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Novel odorant-binding proteins and their expression patterns in grasshopper, *Oedaleus asiaticus*

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ABSTRACT

Insects use olfaction to detect exogenous odors and adapt to environments. In their olfaction systems, odorant-binding proteins (OBPs) are believed to be a key component. The unique OBP system of each species reflects the evolution of chemosensation of insects with habits. Here, we for the first time identified 15 OBPs, OasiOBP1–15, of a grasshopper, *Oedaleus asiaticus*, that lives in the grasslands of Northern China and is closely related to the locust, *Locusta migratoria*. OasiOBP9 and OasiOBP10 are specifically expressed in the antennae. Other OBPs are expressed in the antennae as well as other chemosensory organs, such as the mouthparts and wings. Significantly more OasiOBP7 was detected in male than female antennae, but there are 9 OBPs that were more expressed in female than male antennae by quantitative real-time PCR. Phylogenetic analysis indicated that most of the *O. asiaticus* OBPs are similar to those of *L. migratoria*, but some are substantially different. This indicates that the OBPs originally evolved in a common ancestor, but their unique chemosensory systems are adapted to different ecosystems.

1. Introduction

Insects use olfaction to detect exogenous odors and adapt to environments [1–3]. In their olfaction systems, several families of proteins are involved in chemical signal transduction, including odorant-binding proteins (OBPs), odorant receptors (ORs), and ionotropic receptors (IRs) [4–7].

OBPs are widely expressed in hair-like chemosensory organs of insects [8–10]. Highly expressed OBPs in chemosensory organs indicate that they play an important role in chemoreception [11]. Recent work on changing molecular structure provided strong evidence that LUSH triggers action potential of the olfactory receptor neurons after they are bound by odorant receptors during chemoreception [12]. Therefore, studying insect OBPs is useful for revealing molecular mechanisms of insect olfaction.

A general picture of one species insect OBPs can be revealed using whole-genome sequences [13–15]. Previous results indicated that each insect species has its own specific OBPs, and OBPs may reflect the evolution of chemoreception [16]. Recently, the OBPs of several species were identified by transcriptome sequencing [17–23]. This provides a means by which to compare the repertoire of OBPs between two species to address the evolution of chemosensory mechanisms. However, few studies have focused on the evolution of molecular olfaction between two closely related species within the same family that live in different ecological environments.

The locust, *Locusta migratoria* (Orthoptera, Acrididae), feeds on graminaceous grasses and crops. *Oedaleus asiaticus*, a grasshopper is closely related to *L. migratoria* and also primarily feeds on graminaceous grasses and crops [24].

Unlike *L. migratoria*, which lives in humid environments near rivers, lakes, or seas, *O. asiaticus* has a restricted distribution in arid grasslands of Northern China [25]. Additionally, previous studies on locust chemoreception indicated that locusts may represent a special link in insect olfaction molecular evolution [26]. Therefore, to determine the relationships of olfaction molecular systems between the 2 species, OBPs may provide strong evidence to elucidate the role of chemoreception in the evolution and shaping the geographic distribution of the two species.

Many studies have been conducted on *L. migratoria* molecular olfaction, particularly OBPs [27–29]. However, no study on *O. asiaticus* chemoreception has been reported to date.

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The results of this study report 15 novel OBPs of *O. asiaticus* for the first time, elucidate OBP expression patterns, and provide essential information on molecular olfaction evolution between two closely related species that live in different ecological environments.

2. Materials and methods

2.1. Insects

Adult *O. asiaticus* were collected from Inner Mongolia. Antennae, mouthparts, and wings were collected from both male and female adults, transferred to Eppendorf tubes, and stored at −80 °C until use.

2.2. cDNA library construction, Illumina sequencing, and bioinformatics analysis

Total RNA of male antennae and female antennae was extracted with Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions. The integrity of total RNA was checked using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The mRNA was purified from 16.74 to 18.15 μg total RNA of male and female antennae, respectively, with Oligo (dT) magnetic beads. Mixing with the fragmentation buffer fragmented the mRNA into short fragments. Then, the first-strand cDNA was synthesized using random hexamer-primers, followed by second-strand cDNA synthesis using DNA polymerase, RNaseH, and dNTPs. Short fragments were purified and resolved with EB buffer for end reparation as well as adding poly (A) and then ligated to adaptors. Subsequently, the suitable fragments were selected for PCR amplification as templates. The cDNA library was sequenced on the HisSeq™ 2000 platform using paired-end technology.

De novo assembly was performed with the short reads assembling program Trinity [30]. Trinity combined the reads with a certain length of overlap to form longer fragments called contigs. The reads were then mapped back to contigs with paired-end reads that enable detection of contigs from the same transcripts as well as the distances between these contigs. Then, the contigs were connected to obtain sequences that without Ns and could not be extended on either end. Such sequences were defined as unigenes. Finally, we assembled the unigenes of male and female antennae and eliminated duplication.

2.3. Identification of transcripts encoding putative odorant-binding protein

We screened the unigene sequences against protein databases, including non-redundant (NR), SwissProt, KEGG, COG, using Blastx (*E-value* < 10^{-5}) and the protein function information can be predicted from annotation of the most similar protein in those databases. Then, we used “OBP” and “odorant-binding protein” as key words to screen the annotated sequences. Moreover, to facilitate the identification of putative OBP genes, tBlastn was performed using known OBP sequences of *L. migratoria* as “query” to screen the transcriptome database. All putative OBP genes were manually confirmed using the Blastx program against the NR nucleotide database of the National Center for Biotechnology Information (NCBI) with a cut-off *E-value* = 10^{-5}. The open reading frame (ORF) of the putative OBP genes was predicted using ORF Finder (http://

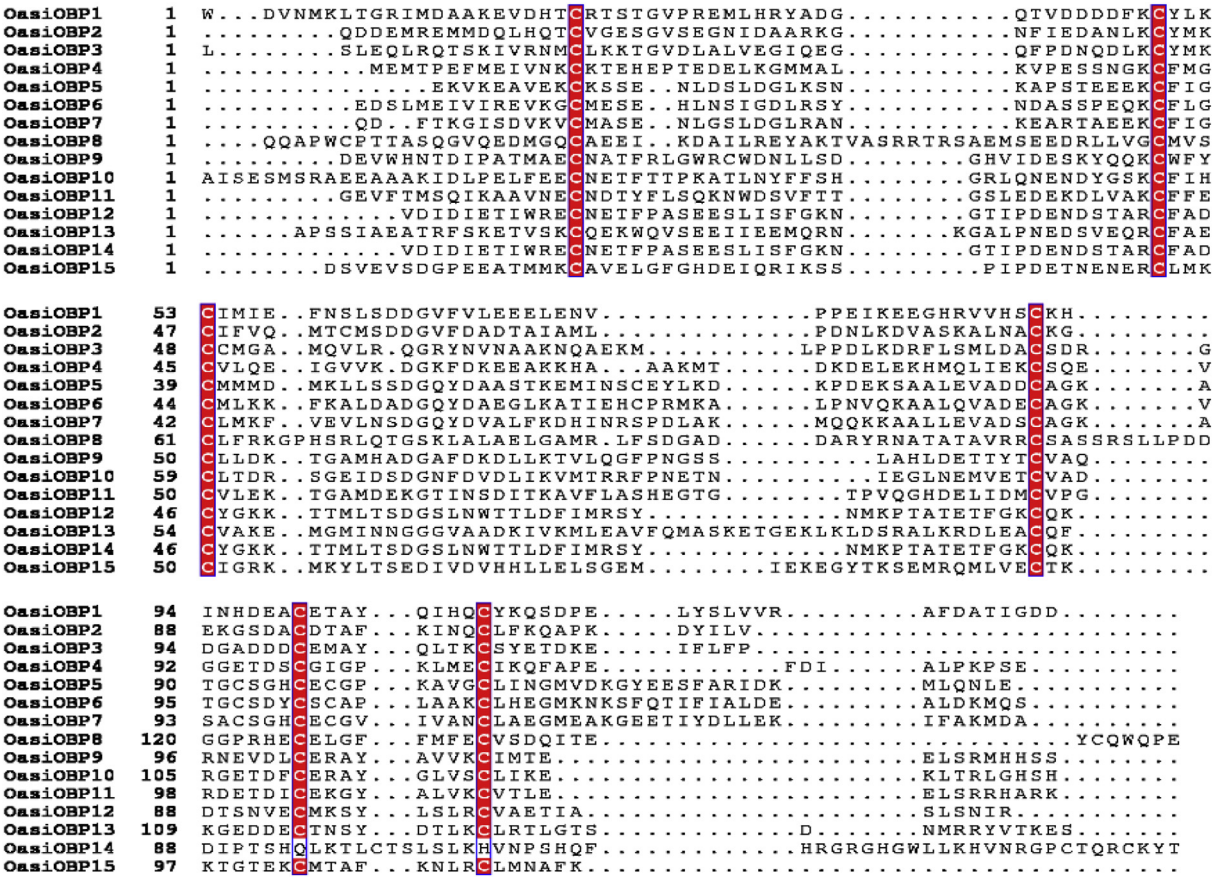

Fig. 1. Alignment of fifteen putative OBPs in *O. asiaticus*. Red boxes show conserved cysteines. GenBank accession numbers are available in Table S2.

Table 1
The consensus(%) of 15 OBP amino acid sequences alignment.

	OBP1	OBP2	OBP3	OBP4	OBP5	OBP6	OBP7	OBP8	OBP9	OBP10	OBP11	OBP12	OBP13	OBP14
0BP2	22.99													
0BP3	18.18	18.18												
0BP4	14.97	19.25	16.58											
0BP5	11.23	12.3	14.44	15.51										
0BP6	12.3	15.51	13.9	16.04	32.62									
0BP7	10.7	11.23	12.3	11.76	38.5	31.55								
0BP8	8.56	8.56	10.16	12.3	9.63	7.49	10.16							
0BP9	10.7	12.3	11.23	13.9	9.63	13.37	11.76	8.02						
OBP10	11.76	13.37	14.44	17.11	14.44	12.83	14.97	10.16	31.55					
OBP11	9.09	8.56	9.09	14.97	12.3	12.83	10.16	12.3	27.27	22.46				
OBP12	7.49	6.95	4.81	9.09	8.56	11.76	10.7	8.56	12.3	13.9	12.3			
OBP13	11.23	13.37	12.83	15.51	11.23	12.83	10.16	9.09	14.44	18.72	14.97	9.09		
OBP14	8.02	7.49	5.35	7.49	8.56	10.16	9.63	8.56	11.23	14.44	13.37	60.96	9.09	
OBP15	8.56	11.76	11.23	9.09	11.23	11.23	7.49	6.42	11.23	9.09	8.02	12.83	10.7	12.83

www.ncbi.nlm.nih.gov/gorf/gorf.html). The signal peptides of the amino acid sequences were predicted using SignalIP4.1 server (http://www.cbs.dtu.dk/services/SignalP/).

2.4. Tissue expression analysis of O. asiaticus OBP genes

Moreover, we analyzed the expression of 15 identified OBPs in different tissues, including antennae, mouthparts, and wings of both male and females by RT-PCR. Total RNA was extracted from antennae, mouthparts, and wings with Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using the first-strand cDNA FastQuant RT Kit (with gDNase) (TIANGEN, China). To validate the specificity of primers used in RT-PCR, PCR product sequencing was performed. We used a 25-μl reaction mixture to conduct PCR under the following conditions: denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, annealing at 59 °C for 30 s (each primer uses a different annealing temperature), extension at 72 °C for 60 s, and then incubation for 7 min at 72 °C.

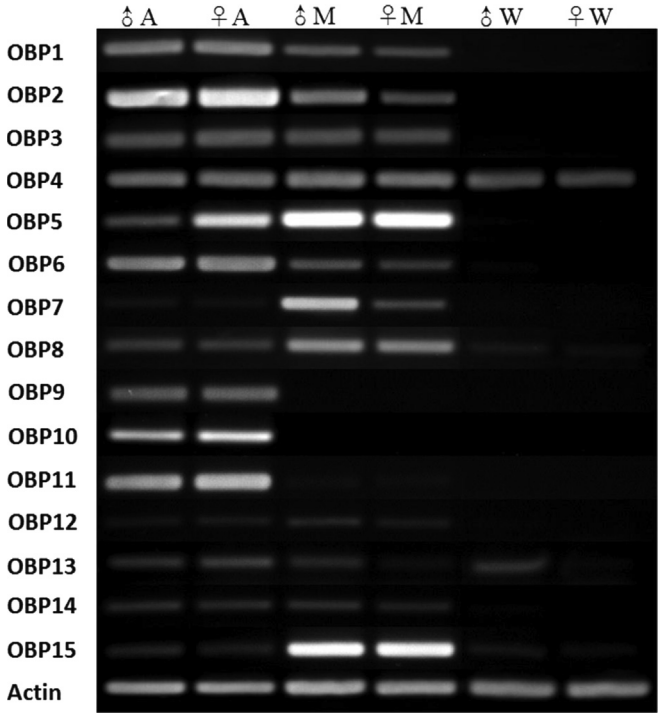

Fig. 2. Tissue expression of fifteen putative OBP genes in *O. asiaticus*. A, antenna; M, mouthparts; W, wings; Actin, *O. asiaticus* actin gene.

2.5. Sex-biased expression of OBP genes in antennae by qRT-PCR

The sex-biased expression of the 15 candidate OBP transcripts were analyzed by qRT-PCR. Total RNA was extracted from antennae as described above. cDNA was synthesized using AMV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Gene-specific primers of 15 OBPs were designed using the Primer Express Software v2.0 (Applied Biosystems, Foster City, CA, USA). All primers used are listed in Table S1. Experiments were performed in the StepOne Plus Real-Time PCR system (Applied Biosystems) using 2 × SYBR green PCR mix (QIAGEN, Hilden, Germany). β-actin was amplified for internal standardization. PCR efficiency and specificity of primers of the target genes were validated in the experiment.

qRT-PCR was performed in a 20-μl reaction mixture, and PCR was conducted under the following conditions: denaturation at 95 °C for 2 min, followed by 40 cycles of 94 °C for 10 s, annealing at 59 °C for 10 s (each primer has itself annealing temperature), and extension at 72 °C for 40 s. At the end of each reaction, the melting curve was analyzed to confirm the specificity of the primers. The expression level of each OBP was calculated by the $2^{-\Delta\Delta CT}$ method to normalize the variation in each reaction. Three biological replicates were performed.

2.6. Phylogenetic analysis of the OBPs between O. asiaticus and other insects

Phylogenetic analysis was performed based on the amino acid sequences of 15 candidate OBPs and 6 other species of insects from 6 different orders. The amino acid sequences used to construct the phylogenetic tree are shown in S1. Because 2 LmigOBP4 and 2 LmigOBP5 were submitted to the NCBI GenBank by different scientists, we renamed LmigOBP4 (OBP4, GenBank: AEX33160.1) as LmigOBP13 and LmigOBP5 (OBP5, GenBank: AEX33161.1) as LmigOBP14. The highly divergent signal peptide sequences were removed before alignment. Sequence alignments of these OBPs were performed in MAFFT version 7 [31]. The tree was constructed using the neighbor-joining method with Poisson correction of distances as implemented in MEGA6 software [32]. Branch support was assessed with 1000 bootstrap replicates.

3. Results

3.1. Identification of OBPs in O. asiaticus

From the transcriptome, we acquired 61629 unigenes with a mean length of 733 bp and the N50 length of 1130 bp. Among the 61629 unigenes, 26064 (42.3%) were annotated. We identified 11 putative OBPs from the annotated sequences and another 4

putative OBPs from the results of tBlastn. A total of 15 putative OBP genes were identified from the transcriptome database, and all of them had the full-length ORF. The 15 putative mature OBPs were aligned in Fig. 1, and all of the putative OBPs of *O. asiaticus* had 6 conserved cysteines. The amino acid sequences of the 15 OBPs ranged from 137 aa to 170 aa, and the molecular weights were calculated from 12.64 kDa to 16.57 kDa. As determined used DNAMAN v6.0, their isoelectric points were acidic and ranged from 4.24 to 5.03, except OBP14, which had a pH of 8.06 (Lynnon Biosoft, Quebec, Canada) (Table S2). The similarity between these OBPs was from 4.81% to 60.96%, which shows high divergence (Table 1).

3.2. Tissue expression of O. asiaticus OBP genes

We then checked whether these putative OBPs were expressed in chemosensory organs by performing RT-PCR after each pair of primers for the PCR has been demonstrated to be specific for each OBP by sequencing the products of each PCR with the primers. Most of these OBPs were specifically detected in antennae and mouthparts, including OBP1, OBP2, OBP3, OBP5, OBP6, OBP7, OBP11, OBP12, and OBP14. This indicates that these proteins may be involved in chemosensing. However, OBP9 and OBP10 were only detected in antennae (Fig. 2), indicating that these proteins may be involved in detection of volatiles only by the antennae. It is interesting that OBP5, OBP7, and OasiOBP15 have much greater expression in mouthparts than in antennae, indicating that these proteins may be involved in detecting food volatiles. Alternatively, OBP4, OBP8, OBP13, and OasiOBP15 were universally detected, and they may be involved in detecting some common chemicals.

3.3. Sex-biased expression of OBP genes in antennae by qRT-PCR

We compared the relative abundance of each OBP transcript between male and female adult *O. asiaticus* antennae. The melting curve showed that the primers used for qRT-PCR were specific. The most important result is that only the OBP7 gene had significantly higher expression in male than female antenna (Fig. 3). By contrast, 9 OBPs, including OBP1, OBP2, OBP3, OBP4, OBP5, OBP8, OBP9, OBP10, and OBP13, had significantly higher expression in females than in males (Fig. 3). For the other OBPs, there was no significant difference of expression levels between males and females (data not shown).

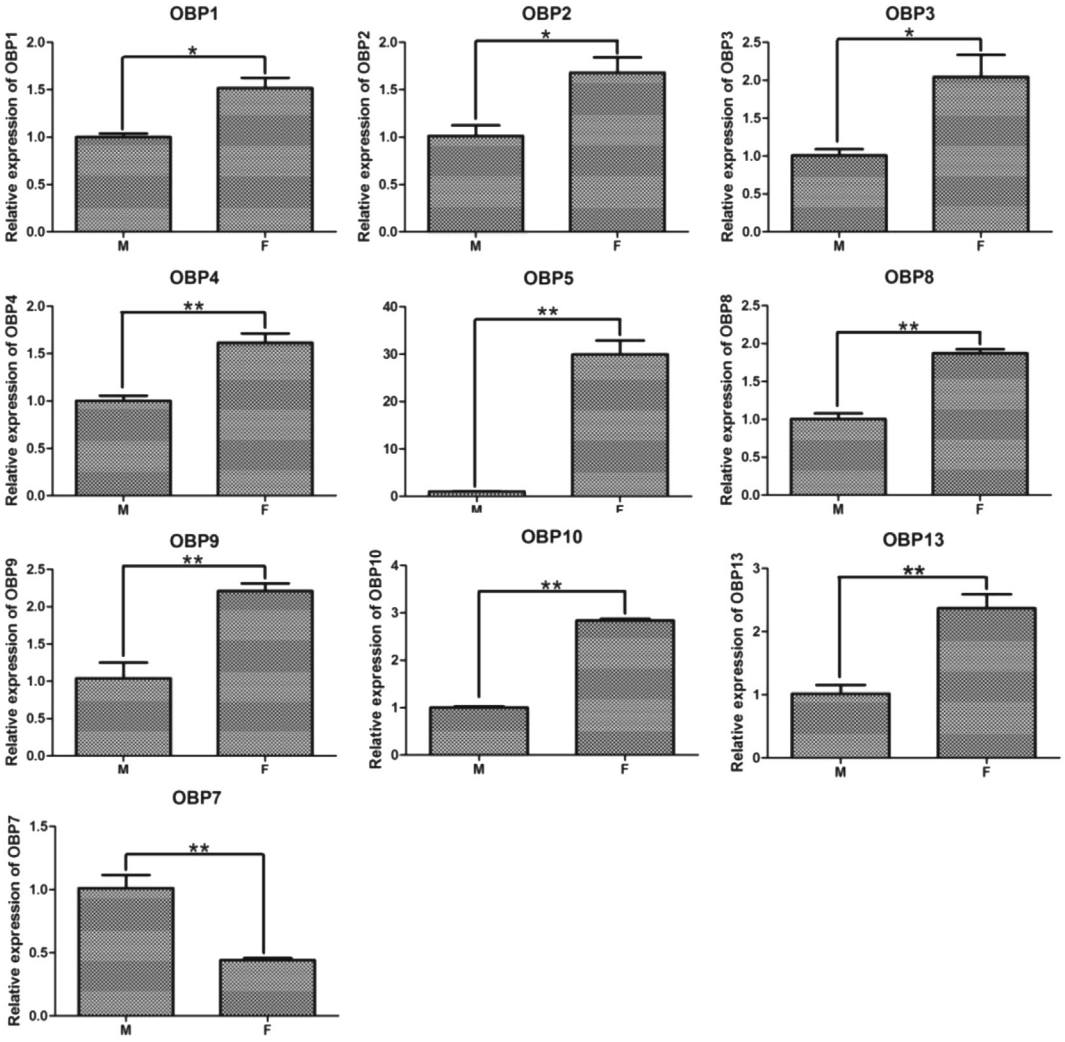

Fig. 3. Sex-bias expression of some OBP genes in grasshopper (*O. asiaticus*) antenna. Only OBP7 genes expressed significantly higher in male antenna than in female. The other genes expressed significantly higher in female antenna than in male in qRT-PCR experiment (t-test; *, $P < 0.05$; **, $P < 0.01$). M, male antennae; F, female antennae. Relative transcript levels were normalized to the internal standard of actin. The bars indicates the standard error of the mean for three independent experiments.

3.4. Phylogenetic analysis of the OBPs between O. asiaticus and other insects

A phylogenetic tree was generated to infer the relationships between 15 OBPs of *O. asiaticus* and 174 OBPs of 6 other insect species from 6 orders (Fig. 4). The figure shows that, of the different classes of OBPs of *O. asiaticus*, OasiOBPs fall into the closest groups with the OBPs of *L. migratoria*, LmigOBPs. In particular, unlike OBPs of other insects in the tree, the OasiOBPs or LmigOBPs show that few OBPs cluster into one group within the same species; that is, OasiOBP or LmigOBP groups are scattered among different clades.

In the tree we found that a total of 10 pairs of OasiOBPs and LmigOBPs were clustered into same clade. The pairs are very similar to each other. These proteins are OasiOBP2 and LmigOBP14 (OBP5), OasiOBP1 and LmigOBP1, OasiOBP3 and LmigOBP9, OasiOBP4 and LmigOBP2, OasiOBP5 and LmigOBP3, OasiOBP7 and LmigOBP11, OasiOBP10 and LmigOBP10, OasiOBP8 and LmigOBP6, OasiOBP9 and LmigOBP13 (OBP4), and OasiOBP11 and LmigOBP5. However, some were not clustered into same clade, such as OasiOBP6, OasiOBP12, OasiOBP13, OasiOBP14, OasiOBP15, LmigOBP4, LmigOBP7, LmigOBP8, and LmigOBP12.

4. Discussion

In this paper, we investigated the antennal transcriptome of *O. asiaticus* adults, and 15 OBP genes were identified from the dataset. We named these OBP genes OasiOBP1–15. However, this is less than the number of OBPs, as 22 OBPs have been reported in *L. migratoria*, for which the genome has been identified [33]. It may be that some OBPs are expressed in other tissues except for antenna, or fewer OBPs are characteristic of *O. asiaticus*, even though both of these species belong to the same family.

Our experiment on expression of these putative *O. asiaticus* OBPs showed that they were all expressed in chemosensory organs, such as the antennae, mouthparts, and wings. This is consistent with previous reports in other insects, which reported that OBPs can be expressed in different tissues [34–36]. However, there is less similarity of these putative OBPs, except between OBP12 and OBP14, as well as their various expression pattern in tissues. This indicates they may play different roles in chemoreception.

It is very interesting that OBP7 has significantly higher expression in male compared with female antennae. This indicates that this protein may be involved in detecting pheromones that are

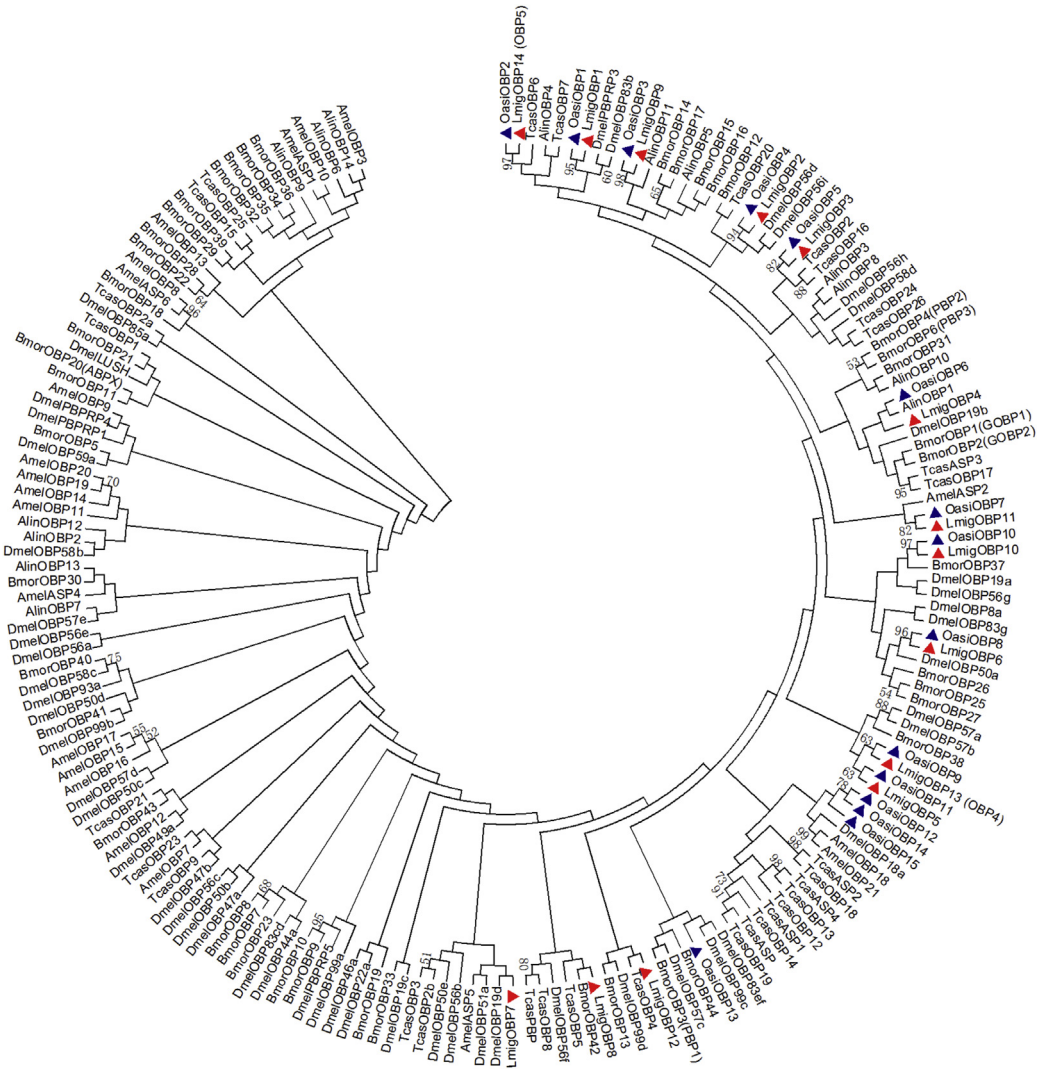

Fig. 4. A phylogenetic tree of OBPs from *O. asiaticus* and *L. migratoria* and other 6 orders of insects. Phylogenetic tree was constructed by neighbor-joining method using the program MEGA 6.0 with 1000 bootstrap replications. Bootstrap values >50% are shown. Blue triangles indicate *O. asiaticus* OBPs, Red triangle indicate *L. migratoria* OBPs. Dmel, *Drosophila melanogaster*; Bmor, *Bombyx mori*; Tcas, *Tribolium castaneum*; Alin, *Adelphocoris lineolatus*; Apis, *Apis mellifera* (the OBPs of the latter 6 species were quoted from S1).

released by females, like which occurs in moths [37]. Additionally, several OBPs are female-biased expression, such as OBP1, OBP2, OBP3, OBP4, OBP5, OBP8, OBP9, OBP10, and OBP13. These may be involved in female-specific chemosensory processes, such as laying eggs [20]. Among these OBPs, OBP5 is barely detected in male antennae and may be involved in recognition of male-specific pheromones. For OasiOBP6, OasiOBP11, OasiOBP12, OasiOBP14, and OasiOBP15, gene expression between males and females showed no difference, and this phenomenon reveals that these OBPs may play the same function in both male and female antennae.

L. migratoria and *Oedaleus asiticus* are both members of Acrididae (Orthoptera). Our phylogenetic analysis revealed that the OasiOBPs were more similar to the LmigOBPs within the same clades than they were OBPs in the same species. This may be because the structure of OBP genes in the insect genome was divergent [38,39]. Alternatively, *L. migratoria* and *O. asiaticus* have quite similar chemosensory systems, which indicates that these systems evolved from a common ancestor. However, there are many differences between the OasiOBPs and LmigOBPs, which may indicate that they evolved their own unique chemosensory systems to adapt to different ecosystems; locust, *L. migratoria* generally breeds in higher humidity and *O. asiaticus* in arid environments. Our results show that few orthopteran insect OBPs cluster into one close group within the same species; that is, each class of OasiOBPs or LmigOBPs scatter into different clades, indicating that Orthopteran chemosensory systems may represent a special link in evolution of insect olfaction.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.03.024.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.024.

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