Journal of Asia-Pacific Entomology 22 (2019) 110–116

Contents lists available at ScienceDirect

Journal of Asia-Pacific Entomology

journal homepage: www.elsevier.com/locate/jape

Full Length Article

# Cloning and expression pattern of odorant receptor 11 in Asian honeybee drones, *Apis cerana* (Hymenoptera, Apidae)

Jun Feng Liu[a,b,1], Le Yang[a,c,1], Mang Li[a], Xu Jiang He[a], Zi Long Wang[a], Zhi Jiang Zeng[a,*]

[a] Honeybee Research Institute, Jiangxi Agricultural University, Nanchang 330045, PR China
[b] Environment and Plant Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Haikou 571101, PR China
[c] Kaihua County Animal Husbandry and Veterinary Bureau, Quzhou 324300, PR China

ARTICLE INFO

*Keywords:*
*Apis cerana*
Odorant receptor 11
Drones
Mating flight
Sexual development

ABSTRACT

Odorant receptors play a crucial role in the special recognition of scent molecules in the honeybee olfaction system. The odorant receptor 11 (*Am*OR11) in western honeybee drones (*Apis mellifera*) has been demonstrated to specifically bind to 9-oxo-2-decenoic acid (9-ODA) of queens. However, little is known regarding the functions of OR11 Asian honeybee drones (*Apis cerana*) in the context of their mating activities. In this study, the odorant receptor 11 gene (*Ac*Or11) from *A. cerana* was cloned, and its expression profiles were examined during two developmental stages (immature and sexually mature) and different physiological statuses (flying and crawling). The cDNA sequence of *Ac*Or11 was highly similar to that of *Am*Or11, and encoded a membrane-coupled protein of 384 amino acids. The results of qRT-PCR indicated that *Ac*Or11 was expressed at higher levels in drone antennae compared to brains, and the expression was significantly up-regulated in sexually mature drone brains compared to immature brains. Interestingly, *Ac*Or11 expression in brains of mature flying drones was dramatically higher than those of mature crawling drones. To our knowledge, this study demonstrate a link between *Ac*Or11 gene expression in the brain of honeybee drones and behavior associated with sexual maturity and mating flight.

## Introduction

Honeybee olfaction is essential for the perception and discrimination of a variety of odor molecules in external environment (Laska et al., 1999; Robertson and Wanner, 2006). This olfaction ability allows honeybees an efficient method of chemical communication inside and outside of their colonies. Furthermore, this ability has been shown to be especially important in mating flights (Slessor et al., 2005; Sandoz et al., 2007). In mating flights, male bees arrive early at drone congregate area (DCA) that in midair. A virgin queen fly through a DCA and release queen mandibular pheromones (QMPs) which mainly including (E)-9-oxodec-2-enoic acid (9-ODA), two enantiomers of (E)-9-hydroxydec-2-enoic acid (9-HDA; 85% (*R*)-(−), 15% (S-(+)), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methoxyphe-nylethanol (HVA) (Butler et al., 1959; Butler, 1971; Gary and Marston, 1971; Slessor et al., 1988; Keeling et al., 2003). On the other hand, drones use their olfaction capabilities, which is believed to be specific to males, to locate virgin queens in mating flights by scanning for and sensing queen pheromones in the air (Brockmann and Brückner, 2001; Wanner et al., 2007). Evidence has indicated that drones can detect QMPs over an extended distance (> 800 m), indicating very high sensitivity of their olfaction (Loper et al., 1993).

Among these, 9-ODA, is one of the predominantly detected compounds of QMPs, which function as a short-range social pheromone attracting workers within the colony, and as a long-range sex pheromone attracting drones at mating flights (Butler, 1971; Gary and Marston, 1971; Boch et al., 1975; Loper et al., 1993; Brockmann et al., 2006). Electrophysiological recordings have demonstrated that drones have a greater proportion of olfactory neurons in their antennae tuned to QMPs compared to workers. More interestingly, drone's antennae are more sensitive to 9-ODA than any other single component of QMPs (Vetter and Visscher, 1997; Brockmann et al., 1998). This is believed to result from the expression of a special odorant receptor in the antennae of honeybees. *A. mellifera* odorant receptor 11 (*Am*Or11) in male bees has been demonstrated to specifically bind 9-ODA (Wanner et al., 2007). Recently, Wu et al. (2016) found that 16 ORs were up-regulated in the sexually matured drones of *A. mellifera* by using high-throughput RNA-Seq. Moreover, a subsequent investigation reported that *Am*Or11

* Corresponding author.
*E-mail address:* bees1965@sina.com (Z.J. Zeng).
[1] These two authors contributed equally to this work.

https://doi.org/10.1016/j.aspen.2018.12.014
Received 22 October 2018; Received in revised form 30 November 2018; Accepted 17 December 2018
Available online 18 December 2018

expression levels are higher in the antennae of sexually mature drones than immature drones (Villar et al., 2015). This observation suggests a likely association between *Am*Or11 and the process of sexual maturation of honeybee drones. In addition, QMPs not only trigger drone mating behavior, but also exert other primer effects on body development of drone bees. Young male-bees exposed to 9-ODA in hive not only result in delayed initial mating flights, but reduce flight duration as well (Villar and Grozinger, 2017). Other evidence has indicated that *Drosophila melanogaster* specific pheromone receptor participate in their regulation of mating behavior both male and female (Kurtovic et al., 2007). In honeybee brains, the antennal lobes (AL) consist of approximately 160–170 glomeruli which correspond with their ORs (Hansson and Anton, 2000; Robertson and Wanner, 2006), although the specific mechanisms of action remain unclear. Therefore, we suspect that odorant receptors expressed in honeybee drones, especially *Am*OR11, may participate in multiple biological functions that aid in the detection of 9-ODA during mating flights, or in the regulation of drone maturation and mating behaviors.

*Apis cerana,* an Asian honeybee species, has been demonstrated to exhibit enhanced searching abilities to locate sparse floral resources (Zeng, 2017), and better colour cognition and orientation learning relative to that of *A. mellifera* (Qin et al., 2012; Zhang et al., 2014). Recently, many *A. cerana* odorant receptor genes (*Ac*Ors) have been preliminarily investigated, including annotated, cloning, characterized, mRNA/protein expression patterns, and localization within the organisms (Zhang et al., 2012; Zhao et al., 2012; Zhao et al., 2013, 2014; Park et al., 2015; Zhao et al., 2015; Zhang et al., 2016; Du et al., 2017a,b; Yang et al., 2017). These mRNA sequences were found to be highly similar with those of *A. mellifera.*Park et al. (2015) have characterized 119 Ors by whole *A. cerana* genome sequencing. The *Ac*Or2 and *Ac*Or3 in Asian honeybee antennae both reveal male-bias, and are expressed at the highest levels at sexual maturity (Zhao et al., 2014; Zhang et al., 2016). However, information is scarce regarding the *A. cerana* odorant receptor 11 (*Ac*Or11) at the time of publication. In this study, we identified the *Ac*Or11gene, determined the gene's DNA sequence, and characterized expression patterns across sexual developmental stages and different physiological statuses in both antenna and brain of *A. cerana* drones. This allows for thorough investigation of the biological functions of *Ac*Or11 in male mating behavior and progression through sexual maturity, and eventually to provide a physiologic background leading to mating flights of honeybee drones.

## Materials and methods

### *Insects*

Three Asian honeybee (*A. cerana*) colonies were maintained at the Honeybee Research Institute of Jiangxi Agricultural University (28.46°N, 115.49°E). Each colony had a mature egg-laying queen and 5 frames.

### *Sample collection*

In first experiment, 30 drones were randomly collected at entrance of the hive, upon returning home, using forceps. The drones were immediately stored in liquid nitrogen for subsequent cloning experiment of target genes. In the second experiment, antennae and brains were collected from 4-day-old (sexually immature) and 14-day-old (sexually mature) drones. This included crawling drones inside of colonies as well as those flying back to the hive. These insects were used for gene expression analysis of the *Ac*Or11 and *Ac*Or2 (odorant receptor co-receptor, ortholog Or83b family) genes. This detailed methods used for our study of collected drones was referenced (Villar et al., 2015). For each group, 30 pairs of antennae and brains each, with 3 biological replicates from 3 different colonies were examined. These studies were conducted during the spring of 2018.

**Table 1**
Primers used to *Ac*Or11gene clone and qRT-PCR.

| Primer names | Primer sequences |
|---|---|
| *Ac*Or11-F1 | 5'-TCACGAACAAGCTTTCATCGG-3' |
| *Ac*Or11-R1 | 5'-GAAAGTGAACAAAGTGCTGTGTACA-3' |
| *Ac*Or11-R2 | 5'-TCAATATCATTTTTGGCTAATCAGA-3' |
| *Ac*Or11-QF | 5'-ATGTGCGGTTTGCTGAAGA-3' |
| *Ac*Or11-QR | 5'-CGAGAAGGTGCCAATGACG-3' |
| *Ac*Or2-QF | 5'-GGATCAGAGGAGGCCAAAAC-3' |
| *Ac*Or2-QR | 5'-CCAACACCGAAGCAAAGAGA-3' |
| *Ac*-actin-QF | 5'-GGCTCCCGAAGAACATCC-3' |
| *Ac*-actin-QR | 5'-TGCGAAACACCGTCACCC-3' |

### *Cloning of the AcOr11 gene*

Total RNA was extracted from 30 pairs of drone antennae using the TransZol reagent (Transgen Biotech, www.transgen.com.cn) according to the manufacturer's instructions, and stored in a freezer at −80 °C until use. Since honeybee odorant receptors are distributed mainly in antennae, and their expression patterns are especially enriched in antennae of honeybee, we therefore used antennae for cloning (Hugh Robertson and Wanner, 2006; Claudianos et al., 2014). The cDNA was synthesized from the total RNA isolated from antennae using the Primer-Script RT reagent Kit (TaKaRa, www.takara-bio.com) according to the manufacturer's instructions.

The primers used to amplify *Ac*Or11 (see Table 1) were designed using the primer premier 5.0 software (Premier Biosoft International Co., Palo Alto, CA) with the input mRNA sequence of the *Am*Or11 gene (GenBank accession: NM_001242962.1) deposited in NCBI. The aforementioned primers were synthesized by Sangon Biotech (Sangon Biotech Shanghai, China Co., Ltd). The PCR thermocycling conditions were as follows: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products were then electrophoretically resolved on a 1.5% agarose gel, and purified using a Gel Extraction Kit (Cwbiotech, www.cwbiotech.bioon.com.cn). Next, the purified products were ligated into an pEASY-T3 Clone Vector, and subsequently transformed into Trans5α Chemically Competent Cell (TransGen Biotech). Positive clones were screened and sequenced by Sangon Biotech.

### *Sequence analysis*

After sequencing, the cDNA sequence of *Ac*Or11 was obtained by assembling forward and reverse sequencing reads using SeqMan program in DNAstar 5.0 software (Lynnon Biosoft, Quebec, Canada). The amino acid sequence was translated by the Bioedit software. Similarity searches were conducted using BLAST programs on the NCBI website (http://www.ncbi.nlm.nih.gov). The isoelectric point (pI) and molecular weights (MW) were computed using Compute pI/MW (http://www.expasy.ch/tools/pi_tool.html). The post-translational modification sites were predicted using PROSITE SCAN (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). The secondary structures were predicted using the SOPMA program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The transmembrane helix (TMH) was predicted by using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM). Alignments of multiple sequences were carried out using ClustalW (Thompson et al., 1994). The phylogenetic tree was constructed using MEGA4.0 (http://www.megasoftware.net/index.php), with a portion of sequences of known *A. mellifera* ORs obtained from GenBank.

### *Expression of the AcOr11 and AcOr2 genes*

Total RNA was isolated from the antennae and brains of drones to determine the expression levels of *Ac*Or11 and *Ac*Or2. Quantitative

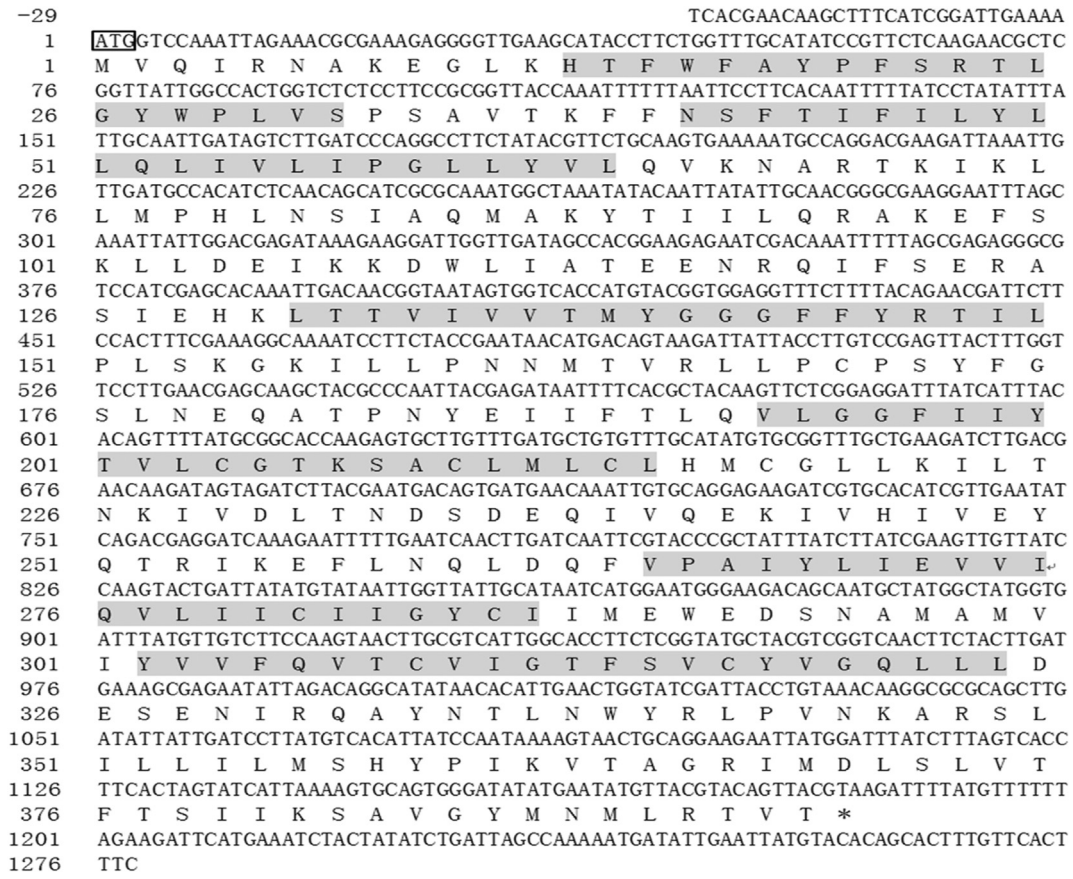


**Fig. 1.** The nucleotide and deduced amino acid sequences of *Ac*Or11. The positions of the nucleotides and amino acids are indicated in the left margin. The start codons used in cloning and sequencing are boxed, and the termination codon is marked with a star. The Shaded amino acid sequences indicate predicted 7-transmembrane (7TM- 6) domains, including TM-I (13–32), TM-II (42–64), TM-III (131–150), TM-IV (193–215), TM-V (265–287) and TM-VI (302–324). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Real-Time PCR (qRT-PCR, ABI 7500 instrument) was performed using the SYBR Premix Ex Taq kit (Takara) in a total reaction volume of 10 µl. The reaction mixture was prepared as follows: 4.2 µl cDNA (water for the negative control) and 0.4 µl of each primer. The primers (see Table 1) for the *Ac*Or11, *Ac*Or2 (GenBank accession: JN792581) and *A. cerana* actin genes (*Ac*-actin, GenBank accession: HM640276.1) were designed respectively to amplify 296, 118 and 195 bp fragments using the primer premier 5.0 software. qRT-PCR was performed with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of 94 °C for 15 s, (*Ac*Or11, 58.9 °C; *Ac*Or2 and *Ac*-actin, 60 °C) for 40 s, 72 °C for 35 s, and a melting curve analysis was conducted to verify the specificity of the amplification. The *Ac*-actin gene was used as the internal control. The relative expression levels of *Ac*Or11 and *Ac*Or2 mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method (Schmittgen and Livak, 2008).

### *Statistical analysis*

Differences in the relative expression of the *Ac*Or11 and *Ac*Or2 genes were determined using a *t*-test analysis in SPSS 17.0 (IBM, Armonk, NY). Values of $P < .05$ were considered significant in all treatments.

## Results

### *Cloning and sequence analysis of the AcOr11gene*

To explore the molecular functions of the *A. cerana* odorant receptor 11, the cDNA sequence of *Ac*Or11 containing the complete coding region was cloned, and the amino acid sequences were predicted by in silico translation. The *Ac*Or11 cDNA (GenBank accession: MG793195) contains a 5′-terminal untranslated region (UTR) of 29 bp, a 3′-terminal UTR of 93 bp, and an open reading frame (ORF) of 1185 bp encoding a polypeptide of 394 amino acids (Fig. 1). The molecular mass of the deduced *Ac*OR11 protein is predicted to be 45.14 kDa, and the calculated isoelectric point (pI) is 8.98. Moreover, the *Ac*OR11 protein belongs to the 7-transmembrane_6 receptor (7TM_6) superfamily, which consist of TM-I (13 − 32), TM-II (42–64), TM-III (131–150), TM-IV (193–215), TM-V (265–287) and TM-VI (302–324) (Fig. 1). The secondary structure was predicted using SOPMA, which consists of 54.31% alpha helices, 22.84% beta sheets, 4.06% turns and 18.78% is random coils. Comparison of the deduced amino acid sequence of *Ac*OR11 (NCBI, BLASTP) to that of *Am*OR11(NP_001229891.1) reveals a high degree of identity 98%. Similarly, alignments with ORs of other Hymenopterans also exhibit high homology with *A. dorsata* OR85b-like (XP_006615208.1 identity, 99%) and *A. florea* OR4-like (XP_003691312.1, identity 97%) (Fig. 2). A phylogenetic tree was constructed using the MEGA 4.0 software using the deduced amino acid sequences of various *A. mellifera* ORs and *Ac*OR11(Fig. 3). Phylogenetic analysis showed that OR11s in *A. mellfera* and *A. cerana* belong to a single subfamily. Moreover, the phylogenetic tree demonstrated that the OR11 exhibits a relatively distant genetic relationship to the OR2 (ortholog OR83b family).

### *Analysis of AcOr11 and AcOr2 expression by qRT-PCR*

The expression profiles of *Ac*Or11 were characterized across different developmental stages and physiological statuses of *A. cerena*

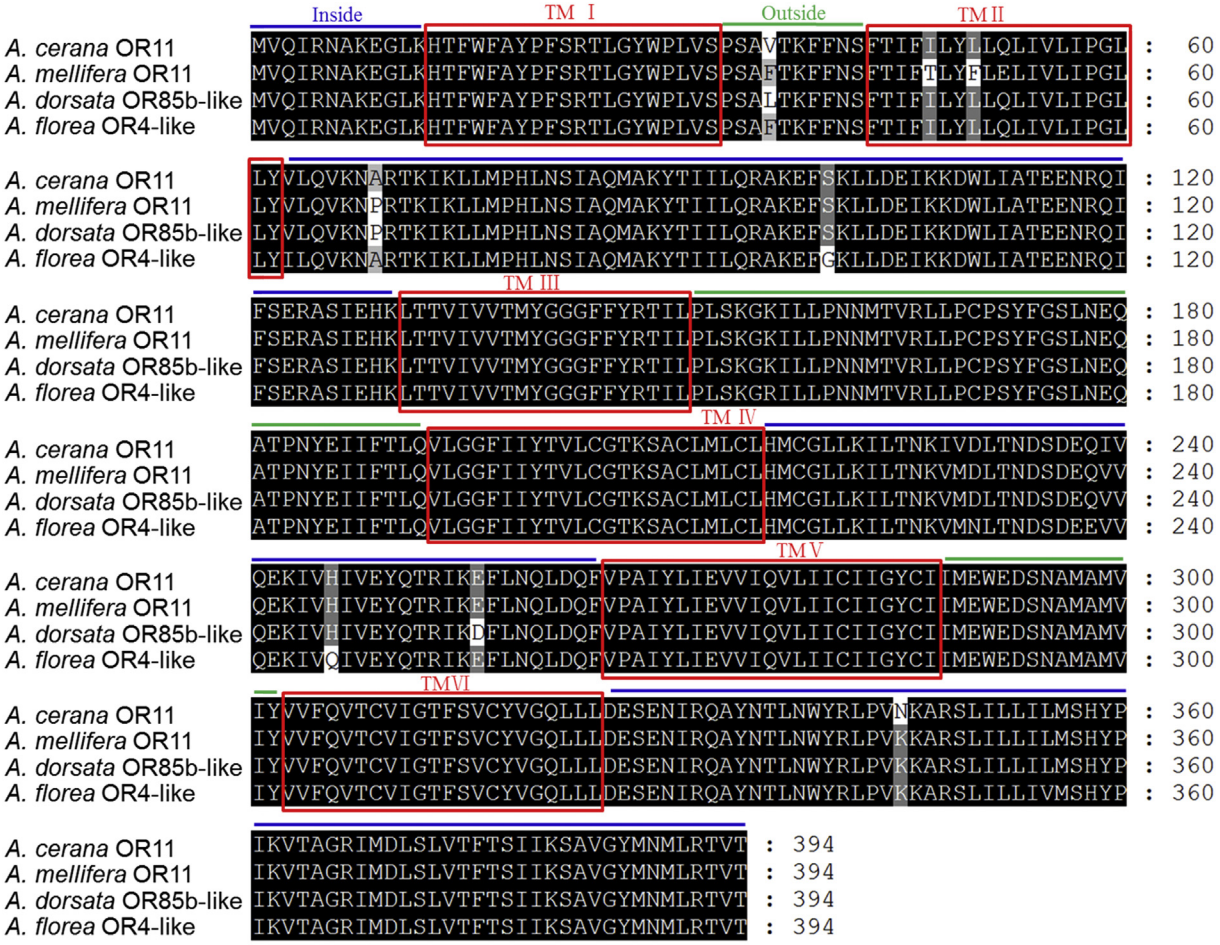


**Fig. 2.** Alignments of *Ac*OR11 with other honeybee OR sequences. Black shade: identity of sequences = 100%; Gray shade: identity of sequences > 75%. The six red boxes of *Ac*OR11 amino acids sequences in respectively represent conserved transmembrane domains from TMI to VI. The blue and green lines respectively reveal inside and outside membrane of *Ac*OR11 protein positions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

using qRT-PCR. The expression profiles of *Ac*Or11 were determined in brains and antennae from immature and mature drones. It was observed that expression of *Ac*Or11 in drone antennae was significantly higher than that of brains ($t = -3.381$, df = 4, $P = .028$, Fig. 4a. left; $t = -5.332$, df = 4, $P = .006$. Fig. 4a. right). In brains, *Ac*Or11 in mature drones was more highly expressed than those of immature drones ($t = -2.883$, df = 4, $P = .045$, Fig. 4b. left), whereas no differences in expression were observed in antennae ($t = -0.620$, df = 4, $P = .569$, Fig. 4b. right). Moreover, *Ac*Or11expression in mature drone brains in flying status was significantly higher than those in crawling status ($t = -2.790$, df = 4, $P = .049$, Fig. 4c. right). However, there was no significant difference between the 2 physiological statuses of immature drones ($t = -0.773$, df = 4, $P = .483$, Fig. 4c. left). In antenna, *Ac*Or11 expression of immature drones was significantly higher in flying status compared to crawling status ($t = -3.516$, df = 4, $P = .025$, Fig. 4d. left), but no differences were observed for the mature stage ($t = -0.150$, df = 4, $P = .888$, Fig. 4d. right). As is presented in Fig. 5, the expression patterns of *Ac*Or11 and *Ac*Or2 were similar in brains and antennae between flying and crawling statuses. The expression level of *Ac*Or2 in mature drone brains in flying status was significantly higher than that in crawling status ($t = -6.350$, df = 4, $P = .003$, Fig. 5a. right). In contrast, between the physiological statuses of immature drones, there were no significant differences ($t = -2.389$, df = 4, $P = .075$, Fig. 5a. left). In antenna, *Ac*Or2 of immature drones was also expressed significantly higher in flying status compared to crawling status ($t = -7.286$, df = 4, $P = .002$, Fig. 5b. left), but was not different in mature drones ($t = 1.994$, df = 4, $P = .117$, Fig. 5b. right).

## Discussion

The proteins of general odorant receptors are highly diverse in insects. This is also true for the conservative co-receptor (OR83b) family (Clyne et al., 1999; Gao and Chess, 1999). In this study, we identified a putative odorant receptor 11 gene in *A. cerana*. The *Ac*Or11 amino acid sequence was observed to share many similar characteristics with that of the *A. mellifera* Or11 orthologue. Interestingly, homologs of the *Ac*Or11 gene was not observed in other insects outside of *Apis*. In general, Ors exhibited a high sequence divergence among insects including classical model insects such as *Anopheles gambiae* and *Drosophila melanogaster* (Hill et al., 2002; Robertson et al., 2003). These observations are consistent with an ancient origin of the OR family. As demonstrated by the phylogenetic tree constructed here, the *Ac*OR11 was more closely related to *Am*OR11 than *Am*OR2 and *Am*OR170 of *A. mellifera*. This result served as a molecular confirmation for the traditional phylogenetic classes of honeybees (Robertson and Wanner, 2006), suggesting that *Ac*OR11 belongs to a typical odorant-receptor protein family in *A. cerana*.

Expression of *Ac*Or11 was assessed in both the antennae and brain of *A. cerana* drones. The data indicated that *Ac*Or11 was expressed at significantly higher levels in the antennae compared to the brain (Fig. 4a). These observations were in agreement with our expected

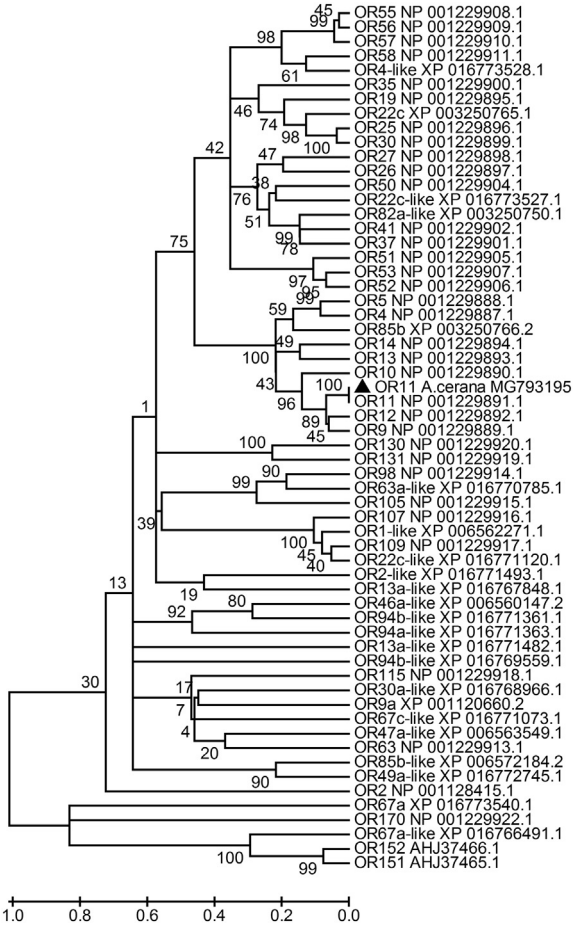


**Fig. 3.** Phylogenetic analysis of amino acid sequences of various *A. mellifera* ORs and *Ac*OR11. Clusters of tandem arrays of *Ac*OR11 (triangle icon) and *Am*ORs on particular chromosomes are indicated by vertical lines on the right. The branch labels correspond to bootstrap values. Bootstrap supported values (%) are based on 1000 replicates, as indicated by the scale bar. The protein accession numbers from NCBI are displayed respectively behind corresponding *A. mellifera* OR proteins.

results, as antennae are the primary sensory organ in honeybees and contain numerous odorant receptors (Akers and Getz, 1992; Joerges et al., 1997). Furthermore, this result is consistent with those published previously (Robertson and Wanner, 2006). In this study, we detected the expression pattern of *Ac*Or11 gene in drone brains under different sexual developmental stages and physiological statuses. Our results clearly indicated that the process of sexual development of honeybee drones strongly effects the expression of *Ac*Or11 in brains. Expression of *Ac*Or11 in brains of mature drones was significantly higher than those of immature drones, whereas there was no difference in antennae between immature and mature drones (Fig. 4b). Both the antennal lobes (AL, the first synaptic processing station), and the mushroom bodies (MB, the multi-sensory integration centers) are the main olfactory brain regions of insects (Szyszka, 2005). The AL is enriched with the terminal axons of odorant receptor neurons (ORNs) and the glomeruli in olfactory sensory neurons (OSNs) (Gao and Chess, 1999; Mombaerts, 1999; Hill et al., 2002; Forêt and Maleszka, 2006). It has been reported that the olfactory glomeruli in young honeybee brains are not well developed, and their volume increases significantly with age (Fahrbach and Robinson, 1995). Therefore, it was hypothesized that the immature drone brains (4 day old drones) were not fully developed, resulting in reduced expression of the *Ac*Or11 gene in their brains, therefore repressing their mating behavior. The flights of sexually immature drones are generally orientation flights rather mating flights (Graham, 2015). Moreover, it was observed that the expression of *Ac*Or11 in brains of flying mature drones was significantly higher than those of the crawling mature drones, but was not different in the antenna (Fig. 4c). In many insects, a number of olfactory neurons express sex pheromone receptors in order to increase sensitivity to respond pheromone in the AL (Graham, 2015), Their function is transferring odor molecules to the sensory receptors distributed on the dendritic. This study indicated that physiological statuses of honeybee drones also correlated with *Ac*Or11 gene expression in brains, reflecting that daily mating flights of mature drones possibly perform as an important role in improving the mating behavior of honeybee drones by regulating the expression of *Ac*Or11 in drone brains. Furthermore, there was no significant difference between flying and crawling immature drone brains, which is likely due to the fact that main outside activity are orientation flights, rather mating flights in immature drones in nature.

Interestingly, expression of *Ac*Or11 was opposite in antenna compared to that of brains. Expression in the antennae of the flying drones was dramatically higher than those of the crawling drones in the immature stage (Fig. 4d). It is unclear how orientation flying activity upregulates *Ac*Or11 gene expression in antennae of immature drones. Perhaps the *Ac*Or11 is employed by immature drones to detect environmental scents for orientation flying, since the olfactory nervous system of young honeybees is most sensitive to environmental odors from inside and outside of their hive (Masson et al., 1993; Sandoz and Menzel, 2001). This is consistent with other studies reporting that many insects utilize floral scents and other environmental odors for orientation (Phelan and Baker, 1987; Hern and Dorn, 1999; Anton et al., 2007). For mature drones, Villar et al. (2015) reported that *Am*Or11 in drone antennae is correlated with mating behavior in response to 9-ODA stimulation. In Fig. 4d, the expression of *Ac*Or11 in mature drone antennae was not affected by physiological status. This could be explained by the fact that Ors expression in mature drone antennae tends to be stable and increases only when stimulated by QMPs, rather than in daily flights. Nevertheless, this phenomenon requires further investigation.

Furthermore, the results of *Ac*Or2 also showed a similar expression pattern in drone brains (Fig. 5a) and antennae (Fig. 5b) at different physiological statuses to that of *Ac*Or11. As the OR2 is the co-receptor of OR11 in honeybees (Wanner et al., 2007), these data serve as additional confirmation of our observations *Ac*Or11.

In summary, *Ac*Or11 from the antennae of *A. cerana* was cloned, and expression patterns were analyzed in drones of different stages of sexual maturity and physiological statuses. The expression of *Ac*Or11 in drone brains was closely correlated with both sexual development and physiological status. This suggests that *Ac*Or11 in brain may have some biological functions involved in the progression to sexual maturity and mating behavior. This study provides an insight into the molecular basis underlying mating flights of *A. cerana* drones.

## Conflict of interest

No conflict of interest exits in the submission of this manuscript.

## Acknowledgments

Zhi Jiang Zeng, Xu Jiang He and Zi Long Wang conceived and designed the experiments. Jun Feng Liu, Le Yang and Mang Li performed the experiments. Jun Feng Liu, Xu Jiang He and Le Yang analyzed the data. Jun Feng Liu, Zhi Jiang Zeng and Xu Jiang He wrote the paper. All authors read and approved the final manuscript. This work is supported by the National Natural Science Foundation of China (31572469), and the Earmarked Fund for China Agriculture Research System (CARS-44-KXJ15).

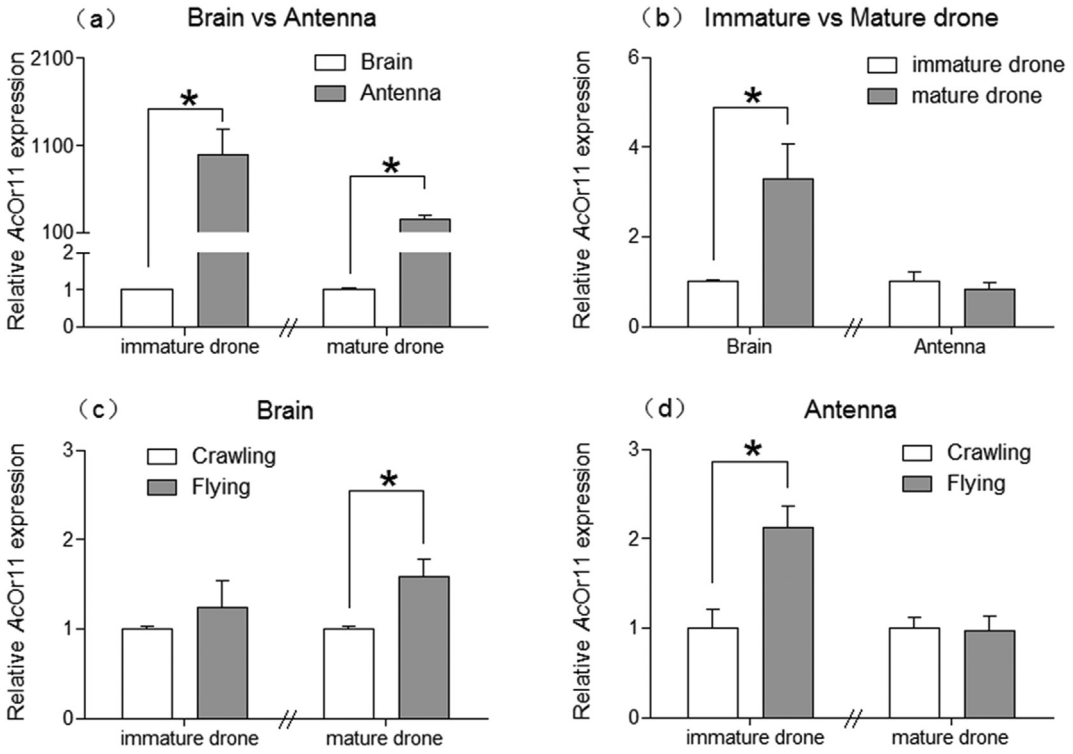


**Fig. 4.** *Ac*Or11 gene expression profiles in *A. cerana* drones. (a) Comparison of *Ac*Or11 expression levels in drone brains versus antennae. The open bars represent the normalized expression level of *Ac*Or11 in antennae. The gray bars represent the relative expression in brains. Drones from two sexually developmental stages (immature and mature) were collected for this analysis respectively, same in (c) and (d). (b) Comparison of *Ac*Or11 expression levels in immature drones versus mature drones. The open bars represent expression level of *Ac*Or11 in immature drones, and the gray bars represent the relative expression in mature drones. Brains and antennae were collected from these immature and mature drones and were analyzed separately. (c) The relative *Ac*Or11 expression pattern in brains of crawling drones versus that of flying drones. The open bars represent expression level of *Ac*Or11 in crawling drone brains, and the gray bars represent the relative expression in flying ones. (d) The relative *Ac*Or11expression pattern in antennae of crawling drones versus that of flying drones. The open bars represent expression level of *Ac*Or11 in crawling drone antennae, and the gray bars represent the relative expression in flying ones. The data was expressed as the mean ± SE, and the "*" indicates a significant difference ($P < .05$).

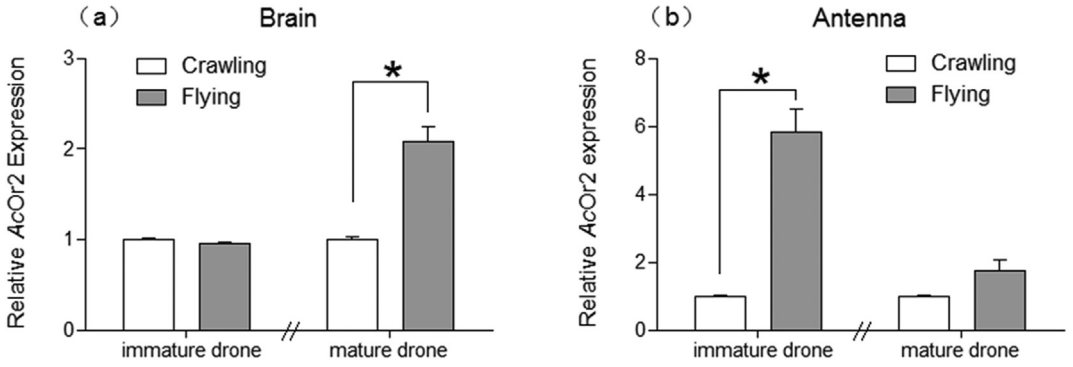


**Fig. 5.** *Ac*Or2 gene expression profiles in *A. cerana* drones. (a) The relative *Ac*Or2 expression pattern in brains of crawling drones versus that of flying drones. The open bars represent expression level of *Ac*Or2 in crawling drone brains, and the gray bars represent the relative expression in flying ones. Immature and mature drones were collected for this analysis respectively, same in (b). (b) The relative *Ac*Or2 expression pattern in antennae of crawling drones versus that of flying drones. The open bars represent expression level of *Ac*Or2 in crawling drone antennae, and the gray bars represent the relative expression in flying drones. The data was expressed as the mean ± SE, and the "*" indicates significant difference ($P < .05$).

## References

Akers, R.P., Getz, W.M., 1992. A test of identified response classes among olfactory receptor neurons in the honey-bee worker. Chem. Senses 17, 191–209. https://doi.org/10.1093/chemse/17.2.191.

Anton, S., Dufour, M.C., Gadenne, C., 2007. Plasticity of olfactory-guided behaviour and its neurobiological basis: Lessons from moths and locusts. Entomol. Exp. Appl. 123, 1–11. https://doi.org/10.1111/j.1570-7458.2007.00516.x.

Boch, R., Shearer, D.A., Young, J.C., 1975. Honey bee pheromones: Field tests of natural and artificial queen substance. J. Chem. Ecol. 1, 133–148. https://doi.org/10.1007/BF00987726.

Brockmann, A., Brückner, D., 2001. Structural differences in the drone olfactory system of two phylogenetically distant *Apis* species, *A. florea* and *A. mellifera*. Naturwissenschaften 88, 78–81. https://doi.org/10.1007/s001140000199.

Brockmann, A., Brückner, D., Crewe, R.M., 1998. The EAG response spectra of workers and drones to queen honeybee mandibular gland components: the evolution of a social signal. Naturwissenschaften 85, 283–285. https://doi.org/10.1007/s001140050500.

Brockmann, A., Dietz, D., Spaethe, J., Tautz, J., 2006. Beyond 9-ODA: SEX pheromone communication in the European honey bee *Apis mellifera* L. J. Chem. Ecol. 32, 657–667. https://doi.org/10.1007/s10886-005-9027-2.

Butler, C.G., 1971. The mating behaviour of the honeybee (*Apis mellifera* L.). J. Entomol. Ser. A Gen. Entomol. 46, 1–11. https://doi.org/10.1111/j.1365-3032.1971.tb00103.x.

Butler, C.G., Callow, R.K., Johnston, N.C., 1959. Extraction and purification of "Queen Substance" from queen bees. Nature 184, 1871. https://doi.org/10.1038/1841871a0.
Claudianos, C., Lim, J., Young, M., Yan, S., Cristino, A.S., Newcomb, R.D., Gunasekaran, N., Reinhard, J., 2014. Odor memories regulate olfactory receptor expression in the sensory periphery. Eur. J. Neurosci. 39, 1642–1654. https://doi.org/10.1111/ejn.12539.
Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., Carlson, J.R., 1999. A novel family of divergent seven-transmembrane proteins. Neuron 22, 327–338. https://doi.org/10.1016/S0896-6273(00)81093-4.
Du, Y.L., Pan, J.F., Wang, S.J., Yang, S., Zhao, H.T., Jiang, Y.S., 2017a. Cloning and expression analysis of odorant receptor gene AcerOr167 in the Chinese honeybee,*Apis cerana cerana* (Hymenoptera:Apidae). J. Environ. Entomol. 39, 39–47. https://doi.org/10.3969/j.issn.1674-0858.2017.01.3.
Du, Y.L., Wang, S.J., Zhao, H.T., Pan, J.F., Yang, S., Guo, L.N., Xu, K., Jiang, Y.S., 2017b. Cloning and temporal-spatial expression profiling of the odorant receptor gene AcerOR113 in the Chinese honeybee. *Apis cerana* cerana. Acta Entomol. Sin. 60, 533–543. https://doi.org/10.16380/j.kcxb.2017.05.005.
Fahrbach, S.E., Robinson, G.E., 1995. Behavioral development in the honey bee: toward the study of learning under natural conditions. Learn. Mem. 2, 199–224. https://doi.org/10.1101/lm.2.5.199.
Forêt, S., Maleszka, R., 2006. Function and evolution of a gene family encoding odorant binding-like proteins in a social insect, the honey bee (*Apis mellifera*). Genome Res. 16, 1404–1413. https://doi.org/10.1101/gr.5075706.
Gao, Q., Chess, A., 1999. Identification of candidate *Drosophila* olfactory receptors from genomic DNA sequence. Genomics 60, 31–39. https://doi.org/10.1006/geno.1999.5894.
Gary, N.E., Marston, J., 1971. Mating behaviour of drone honey bees with queen models (*Apis mellifera* L.). Anim. Behav. 19, 299–304. https://doi.org/10.1016/S0003-3472(71)80010-6.
Graham, B.M., 2015. The hive and the honey bee. Dadant & Sons Press, Hamilton, Illnois, USA.
Hansson, B.S., Anton, S., 2000. Function and morphology of the antennal lobe: new developments. Annu. Rev. Entomol. 45, 203–231. https://doi.org/10.1146/annurev.ento.45.1.203.
Hern, A., Dorn, S., 1999. Sexual dimorphism in the olfactory orientation of adult *Cydia pomonella* in response to α-farnesene. Entomol. Exp. Appl. 92, 63–72. https://doi.org/10.1023/A:1003756428631.
Hill, C.A., Fox, A.N., Pitts, R.J., Kent, L.B., Tan, P.L., Chrystal, M.A., Cravchik, A., Collins, F.H., Robertson, H.M., Zwiebel, L.J., 2002. G protein coupled receptors in *Anopheles gambiae*. Science (80-.) 298, 176–178. https://doi.org/10.1126/science.1076196.
Joerges, J., Küttner, A., Galizia, C.G., Menzel, R., 1997. Representations of odours and odour mixtures visualized in the honeybee brain. Nature 387, 285–288. https://doi.org/10.1038/387285a0.
Keeling, C.I., Slessor, K.N., Higo, H. a, Winston, M.L., 2003. New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. Proc. Natl. Acad. Sci. U. S. A. 100, 4486–4491. https://doi.org/10.1073/pnas.0836984100.
Kurtovic, A., Widmer, A., Dickson, B.J., 2007. A single class of olfactory neurons mediates behavioural responses to a *Drosophila* sex pheromone. Nature 446, 542–546. https://doi.org/10.1038/nature05672.
Laska, M., Galizia, C.G., Giurfa, M., Menzel, R., 1999. Olfactory discrimination ability and odor-structure-activity relationships in honeybees. Chem. Senses 24, 429–438. https://doi.org/10.1093/chemse/24.4.429.
Loper, G.M., Wolf, W.W., Taylor, O.R., 1993. Radar detection of drones responding to honeybee queen pheromone. J. Chem. Ecol. 19, 1929–1938. https://doi.org/10.1007/BF00983797.
Masson, C., Pham Delegue, M.H., Fonta, C., Gascuel, J., Arnold, G., Nicolas, G., Kerszberg, M., 1993. Recent advances in the concept of adaptation to natural odour signals in the honeybee, *Apis mellifera* L. recent progress in the neurobiology of honeybees. Apidologie 24, 169–194. https://doi.org/10.1051/apido:19930302.
Mombaerts, P., 1999. Seven-transmembrane proteins as odorant and chemosensory receptors. Science (80-.) 286, 707–711. https://doi.org/10.1126/science.286.5440.707.
Park, D., Jung, J.W., Choi, B.-S., Jayakodi, M., Lee, J., Lim, J., Yu, Y., Choi, Y.-S., Lee, M., Park, Y., Choi, I., Yang, T., Edwards, O.R., Nah, G., Kwon, H.W., 2015. Uncovering the novel characteristics of Asian honey bee, *Apis cerana*, by whole genome sequencing. BMC Genomics 16 (1). https://doi.org/10.1186/1471-2164-16-1.
Phelan, P.L., Baker, T.C., 1987. An Attracticide for Control of *Amyelois transitella* (Lepidoptera: Pyralidae) in Almonds. J. Econ. Entomol. 80, 779–783. https://doi.org/10.1093/jee/80.4.779.
Qin, Q.H., He, X.J., Tian, L.Q., Zhang, S.W., Zeng, Z.J., 2012. Comparison of learning and memory of *Apis cerana* and *Apis mellifera*. J. Comp. Physiol. A. 198, 777–786. https://doi.org/10.1007/s00359-012-0747-9.
Robertson, H.M., Wanner, K.W., 2006. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. Genome Res. 16, 1395–1403. https://doi.org/10.1101/gr.5057506.
Robertson, H.M., Warr, C.G., Carlson, J.R., 2003. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 100 (Suppl), 14537–14542. https://doi.org/10.1073/pnas.2335847100.
Sandoz, J.C., Menzel, R., 2001. Side-specificity of olfactory learning in the honeybee: generalization between odors and sides. Learn. Mem. 8, 286–294. https://doi.org/10.1101/lm.41401.an.
Sandoz, J.-C., Deisig, N., de Brito Sanchez, M.G., Giurfa, M., 2007. Understanding the logics of pheromone processing in the honeybee brain: from labeled-lines to across-fiber patterns. Front. Behav. Neurosci. 1 (5). https://doi.org/10.3389/neuro.08.005.2007.
Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.
Slessor, K.N., Kaminski, L.-A., King, G.G.S., Borden, J.H., Winston, M.L., 1988. Semiochemical basis of the retinue response to queen honey bees. Nature. https://doi.org/10.1038/332354a0.
Slessor, K.N., Winston, M.L., Le Conte, Y., 2005. Pheromone communication in the honeybee (*Apis mellifera* L.). J. Chem. Ecol. 31, 2731–2745. https://doi.org/10.1007/s10886-005-7623-9.
Szyszka, P., 2005. Sparsening and temporal sharpening of olfactory representations in the honeybee mushroom bodies. J. Neurophysiol. 94, 3303–3313. https://doi.org/10.1152/jn.00397.2005.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680. https://doi.org/10.1093/nar/22.22.4673.
Vetter, R.S., Visscher, P.K., 1997. Influence of Age on Antennal Response of Male Honey Bees, *Apis mellifera*, to Queen Mandibular Pheromone and Alarm Pheromone Component. J. Chem. Ecol. 23, 1867–1880. https://doi.org/10.1023/B:JOEC.0000006456.90528.94.
Villar, G., Grozinger, C.M., 2017. Primer effects of the honeybee, *Apis mellifera*, queen pheromone 9-ODA on drones. Anim. Behav. 127, 271–279. https://doi.org/10.1016/j.anbehav.2017.03.023.
Villar, G., Baker, T.C., Patch, H.M., Grozinger, C.M., 2015. Neurophysiological mechanisms underlying sex- and maturation-related variation in pheromone responses in honey bees (*Apis mellifera*). J. Comp. Physiol. A. 201, 731–739. https://doi.org/10.1007/s00359-015-1006-7.
Wanner, K.W., Nichols, A.S., Walden, K.K.O., Brockmann, A., Luetje, C.W., Robertson, H.M., 2007. A honey bee odorant receptor for the queen substance 9-oxo-2-decenoic acid. Proc. Natl. Acad. Sci. U. S. A. 104, 14383–14388. https://doi.org/10.1073/pnas.0705459104.
Wu, X.B., Wang, Z.L., Gan, H.Y., Li, S.Y., Zeng, Z.J., 2016. Transcriptome comparison between newly emerged and sexually matured bees of *Apis mellifera*. J. Asia Pac. Entomol. 19, 893–897. https://doi.org/10.1016/j.aspen.2016.08.002.
Yang, L., Zhang, L.Z., Zeng, Z.J., 2017. Cloning and sequencing analysis of Odour receptor 170 in honeybees, *Apis cerana cerana*. J. Environ. Entomol. 39, 48–54. https://doi.org/10.3969/j.issn.1674-0858.2017.01.4.
Zeng, Z.-J., 2017. Apiculture. China Agriculture Press, Beijing.
Zhang, L.Y., Xie, B.H., Ni, C.X., Zhao, L., Li, H.L., Shang, H.W., 2012. Cloning, expression and subcellar localization of the olfactory co-receptor Orco gen in the Chinese honeybee, *Apis cerana cerana*(Hymentoptera: Apidae). Acta Entomol. Sin. 55, 1246–1254. https://doi.org/10.16380/j.kcxb.2012.11.002.
Zhang, L.Z., Zhang, S.W., Wang, Z.L., Yan, W.Y., Zeng, Z.J., 2014. Cross-modal interaction between visual and olfactory learning in *Apis cerana*. J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol. 200, 899–909. https://doi.org/10.1007/s00359-014-0934-y.
Zhang, Z.Y., Yang, S.S., Zhao, H.T., Jiang, Y.S., 2016. Expression and localization analysis of olfactory receptor AcerOrco in the Chinese honeybee,*Apis cerana cerana* (Hymentoptera: Apidae). Acta Entomol. Sin. 59, 185–191. https://doi.org/10.16380/j.kcxb.2016.02.008.
Zhao, H.T., Gao, P.F., Zhang, G.X., Ma, W.H., Jiang, Y.S., 2012. Gene cloning and sequence analysis of Or1 and Or2in *Apis cerana*. Chin. J. Appl. Entomol. 49, 1117–1124.
Zhao, H.T., Gao, P.F., Zhang, C.X., Ma, W.H., Jiang, Y.S., 2013. Molecular identification and expressive characterization of an olfactory co-receptor gene in the Asian Honeybee, *Apis cerana cerana*. J. Insect Sci. 13, 1–14. https://doi.org/10.1673/031.013.8001.
Zhao, H.T., Gao, P.F., Du, H. yan, Ma, W.H., Tian, S.H., Jiang, Y.S., 2014. Molecular characterization and differential expression of two duplicated odorant receptor genes, AcerOr1 and AcerOr3, in *Apis cerana cerana*. J. Genet. 93, 53–61. https://doi.org/10.1007/s12041-014-0332-9.
Zhao, H.T., Gao, P.F., Zhang, G.X., Tian, S. hao, Yang, S.S., Meng, J., Jiang, Y.S., 2015. Expression and Localization Analysis of the Odorant Receptor Gene Orco in Drones Antennae of *Apis cerana cerana*. Sci. Agric. Sin. 48, 796–803. https://doi.org/10.3864/j.issn.0578-1752.2015.04.17.