

# Sex-biased olfactory gene expression in the antennae of the adzuki bean beetle, *Callosobruchus chinensis*

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## Abstract

The adzuki bean beetle, *Callosobruchus chinensis* (L.) (Coleoptera Bruchidae), is a major pest of economically important leguminous grains. Since chemosensory genes play key roles in insect behaviour, they can potentially be used as new targets for pest control. However, the molecular basis of olfaction is unknown in *C. chinensis*. In this study, we sequenced the antennal transcriptomes of male and female *C. chinensis* and estimated of gene expression showed differences in up- and down-regulation of 117 and 98 genes, respectively, between female and male antennae. Among these genes, 1 odorant binding protein (OBP), 1 sensory neuron membrane protein (SNMP) and 1 gustatory receptor (GR) are male antennae-specific expression, whereas 2 OBPs, 23 odorant receptors (ORs) and 14 GRs are female antennae-enriched expression. Quantitative real-time RT-PCR analyses validated strong male antennal-biased expression of seven genes (OBP3, OR1, OR11, OR64, OR89, OR12, and GR5), whereas five genes (OBP4, OR7, OR5, GR15, and SNMP1) were especially expressed to dominantly expressed in female antennae. Sex biases gene expression described here provides important insights in gene functionalization. Meanwhile, our work allows for further functional studies of pheromone and general odorant detection genes, possible meaningful targets for pest management.

**Key words:** differentially expressed gene, transcriptomes, RNA-Seq, olfactory, sex-biased expression.

## Introduction

The adzuki bean weevil, *Callosobruchus chinensis* (L.) (Coleoptera Bruchidae), is a major pest of economically important leguminous grains, such as cowpeas, mung bean, soybean and other important beans. It is widely distributed across the tropical and subtropical regions of the world (Duan *et al.*, 2016). Bruchid infestations start in the field, and in most cases, continue in storage. The females lay their eggs directly on the surface of the pods in the field or seeds in storage, and the larvae then penetrate and feed on the beans (Zhang *et al.*, 2017). Due to the high fertility, ability to re-infest and short generation times, even low initial infestation rates can lead to tremendous damage (Yamane *et al.*, 2013). It causes not only quantitative but also qualitative losses like nutritive loss and germination loss (Mainali *et al.*, 2015).

For a long time, the prevention and control of the beetles has been mainly depended on chemical pesticide which brings about potential danger to health of people and environment. Many strategies to delay or prevent the onset of resistance to chemical insecticides (Hsu *et al.*, 2006; Jin *et al.*, 2011; Liu *et al.*, 2016), and olfactory-mediated methods of control are preferred. The olfactory recognition system of insect is extremely important in wide ranges of behaviours including feeding, mating, reproduction, and predator avoidance, all of which are paramount for their survival (Leal *et al.*, 2013; Li *et al.*, 2015). Studies of the molecular mechanisms of the olfactory system have provided new prospects for integrated pest management (Hu *et al.*, 2016).

The insect antenna is a highly sophisticated sensory organ, usually covered with several different types of sensilla for the detection of signals coming from the environment

(Ortega *et al.*, 2019). Diverse olfactory proteins involved in olfaction include odorant-binding proteins (OBPs), odorant receptors (ORs), chemosensory protein (CSPs), sensory neuron membrane proteins (SNMPs) and ionotropic receptors (IRs). They play an important role in the signal transduction process. OBPs and CSPs are both binding-protein families, and they are involved in the first step of recognition of chemical signals. After being released from the OBPs or CSPs, the signals are then transferred to the receptors, ORs and IRs. After the activation of the olfactory receptor, the semiochemicals are removed and degraded by the odorant-degrading enzymes (Leal, 2013). The SNMPs are expressed in the olfactory sensory neurons and hypothesize to play an important role in olfaction and gestation (Liu *et al.*, 2013). Olfactory-related genes have been identified based on sequence similarity to previously reported genes from several Coleoptera species (Mitchell *et al.*, 2012; Andersson *et al.*, 2013; Chen *et al.*, 2014; Gu *et al.*, 2015; Antony *et al.*, 2016; Bin *et al.*, 2017), yet their precise functions remain largely unclear. Examination of gene expression profiles has been treated as an important indicator of chemosensory genes function, particularly the tissue or sex distribution (Gong *et al.*, 2009; Olivier *et al.*, 2011).

Next-generation sequencing provides a platform for measuring gene expression in a manner that is more sensitive than traditional hybridization experiments (Wilhelm and Landry, 2009). It has become widely used to detect differentially expressed genes (DEGs) in variety of organisms in recent years (Xu *et al.*, 2013; Dai *et al.*, 2013; Yang *et al.*, 2015; Jin *et al.*, 2016; Zhang *et al.*, 2017). We reported the antennal transcriptomes and a total of 140 olfactory genes were identified from antennal transcriptome of *C. chinensis*. In order to search differential expression of

olfactory genes closely associated with sex, here, we present a further analysis of DEGs in the antennal transcriptome of male and female *C. chinensis*. Moreover, quantitative real-time PCR (qPCR) is performed to verify the expression values of DEGs. The results will provide a foundation for future functional studies of olfactory genes which might be meaningful targets for pest management in *C. chinensis*.

## Materials and methods

### Insect rearing and antennae collection

*C. chinensis* were obtained from a laboratory colony at the Institute of Plant Protection, Shanxi Agricultural University. They were fed on mung beans at  $26 \pm 1$  °C, RH  $75 \pm 5\%$ , 16L/8D photoperiod. Exactly 200 newly emerged beetles (100 males and 100 females; 1-2-d-old; unmated) were collected (three biological replicates of each sample). Antennae of adult were dissected (males and females were dissected separately) using micro scissors and were immediately frozen in liquid nitrogen, then stored at  $-80$  °C until required for later analyses. Antennae of both sexes were also collected in the same way again for subsequent RT-qPCR validation of gene expression.

### RNA extraction

Total RNA was isolated from antennas using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. After digestion of residual DNA with DNase I, the concentration and the quantity of RNA were determined using Qubit 2.0 Fluorometer (Life) and 1.2% agarose electrophoresis. The integrity of the isolated total RNA was assessed using an Agilent 2100 bioanalyzer (Agilent). Extracted total RNA was stored at  $-70$  °C for later use.

### cDNA library preparation and sequencing

Total RNA samples of male and female antennae in three biological replicates were prepared. Then they were submitted to Biomarker Technology Company (Beijing, China) for cDNA library preparation and sequencing. The cDNA library was sequenced using the Illumina HiSeq 2500 platform.

### Sequence analysis and assembly

Clean reads were obtained by removing sequencing joints, poly-N regions, and low-quality reads. The Q20, Q30, GC content, and repetitive sequences were calculated, and all downstream analyses were performed on high-quality clean data (Q30 > 85%). The transcriptome assembly program Trinity (Grabherr *et al.*, 2011) was used to generate transcripts, based on clean and high-quality data. Next, the contigs were assembled into transcripts by performing pair-end joining and gap filling, and then they were subsequently clustered to obtain unigenes. In this research, all Illumina sequencing data was submitted to the SRA of NCBI (accession number: SRP119884).

### Gene annotation and chemosensory gene identification

Unigene sequences were compared in the following databases using BLAST software to obtain annotation information for all unigenes. Based on BLAST parameters, the unigenes with an E-value of less than  $10^{-5}$  were selected. Sequences of selected unigenes were aligned within databases, including Nr, Swiss-Prot, KEGG, GO, and COG.

To identify the putative chemosensory genes of *C. chinensis*, sequences whose annotations corresponded best to OBPs, ORs, GRs or SNMPs were retained as candidate genes. These genes were manually revised by further analysis using the BLASTn at NCBI. The nucleotide sequences of all olfactory genes that were identified from *C. chinensis* antennal transcriptomes were named according to sequence homology analysis and numbered arbitrarily.

### Analysis of differentially expressed genes

To compare gene expression levels between the male and female RNA libraries in the *C. chinensis*, the relative transcript level of each expressed gene was calculated by the RPKM (Reads Per Kilobase per Million mapped reads) algorithm (Mortazavi *et al.*, 2008). The *P*-value threshold was determined by the FDR to account for multiple test of significance. During this screening criteria, a threshold for FDR of < 0.01 and an absolute expression value of  $\log^2FC$  (fold change)  $\geq 2$  were used to determine significant differences in gene expression. As a result, we acquired all DEGs and their RPKM values. The differentially expressed genes were analysed for hierarchical clustering (Murtagh *et al.*, 2014). After be identified by screening, the DEGs were analysed by GO function enrichment and COG classification analysis.

### Quantitative RT-PCR (qRT-PCR) analysis

qPCR for RNA sequencing samples from different sexes was performed in order to verify the expression values of the differentially expressed genes identified from our transcriptome. Specific primers were designed using Primer Premier 5.0. The gene of  $\beta$ -tubulin was used as an internal control. The qRT-PCR was performed using a SYBR Premix Ex Taq™ Kit (Takara, Dalian, China) according to the manufacturer's instructions. The thermal cycle conditions were as follows: 95 °C for 3 minutes, 40 cycles of 95 °C for 10 seconds, and 58 °C for 30 seconds. Three biological replicates were performed for each tested gene, and three technical replicates were performed for each biological replicate. Negative controls were non-template reactions (replacing cDNA with ddH<sub>2</sub>O). Relative quantification was analysed using the  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

## Results

### Sequence analysis and assembly

After filtering the raw reads, six non-normalized cDNA libraries of the *C. chinensis* antennae were constructed, representing the samples from females and males. The six libraries were sequenced and the clean data reached

**Table 1.** Summary of the raw reads for the six samples of antennal of *C. chinensis*.

Samples	Read Number	Base Number	GC (%)	Q30 (%)
T1-1	24,616,293	7,199,400,096	41.54	86.40
T1-2	27,446,452	8,042,179,414	41.50	85.04
T1-3	32,111,589	9,404,493,446	42.12	85.46
T2-1	30,956,077	9,081,198,314	41.49	85.66
T2-2	31,744,803	9,314,790,022	41.53	85.93
T2-3	27,459,060	8,052,995,494	42.43	86.19

T1-1, T1-2, T1-3: biological repetition samples of female; T2-1, T2-2, T2-3: biological repetition samples of male.

**Table 2.** An overview of the sequencing and assembly process.

Length Range	Transcript	Unigene
200-300	37,151 (28.90%)	33,820 (40.49%)
300-500	25,095 (19.52%)	20,159 (24.13%)
500-1000	23,543 (18.32%)	14,481 (17.34%)
1000-2000	20,081 (15.62%)	7,926 (9.49%)
2000+	22,658 (17.63%)	7,149 (8.56%)
Total Number	128,528	83,535
Total Length	148,371,160	63,587,354
N50 Length	2,397	1,492
Mean Length	1154.39	761.21

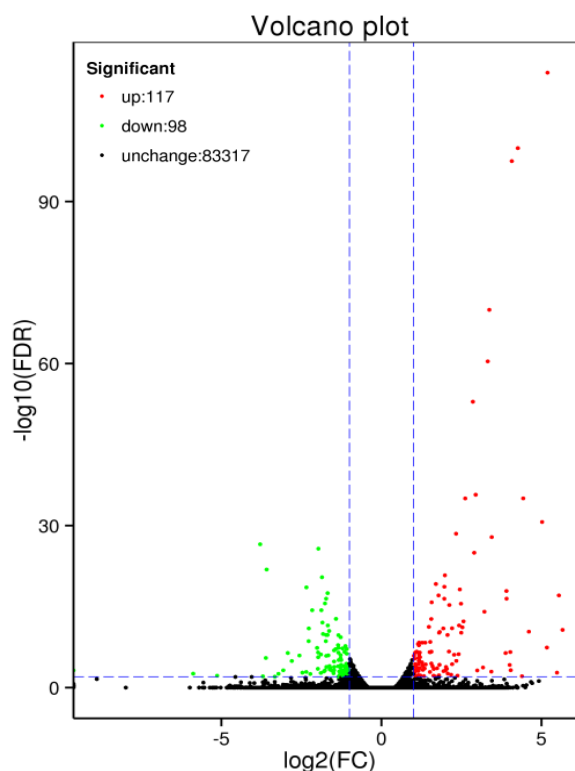
7.2 Gb in each library (table 1). More than 85.04% of the data yielded a high-quality score (Q30). GC content from these clean data reached more than 41.49%. These clean reads were assembled into 128,528 transcripts. After merging and clustering, 83,535 unigenes were acquired with a mean length of 761 nt and N50 length of 1,492 nt. Among these unigenes, 29,556 (35.39%) were longer than 500 bp and 15,075 (18.05%) were longer than 1 kb (table 2).

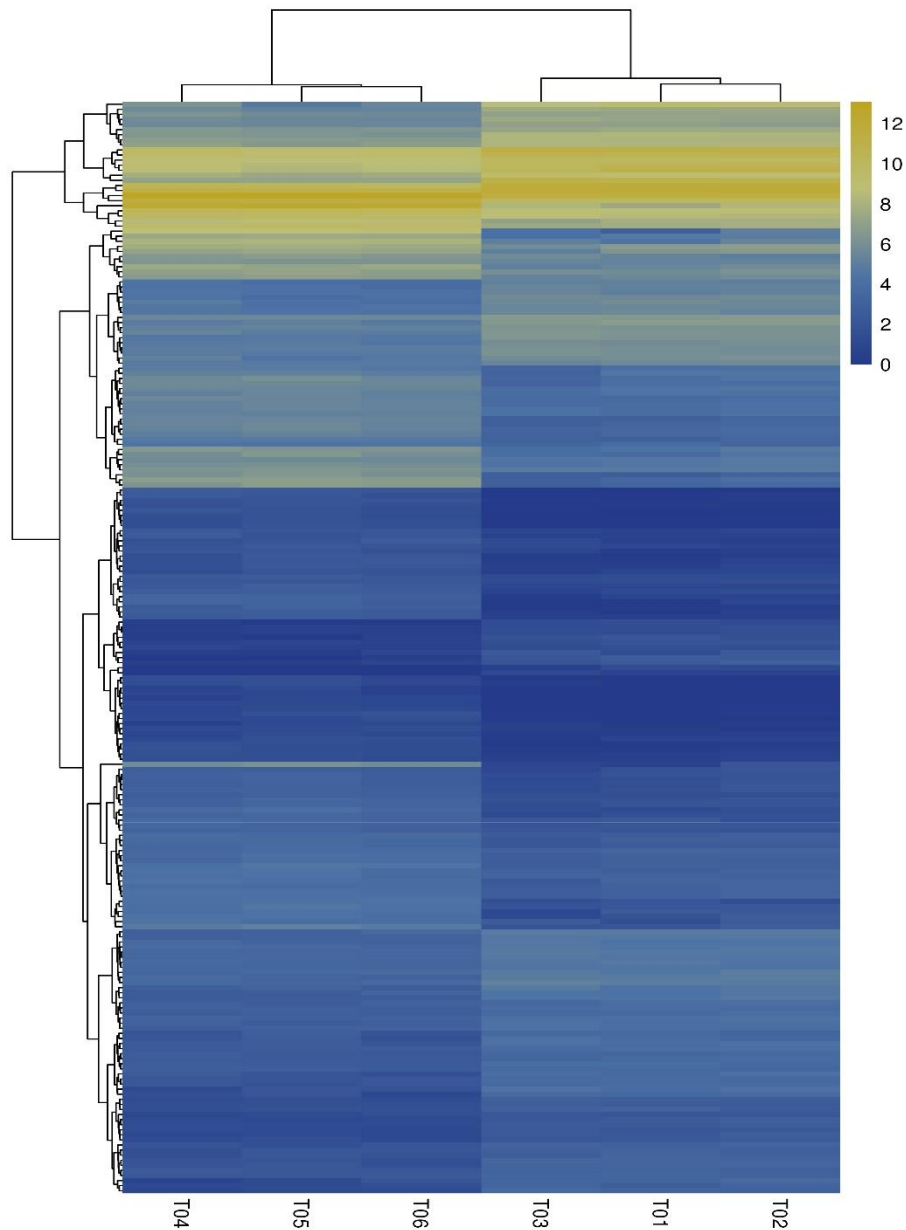
### Functional annotation of genes

To annotate the unigenes, blastx alignment against the databases of nr, GO, KEGG, Swiss-Prot, and COG was performed. The result showed that a total of 22,148 known unigenes were identified by blastx, which represents 26.51% of all genes (table 3). Among these genes, there were 18,744 (22.4%) nr database annotated unigenes. The Swissprot database contained 10,325 unigenes (12.36%). A total of 140 olfactory genes, including 12 OBPs, 116 ORs, 4 CSPs, 7 SNMPs and 1 IR, were identified from antennal transcriptome of *C. chinensis*.

**Table 3.** Annotated unigene of antennal transcriptome of *C. chinensis*.

Annotated database	Number of annotated unigene	Percentage of annotated unigene	300<=length<1000	length>=1000
COG	6,794	8.13	2,133	3,629
GO	9,084	10.87	2,722	5,128
KEGG	8,338	9.98	2,498	4,709
KOG	12,558	15.03	3,673	7,130
Swiss-Prot	10,325	12.36	2,978	6,298
eggNOG	15,787	18.90	6,075	6,324
NR	18,744	22.44	6,146	10,091
All	22,148	26.51	7,604	10,455

**Figure 1.** Volcano plot of DEGs from samples of male and female *C. chinensis*. X-axis values correspond to log<sub>2</sub> (fold change). Y-axis values represent  $-\log_{10}$  (false discovery rate). Green points represent significantly down-regulated genes, black points represent no difference in genes, and red points represent significantly up-regulated genes.



**Figure 2.** Clustering gene expression pattern of DEGs. Each column represents one sample, each row represents one gene, and colours represent the expression level, FPKM with  $\log_2$  in sample genes. The colour from yellow to blue indicates high (yellow) to low (blue) expression levels.

#### Analysis of differentially gene expression

A total of 215 genes, representing 0.96% (215/22,148) of the total unigenes, were significantly differentially expressed in male and female antennae. Among the DEGs in both sexes, 117 genes were up-regulated, and 98 genes were down-regulated in males. Volcano plots were plotted to illustrate the distribution of significantly regulated genes according to the  $\log_2FC$  and FDR (figure 1). To visualize the expression patterns of these DEGs in male and female antennae, a heatmap was constructed using RPKM values (figure 2). The profile of male antennae was obviously different with female antennae. Based on the heatmap, the correlation between biological replicates was well established.

Among all olfactory genes (figure 3), 42 chemosensory-related DEGs were screened, including 3 OBPs, 23

ORs, 15 GRs and 1 SNMP. Among these genes, 1 OBP, 1 SNMP and 1 GR showed male antennal-specific expression, whereas 2 OBPs, 23 ORs and 14 GRs showed female antennal-enriched expression.

#### Functional enrichment analysis of DEGs

Among the DEGs in both sexes, 126 genes could be functionally annotated against public protein databases. Corresponding BLASTx annotations obtained for the putatively 58 male-biased transcripts and 68 female biased antennal transcripts. In order to analyse the DEGs at the functional level, GO enrichment analyses were performed. The 54 DEGs were categorized into 35 functional groups based on sequence homology, which included 18 for biological processes, 9 for cellular components, and 8 for molecular function annotations. Among the 35 functional



classes, cell killing, membrane-enclosed lumen, extracellular regions, extracellular region parts, synapse parts, extracellular matrixes, electron carrier activities and protein binding transcription factor activities presented the largest differences between DEG unigene and all unigenes (figure 4). The 39 DEGs were assigned to the COG database, which could be grouped into 17 categories. The largest category was “general functional prediction only”, followed by “secondary metabolites biosynthesis, transport and catabolism”, and “amino acid transport and metabolism” (figure 5).

### Validation of DEGs by qPCR

To verify the accuracy of the RNA-Seq data, 12 randomly selected chemosensory-related DEGs were validated by qRT-PCR. The twelve DEGs includes seven genes (OBP3, OR1, OR11, OR64, OR89, OR12, and GR5) were highly expressed in male antennae and five genes (OBP4, OR7, OR5, GR15, and SNMP1) were relatively enriched in female antennae. As in the qRT-PCR results, the expression patterns of all the selected genes were completely consistent with transcriptome analysis (figure 6). It indicated that the expression data from RNA-Seq analysis well matched those from qPCR and therefore is reliable.

### Discussion

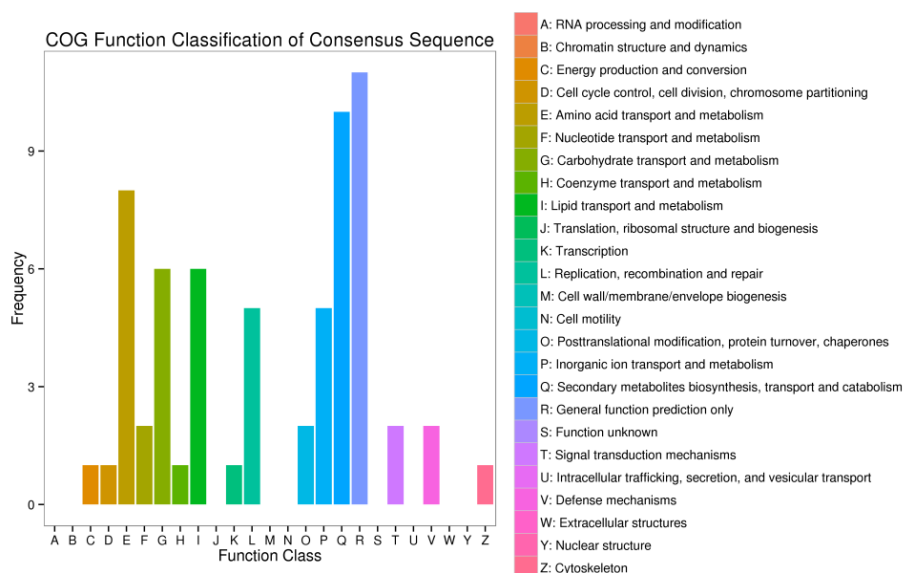
RNA-Seq is currently a powerful tool for transcriptome studies, especially in non-model insect species whose genomic sequences are yet to be determined (Hornett and Wheat, 2012; Fondevilla *et al.* 2015). Comparative transcriptomics analysis has been widely applied to investigate differentially expressed genes under different types of conditions. In this study, we used RNA-Seq to compare the antennal transcriptome of male and female *C. chinensis*.

Investigations on the molecular mechanisms of olfaction in *C. chinensis* provide insight into chemoreception. To date, olfactory genes have been studied in many species

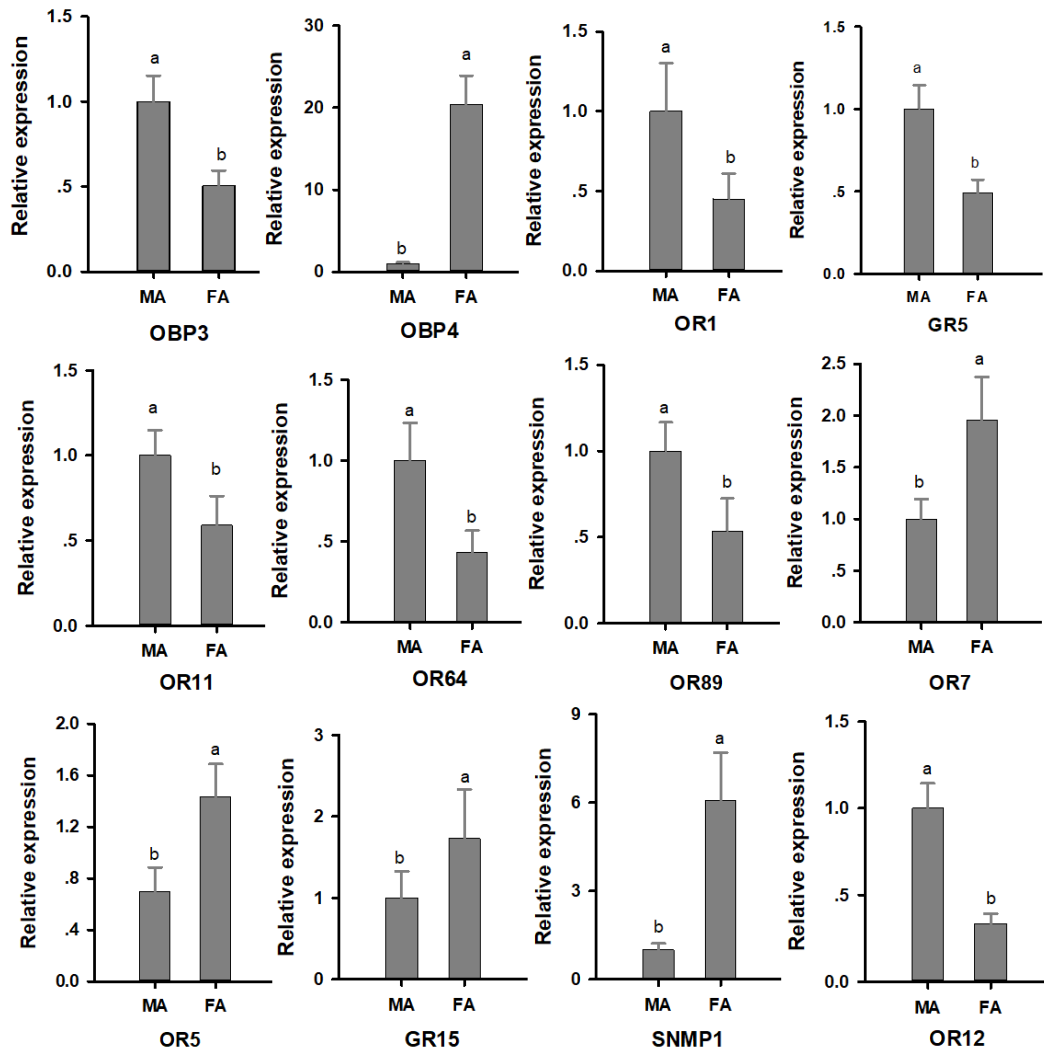
of Coleoptera. However, the most number (116) of putative ORs are identified in *C. chinensis*. The number of 12 OBPs is less than those identified from the antennal transcriptome of in *Anoplophora chinensis* (Forster) (n = 46), *Cylas formicarius* (F.) (n = 33) and *Tenebrio molitor* L. (n = 19) (Su *et al.*, 2015; Wang *et al.*, 2017; Bin *et al.*, 2017). The 4 CSPs predicted in *C. chinensis* transcriptome is also less than the reported 16 genes in *A. chinensis*, 19 genes in *Monochamus alternatus* Hope (Wang *et al.*, 2014) and 20 genes in *Tribolium castaneum* (Herbst) (Tribolium Genome Sequencing Consortium, 2008). While in our study, we did not identify any ODEs, suggesting that they are slowly expressed in *C. chinensis* and that some are species-specific. Similar results were found in *A. chinensis* and *C. formicarius*.

Comparison of sexual variation in gene expression in antennal tissues conducted in this experiment obtained 215 DEGs, which included the up-regulated genes (1 OBPs and 1 SNMPs) and the down genes (2 OBPs, 23 ORs and 15 GRs) in male antennae. Among these DEGs, 126 genes could be functionally annotated through the homology-based comparison against public databases, while no functional annotation was found for 41.4% of the all DEGs, either due to a match with a protein of unknown function or because no homologous nucleotide sequence was found in the database (Lu *et al.*, 2011). These genes may have great importance for further research. Several novel DEGs were also identified such as putative senescence genes and seminal fluid-encoding genes. They likely represents valuable candidate genes that can be used to further the understanding of cellular processes that vary between the sexes, however further investigations are needed to demonstrate obvious links between statistical and biological significance, which are beyond the scope of the present research.

Our results showed that one OBP displayed male-biased expression that may play essential roles in the detection of sex pheromones. Comparatively, the expression of the other two OBPs in female antennae were higher than those



**Figure 5.** COG function classification of consensus sequences. The categories of the COG are shown on the horizontal axis and gene numbers are plotted on the vertical axis.



**Figure 6.** Q-PCR for 12 differentially expressed genes (DEGs). Relative gene expression was normalized by comparison with the expression of  $\beta$ -tubulin. MA: male antennae; FA: female antennae. The standard error is represented by the error bar, and the different small letters above each bar denote significant differences ( $P < 0.05$ ).

in male antennae, which suggests that they may play important roles in the detection of general odorants such as host plant volatiles. Similar results were found in *C. formicarius*. The 23 ORs showed a higher RPKM in the female antennae than in the male antennae in *C. chinensis*. These female-biased ORs appear to be involved in female specific behaviours such as egg-laying-related odorant detection (Pelletier *et al.*, 2010; Yan *et al.*, 2015). The RPKM value analysis revealed no differences between male and female IRs. It was speculated that the IRs were relatively highly conserved.

## Conclusion

The olfactory system plays a role in the survival and maintenance of species, including insects. We obtained substantial molecular information on the combined male and female antennal transcriptome of *C. chinensis* using RNA-seq technology. Based on the transcriptomic analysis, 140 olfactory genes were identified. Among these, 42 chemosensory-related DEGs were screened,

including 3 OBPs, 23 ORs, 15 GRs and 1 SNMP, and their expression levels were measured based on the transcriptomic data, and validated by RT-qPCR. These results will be fundamental for future functional studies of olfactory-related genes in *C. chinensis*. Linking the molecular information presented to the available chemical and ecological knowledge will clarify the olfactory mechanisms of *C. chinensis*, and provide new targets for pest management in the future.

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