RESEARCH ARTICLE

Molecular characterization and differential expression of two duplicated odorant receptor genes, *AcerOr1* and *AcerOr3*, in *Apis cerana cerana*

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Abstract

Insects use olfaction to recognize a wide range of volatile cues, to locate food sources, mates, hosts and oviposition sites. These chemical volatiles are perceived by odorant receptors (ORs) expressed on the dendritic membrane of olfactory neurons, most of which are housed within the chemosensilla of antennae. Most insect ORs are tandemly arrayed on chromosomes and some of them are formed by gene duplication. Here, we identified a pair of duplicated *Or* genes, *AcerOr1* and *AcerOr3*, from the antennae of the Asian honeybee, *Apis cerana cerana*, and reported their molecular characterization and temporal expression profiles. The results showed that these two genes shared high similarity both in sequence and the gene structure. Quantitative real-time PCR analysis of temporal expression pattern indicated that in drones the expression pattern of these two genes were very similar. The transcripts expressed weakly in larvae and pupae, then increased gradually in adults. In workers, the expression level of *AcerOr1* changed more drastically and expressed higher than that of *AcerOr3*. However, both reached their highest expression level in one-day-old adults. In addition, the expression profiles between different sexes revealed that *AcerOr3* appear to be expressed biased in male antennae. These results suggest that AcerOr1 may perceive odours of floral scents, while *AcerOr3* may detect odours critical to male behaviour, such as the queen substance cues.

[Zhao H., Gao P., Du H., Ma W., Tian S. and Jiang Y. 2014 Molecular characterization and differential expression of two duplicated odorant receptor genes, *AcerOr1* and *AcerOr3*, in *Apis cerana cerana*. *J. Genet*. **93**, 53–61]

Introduction

Olfactory system of insects plays an important role in detecting volatile chemical cues in the environment and is consequently used for identification of food sources, mating, reproduction, avoidance of predators, and kin communication. Peripheral olfactory information is detected by olfactory receptor neurons, most of which are housed in the chemosensilla of insect's antennae (Kwon *et al.* 2006; Van der Goes van Naters and Carlson 2006) and the odorant receptors (ORs) that are located on the dendritic membrane of ORNs (Jones *et al.* 2005; Benton *et al.* 2007).

Insect ORs appear to have no sequence similarity to those of vertebrates (Robertson *et al.* 2003; Hallem *et al.* 2004) and have evolved a membrane topology reversed from that of typical G protein-coupled receptors with an intracellular NH_2-terminus and an extracellular COOH-terminus (Benton *et al.* 2006; Lundin *et al.* 2007; Smart *et al.* 2008). Insect ORs display low sequence identity within and among species except for the odorant receptor coreceptor (Orco) orthologues and ORs form dimers with Orco to carry out the function of olfactory signal transduction (Neuhaus *et al.* 2005; Benton *et al.* 2006; Sato *et al.* 2008; Wicher *et al.* 2008).

The *Or* genes are widely distributed throughout the insect genome (Clyne *et al.* 1997) and they constitute a large gene family. For example, *Drosophila melanogaster*, *Anopheles gambiae*, *Bombyx mori*, *Tribolium castaneum* and *Nasonia vitripennis* have 62, 79, 60, 341 and 301 *Or* genes, respectively (Gao and Chess 1999; Hill *et al.* 2002; Wanner *et al.* 2007; Engsontia *et al.* 2007; Robertson *et al.* 2010). The evolutionary origin of the insect ORs is mysterious: the *Or* families are highly diverged with some gene duplication and deletion, suggesting an ancient origin and rapid evolution of this gene family (Clyne *et al.* 1999; Nei *et al.* 2008).

The honeybees (*Apis mellifera*), a key model in the study of the olfactory perception and learning, are social insects and in this eusocial colony the queen, worker bees and drones employ their sensitive olfactory sense primarily to reproduce,

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Keywords. odorant receptor; duplicated genes; sequence; *Apis cerana cerana*.

seek out food, communicate with one another and transmit important information to members effectively.

Sequencing of the honeybee genome (Weinstock *et al.* 2006) has facilitated the identification of chemoreceptors in this species and the study of chemosensory evolution across the genus. More recently, 170 *Or* genes, including seven pseudogenes, were discovered in the *A. mellifera* genome (Robertson and Wanner 2006). The expansion of the *Or* gene family may be related to the remarkable olfactory ability of bees to recognize various odours or pheromones emitted from external and internal environments. Phylogenetic analysis showed that the majority of *Or* genes within this large family are tandemly arrayed, and a 60-gene array is located on chromosome 2. Within this gene cluster, two genes, *AmelOr1* and *AmelOr3*, are closely related to each other but are relatively divergent from the others (Robertson and Wanner 2006).

Although the honeybee *Or* genes have been identified, the specific characterization of individual genes has not been done, with the exception of the *Orco* gene (Krieger *et al.* 2003; Zhao *et al.* 2013). Here, we identified the *AcerOr1* and *AcerOr3* genes from both DNA and cDNA in *Apis cerana cerana* and quantified their expression pattern in different stages and sexes to investigate their potential function in olfactory recognition.

Materials and methods

Samples

Apis cerana cerana was obtained from a laboratory colony in Shanxi Agriculture University, Shanxi, China. Dissected thoraxes and antennae from worker bees were used for gene cloning. Pooled samples were collected respectively from larvae, pupae, and antennae of adults at different developmental stages in both workers and drones for mRNA expression pattern analysis.

RNA isolation and full-length cDNA cloning

Antennae were dissected from 50 workers and immediately frozen in liquid nitrogen. Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, USA) following the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScript® RT Reagent Kit (Takara, Dalian, China). Primers were designed according to the *Or1* and *Or3* gene sequences of *A. mellifera* in GenBank (GenBank acc. no. XM_001121080, XM_003250721). The internal sequences of the two genes were amplified under the following PCR conditions: 3 min at 94°C followed by 35 cycles of 30 s at 94°C, 1 min at 50–58°C, 1 min at 72°C and finally 8 min elongation at 72°C. Primers used in this assay are shown in table 1. PCR products were purified and cloned into a pGM-T vector (Tiangen, Beijing, China), and sequenced at Huada Gene Research Center (Beijing, China). The 5′ and 3′ flanking regions were obtained with a SMARTer™ RACE cDNA Amplification Kit (Clontech, Palo Alto, USA). Gene-specific primers (GSP) (listed in table 1) were designed based on the sequences obtained above. 10× Universal Primer A Mix (UPM, Clontech, Palo Alto, USA) was used as the upper primer for 3′ RACE and the lower primer for 5′ RACE. PCR conditions were as follows: 94°C for 3 min followed by 33 cycles of 94°C for 30 s, 55–60°C for 40 s, and 72°C for 1 min followed by final extension at 72°C for 6 min. PCR products were cloned and sequenced. The full-length cDNAs were obtained by assembling the fragments of the internal sequences and the 5′ and 3′ segments.

DNA extraction and intron cloning

Total DNA was extracted from the thorax of a single individual bee using the method described by Smith and Hagen (1996). To clone full introns, specific primers for both gene were designed according to the full-length cDNAs acquired in above experiment (primers are listed in table 1). The PCR conditions were as follows: 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 53–58°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 8 min. Each fragment was cloned and sequenced, then the total introns were assembled with the cDNA sequences.

Sequence alignment and phylogenetic analysis

Homologous amino acid sequences were searched using BLAST (www.ncbi.nlm.nih.gov/BLAST). These sequences and the two genes identified above were aligned in Clustal W (Thompson *et al.* 1994), and the per cent identity values of each sequence pair were acquired in the MegAlign program DNASTAR Lasergene 7.1 (Madison, USA). Phylogenetic analysis was conducted using MEGA 4.0 (Tamura *et al.* 2007) according to the neighbour-joining (NJ) algorithms and bootstrap values were calculated from 1000 replications.

Transmembrane domain prediction

Transmembrane domains of the two ORs were predicted with TMHMM 2.0 (Krogh *et al.* 2001) at the server (http://www.cbs.dtu.dk/services/TMHMM/) and TMPred (Hofmann and Stoffel 1993) at the server (http://www.ch.embnet.org/software/TMPRED_form.html).

Developmental expression analysis by qRT-PCR

The total antennae RNA from larvae (2, 4, 6 days old), pupae (5, 10 days old), adult worker bees (1, 5, 10, 15, 20, 25, 30, 35 and 40 days old), and uncapped larvae (4 days old larvae) , capped larvae (6 days old larvae), pupae to eclosion (10 days old pupae), adult drones (1, 5, 10, 15, 20, 25, 30 days old) was extracted for expression profiling. Primers were designed according to the two full-length sequences obtained from RACE PCR. To adjust for the variations in the quantity of input cDNA, the housekeeping gene *Rps18* was

Table 1. Primers for nucleotide sequences used in this study.

Purpose	Primers	Sequence (5′–3′)
cDNA cloning	Or1 F[a]	GATCCGATTTTGGTTGATGG
	Or1 R[b]	TATCTTGGCCCCAGACAGAC
	Or3 F	GGTCGGAATCTGGCCAAGGAGG
	Or3 R	TGGCACCAGACAGGCTTGATG
5′ RACE	Or1 GSP[c]	CGATCTTCTTCGCATTCCACGTCT
	Or3 GSP	CGCCAAAAGGCTGAAAACCTGGGC
3′ RACE	Or1 GSP	GGTACGATTTTCCAACGGAAGTGG
	Or3 GSP	CCATCAAGCCTGTCTGGTGCCAAG
DNA cloning	Or1 F1	CAAGGAGGACAACACGACTCA
	Or1 R1	TGCTCAGTGATTCTCCAACCC
	Or1 F2	CACGGGTTGGAGAATCACT
	Or1 R2	GTAGGCTGCCGAAGTTTT
	Or3 F1	AGATTTGCGTGGCCGCATCG
	Or3 R1	TGGCACCAGACAGGCTTGATG
	Or3 F2	CCATCAAGCCTGTCTGGTGCC
	Or3 R2	AATTTAGATATGCTGCTGAAG
qRT-PCR	Or1 F	AGGATTCGCCGATTTACGAG
	Or1 R	CGCAGCAGTGCATGGTTATAG
	Or3 F	AGCCGCCCAGGTTTTCAGCC
	Or3 R	CCGATCCTCTTCGCATTCCACG
	Rps 18 F	GATTCCCGATTGGTTTTTGA
	Rps 18 R	CCCAATAATGACGCAAACCT

used as an internal control (Scharlaken *et al.* 2008). Primers were designed based on the *A. mellifera Rps18* sequence (XM_625101). PCR-grade water was used as a negative control. All primer pairs are shown in table 1. qRT-PCR was run on an Mx3000P real-time PCR system (Stratagene, La Jolla, USA) using the SYBR Premix Ex Taq^{TM} kit (Takara, Beijing, China). All samples were tested in triplicate. PCR conditions were as follows: 95°C for 20 s followed by 45 cycles of 95°C for 15 s and 60°C for 20 s, followed by 95°C for 30 s, 60°C for 30 s, and 95°C for 30 s to obtain the dissociation curve. Relative quantification was analysed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). We used the SPSS software (IBM, Chicago, USA) for statistical analysis.

Results

Characterization of DNA and cDNA sequences

The full-length cDNA sequences and total introns of *AcerOr1* and *AcerOr3* were identified and the corresponding sequences are available in GenBank (cDNA accession no.: JN792580, JX049410; DNA accession no.: JN544932, JX258126).

The entire *AcerOr1* gene sequence was 2072 bp and consisted of an open reading frame (ORF) of 1215 bp, a 5′-UTR of 93 bp, a 3′-UTR of 199 bp, and four introns of 67–333 nt in length. The 3′-UTR of the *AcerOr1* gene contained the polyadenylation signal AATAAA 148 bp downstream of the stop codon and an expected poly(A) tail 23 bp further downstream from the polyadenylation signal (figure 1). Similarly, the entire *AcerOr3* gene was 1891 bp, consisting of an ORF of 1209 bp, a 5′-UTR of 85 bp, a short 3′-UTR of 26 bp, and four introns of 70–332 nt in length. The polyadenylation signal in *AcerOr3* slightly overlapped with its stop codon (figure 2). The comparison of the two gene structures is shown in figure 3.

Nucleotide sequence comparison

Comparison of the nucleotide sequences of *AcerOr1* and *AcerOr3* revealed a high level of conservation in their sequences (with the exception of the poly(A) tail), and the nucleotide identity was 77.3%. In addition, the introns exhibited 64.5% identity between the two genes. The 5′ flanking sequences shared 73.1% identity. The average AT content of *AcerOr1* and *AcerOr3* was 81.4 and 78.5% for the introns and 62.4 and 60.6% for the exons, respectively. This suggests the existence of codon usage preferences.

Deduced amino acid sequence analysis

In total, 404 and 402 amino acids were deduced from the coding regions of *AcerOr1* and *AcerOr3*, respectively. Their protein molecular weights about 47 kDa. Using BLAST, we searched several homologous amino acid sequences from other hymenopteran insect species. Multiple sequence alignments revealed that these proteins were fairly conserved (figure 4a). The pair-wise amino acid identity values across the multiple sequences are listed in table 2. The two ORs identified in this study, AcerOr1 and AcerOr3, shared 82.7% identity with each other. When compared with other homologous proteins, the identities ranged from 81.8% to 98.2% within the genus and 51.7 to 84.4% between the genera.

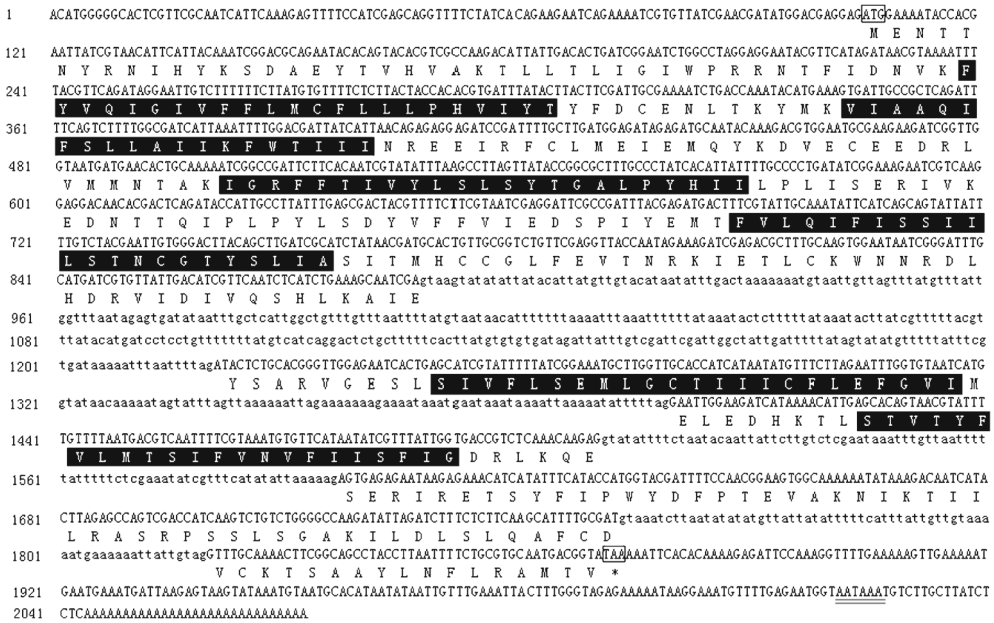

Figure 1. The genomic and cDNA sequences of *AcerOr1*. Uppercase letters represent exons and lowercase letters represent introns. The deduced amino acid sequence of *AcerOr1* is shown below the coding regions. Start and stop codons are boxed. Double underline indicates the polyadenylation signal. The shaded aa sequence represent the TM domains.

The phylogenetic tree revealed that two paralogous genes also exist in the genomes of most other species in addition to *A. c. cerana*, but there was only one isogenous gene in *Megachile rotundata* and *Nasonia vitripennis* (figure 4b). This may have arisen from the gene duplication or loss.

Transmembrane domain analysis

The prediction results showed that both receptors contained six transmembrane domains. In addition, the transmembrane orientation and location of the two receptors were similar,

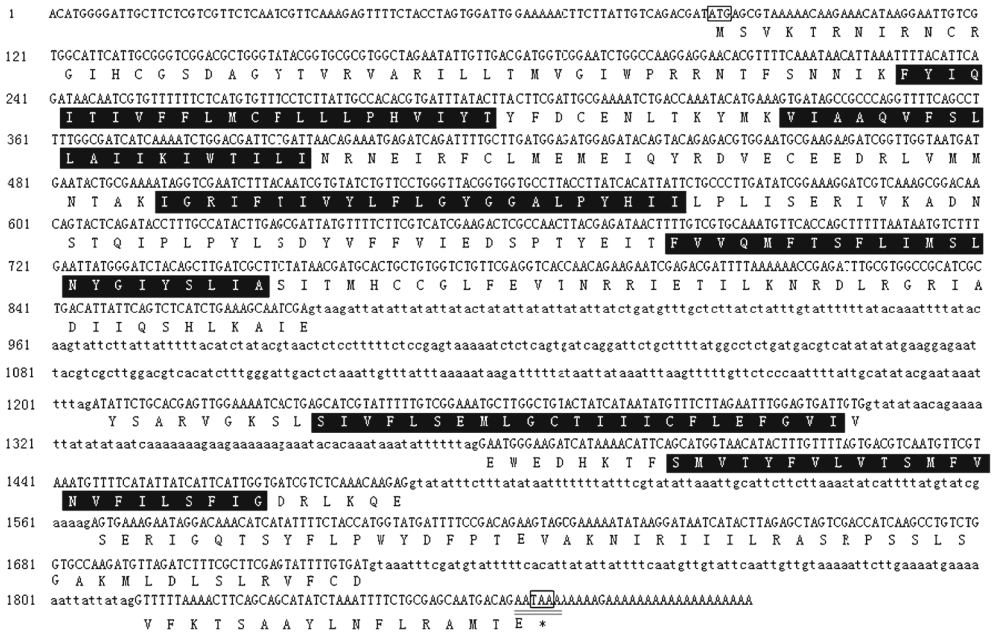

Figure 2. The genomic and cDNA sequences of *AcerOr3*. Uppercase letters represent exons and lowercase letters represent introns. The deduced amino acid sequence of *AcerOr1* is shown below the coding regions. Start and stop codons are boxed. Double underline indicates the polyadenylation signal. The shaded aa sequences represent the TM domains.

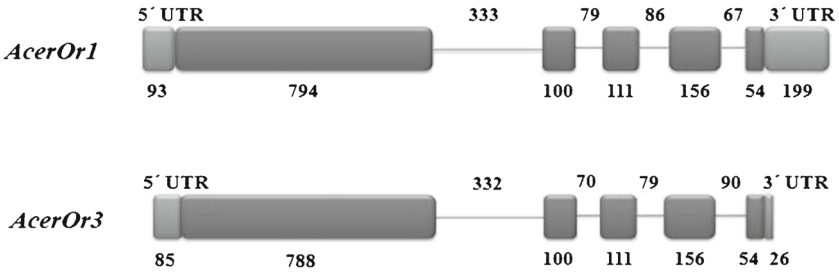

Figure 3. Schematic structure comparison of the *AcerOr1* and *AcerOr3* genes. Exons are shown as boxes in which both ends represent untranslated regions. The lines connecting ORFs represent introns. Numbers represent the size of each exon and intron.

and the N-terminus were both intracellular, as observed in other insect ORs (Bengtsson *et al.* 2012).

mRNA expression profiles at different developmental stages

mRNA temporal expression patterns of *AcerOr1* and *AcerOr3* in both sexes are presented in figure 5. *AcerOr1* transcripts were relatively low in uncapped worker bee larvae but then increased notably in capped larvae and pupae, and reached the highest level on the day after eclosion. The expression level decreased at day 5 and remained low from then on, with the exception of a slight increase at day 21. Levels were relatively stable in other time. mRNA expression levels of *AcerOr3* were more moderately but lower throughout the developmental stages than *AcerOr1*; they were slightly higher in larvae and pupae than in adults. The highest level also occurred on the first day after eclosion (figure 5a). In drones, *AcerOr3* expression levels were relatively higher than those of *AcerOr1*. However, the expression trend in both genes was very similar: lower in larvae and pupae, and then increasing gradually in adults, and finally reaching their highest level at the end of the sampling stage (figure 5b).

In addition, two sampling time points, day 1 and day 30 of adult bees, were selected for comparing the expression levels of *AerOr1* and *AcerOr3* between workers and drones. In figure 6, we can observe there are clear differences between the expression patterns of the two genes. For *AerOr1* gene, the expression level of one-day-old adult bees was significantly higher in workers than in drones, while it was higher in drones than in workers at 30-day-old ($P < 0.01$) (figure 6a). For *AcerOr3* gene, the transcripts were both significantly higher in drones at the two developmental stages (figure 6b). We can infer that *AcerOr3* expression was male-biased from these results.

Discussion

AcerOr1 and AcerOr3 are duplicated genes

Insect odorant receptor genes are widely distributed throughout the genome but also are often found in clusters. Genes within a cluster often exhibit a high degree of sequence identity to each other, suggesting that some of the ancestral *Or* genes have undergone duplication events (Robertson *et al.* 2003; Zhou *et al.* 2012).

Duplicated genes are usually generated by unequal crossingover during meiosis. It is believed that gene duplication is a necessary source of material for the origin of evolutionary novelty (Lynch and Conery 2000). The two odorant receptor genes identified in this study, *AcerOr1* and *AcerOr3,* can be regarded as duplicated genes. First, the two genes are quite similar in sequence (77.3% identity on DNA level and 82.7% identity on protein level). Second, their overall gene structures are highly similar too; both genes consist of five exons and four introns and have similar exon/intron boundary positions and exon sizes. In addition, the amino acid sequences of the two genes encode ~400 amino acid residues, and both contain six transmembrane domains with identical orientation. Moreover, their orthologous genes in *A. mellifera*, *AmelOr1,* and *AmelOr3*, are located on the same chromosome and tandemly arrayed in the genome (Robertson and Wanner 2006). This suggests that *AcerOr1* and *AcerOr3* might be neighbouring duplicated genes in the genome of *A. cerana* as well, although confirmation will require the complete sequencing of the *A. cerana* genome.

The BLAST and homology comparison results revealed that the duplication that led to the *AcerOr1* and *AcerOr3* pair seems to be unique to the Apidae lineage, as suggested by the phylogenetic reconstruction. This duplication seems not to have occurred in the sister group to the Apidae, the Megachilidae, since we only discover one gene from *M. rotundata*. Further support that this duplication is a later event in the Apidae would stream from the fact that the much more distant *N. vitripennis* (a Chalcidoidea) only has one putative orthologue NvitOr2. This result supports the view that the birth-and-death model is the major mechanism of evolution in the insect *Or* family (Roelofs and Rooney 2003; Sánchez-Gracia *et al.* 2009). This copy number variation between species is also suggested to have a significant role in adaptation and could provide raw material for genes with new functions (Innan and Kondrashov 2010).

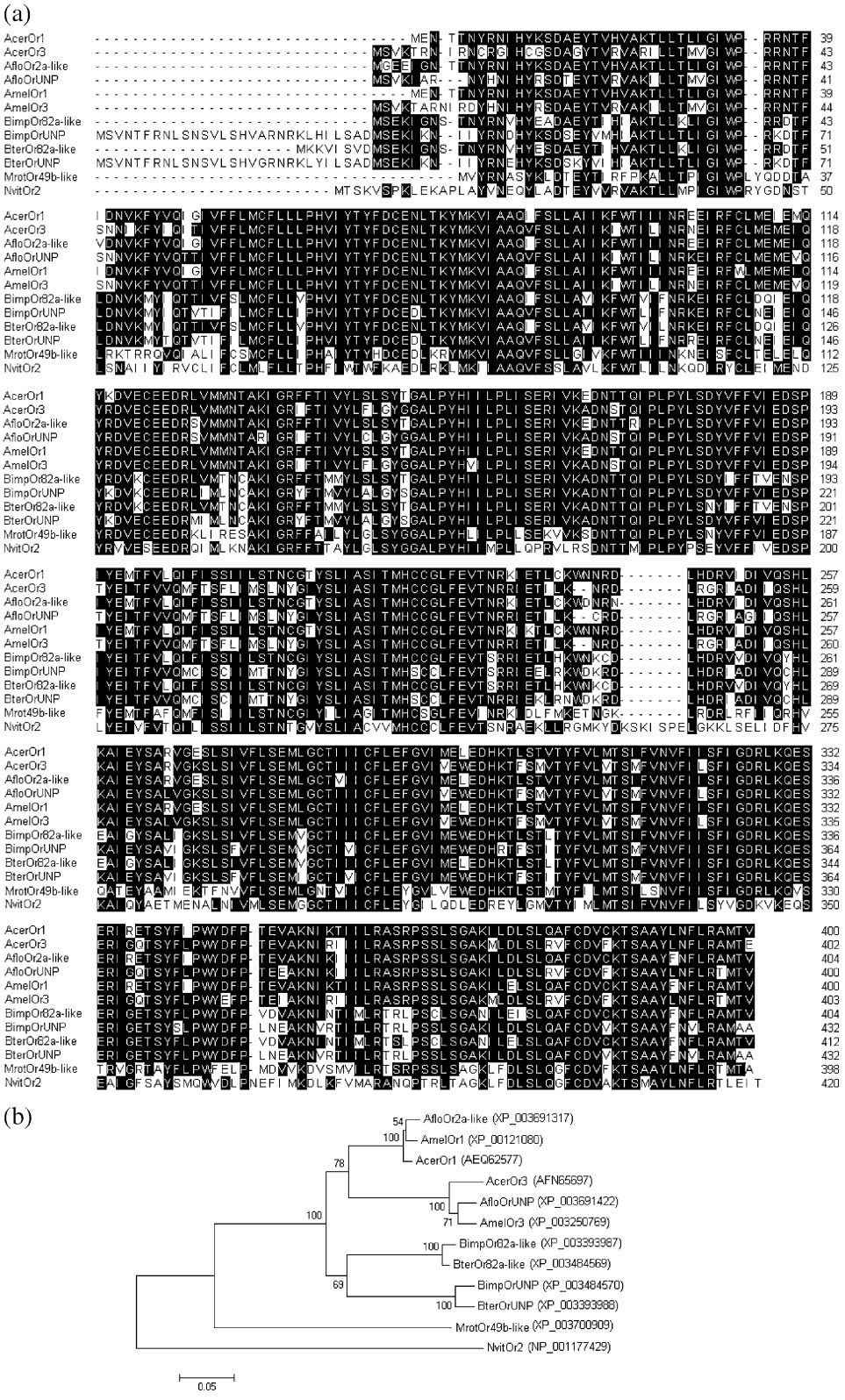

Figure 4. Comparison of the deduced *AcerOr1* and *AcerOr3* amino acid sequences with homologous proteins from other Hymenoptera species. (a) Alignment of amino acid sequences. Identical amino acid residues are shaded in black. (b) Phylogeny tree constructed by the NJ method. The branch labels are bootstrap values (%), and the scale bar is 0.05. UPN stands for uncharacterized proteins. Abbreviations are: Acer, *Apis cerana*; Aflo, *Apis florea*; Amel, *Apis mellifera*; Bimp, *Bombus impatiens*; Bter, *Bombus terrestris*; Mrot, *Megachile rotundata*, and Nvit, *Nasonia vitripennis*.

Table 2. The identity matrix of *AcerOr1* and *AcerOr3* and homologous amino acid sequences from other Hymenoptera species.

	a	b	c	d	e	f	g	h	i	j	k	l
a	100											
b	82.1	100										
c	96.5	81.8	100									
d	82.2	93.2	82.5	100								
e	98.2	82.4	97.0	82.3	100							
f	81.8	95.0	81.3	96.5	81.9	100						
g	83.4	74.6	82.9	75.2	84.0	74.4	100					
h	78.7	75.8	77.9	76.5	78.2	75.8	81.6	100				
i	84.4	75.1	83.9	75.8	85.0	74.9	98.3	79.8	100			
j	78.9	76.1	78.4	76.8	78.4	76.1	80.4	96.5	78.6	100		
k	66.4	63.7	66.4	64.4	65.9	65.0	62.4	62.6	62.9	63.4	100	
l	54.2	51.7	53.7	53.5	54.2	52.6	53.7	52.8	53.9	52.6	54	100

Potential roles of AcerOr1 and AcerOr3

Honeybee has a remarkably large odorant receptor family. This family might have evolved not only to permit the elaborate social chemical communication system but also the subtle ability of the honeybee to differentiate diverse floral odours (Robertson and Wanner 2006).

It may take a long time for duplicated genes to generate functional differentiation, but divergence in quantitative expression can occur quite soon after the genes originate

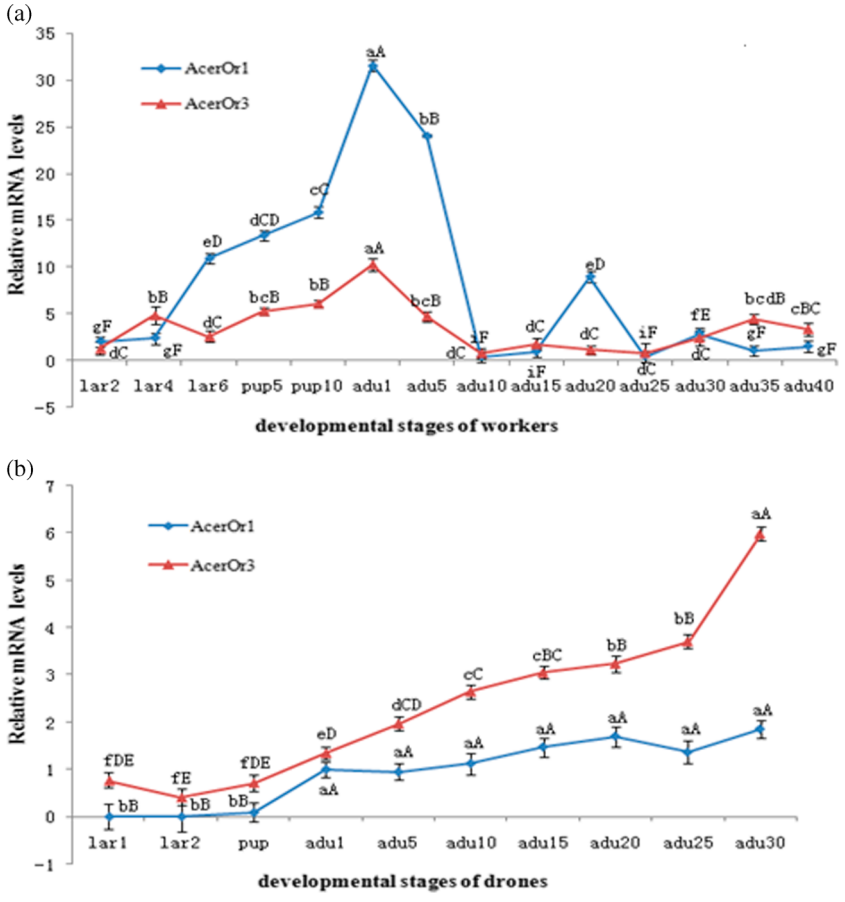

Figure 5. Relative amounts of *AcerOr1* and *AcerOr3* mRNA in workers and drones at different developmental stages. The y-axis is the relative level as determined by the $2^{-\Delta\Delta Ct}$ method. (a) Comparison of *AcerOr1* and *AcerOr3* expression levels in worker bees. lar2 and lar4 are 2 and 4-day-old uncapped larvae, respectively; lar6 are 6-day-old capped larvae; pup5 and pup10 are 5 and 10-day-old pupae, respectively; and adu1 to adu40 are adult days after eclosion. (b) Comparison of *AcerOr1* and *AcerOr3* expression levels in drones. lar1 are uncapped larvae; lar2 are capped larvae; pup are pupae to eclosion; adu1 to adu30 are adult days after eclosion. Data are shown as the mean ± SEM.

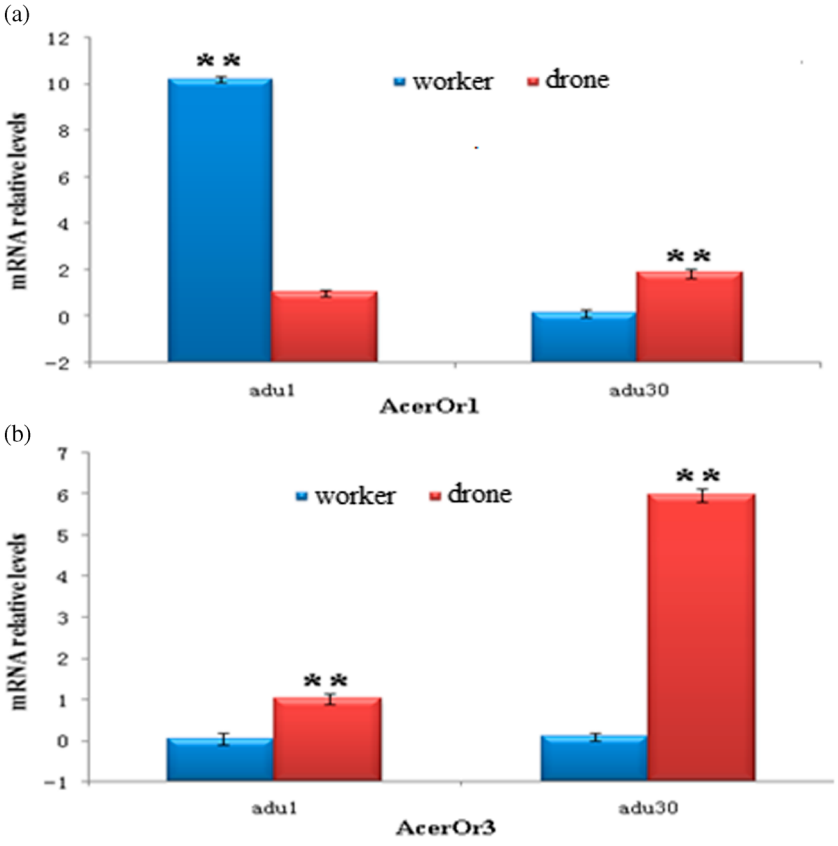

Figure 6. Expression differences of *AcerOr1* and *AcerOr3* between workers and drones adu1 and adu30 are one-day-old adult and 30-day-old adult bees. Data are shown as the mean ± SEM.

(Gu *et al.* 2002; Chain *et al.* 2008). The qRT-PCR analysis revealed that the *AcerOr1* and *AcerOr3* genes expressed in both worker and drone antennae, but their expression profiles were different in sexes and developmental stages. Observed from the temporal expression profiles, in drones, the two genes expressed not very drastically and exhibited an upward trend throughout the developmental stages. It is possibly due to that the main tasks of drones are to be ready to mate with a receptive queen. In workers, *AcerOr3* mRNA expression levels remained relatively stable compared with *AcerOr1*. However, the peak level for both genes occurred on the day that the pupae underwent eclosion. This may be attributed to the newly emerged bees receiving many kinds of chemical cues, such as pheromones and odours originating from the colony. In addition, it is worth noting that *AcerOr1* transcripts in 20-day-old adults stood out compared with those expressed after day 5. Since mature bees become foragers and begin collecting nectar and pollen at approximately three weeks old (Mark 1987; Yang 1998), we suggest that *AcerOr1* may be associated with the perception of floral scents volatiles. Analysed from the sexual expression profiles, *AcerOr3* gene was expressed at moderate male-biased ratio. This result suggests that AcerOr3 may detect odours critical to male behaviour, such as the queen substance cues.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31272513) and the Graduate Outstanding Innovation Foundation of Shanxi province (20123052).

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Received 25 July 2013, in revised form 25 September 2013; accepted 1 October 2013
Published on the Web: 28 March 2014