculabialis* exhibited distinct types of antennal sensilla and expression patterns of olfactory genes. These results establish a foundation for future studies on the functions of antennal sensilla and olfactory genes in *R. aculabialis*, in order to understand how the olfactory system of termites is involved in the adaptability of social organizations.

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Author Contributions

Conceptualization, X.S., C.Y. and N.S.; methodology, C.Y. and N.S.; formal analysis, C.Y., N.S. and W. Z.; investigation, N.S., C.Y., W.Z., T.W., Y.W., X.Z., Z.S. and L.X.; writing – original draft, N.S., C.Y. and X.S.; writing review, X.S.; editing, C.Y.

Data Availability

All raw sequence reads have been deposited in the NCBI SRA database and are accessible through SRA accession number SRP199695. The assembled gene sequences have been deposited in the NCBI TSA database under accession number GHMS00000000.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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