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De novo analysis of the oriental armyworm *Mythimna separata* antennal transcriptome and expression patterns of odorant-binding proteins

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ABSTRACT

To better understand the olfactory mechanisms in the oriental armyworm *Mythimna separate*, one of the most serious pests of cereals, an antennal transcriptome was constructed in this study. A total of 130 olfactory related transcripts were identified. These transcripts were predicted to encode 32 odorant-binding proteins (OBPs), 16 chemosensory proteins (CSPs), 71 olfactory receptors (ORs), 8 ionotropic receptors (IRs), 1 gustatory receptor (GR) and 2 sensory neuron membrane proteins (SNMPs). Q-PCR analysis of the temporal expression profiles of seven *OBPs* in different tissues indicated that, except for *MsepOBP19* which was highly expressed in the wings of 0-day-old adult and *MsepOBP20* which was low expressed in all tissues, other tested *MsepOBPs* were significantly more highly expressed in the antenna than in the head (antenna excluded), thorax, abdomen, legs and wings. The expression levels of *MsepOBPs* were diverse in different life stages (differed on eclosion days). *MsepOBP5* exhibited female-biased expression in 0- and 5-day-old adult, while no gender bias in 1- and 3-day-old adult was detected and similar expression profiles were found for *MsepOBP7*, *20*, *24* and *26*. In addition, we found that although the expression of *MsepOBP22* was female biased in 0- and 5-day-old adult, in the 3-day-old adult it was male-biased. Our findings established a foundation for future studies of the functions of olfactory proteins in *M. separata*.

1. Introduction

The chemosensory system of insects can detect and recognize semiochemicals to locate hosts, mates and oviposition sites (Fatouros et al., 2008; Brigaud et al., 2009; Penaflor et al., 2011; Lu et al., 2015). The chemosensory system includes ligand-binding proteins and membrane receptors (Sánchez-Gracia et al., 2009). Ligand-binding proteins consist of odorant-binding proteins (OBPs) and chemosensory proteins (CSPs). The OBPs are grouped into pheromone binding proteins (PBPs) (Vogt and Riddiford, 1981), general odorant binding proteins (GOBPs) (Vogt et al., 1991) and antennal binding proteins X (ABPX) (Krieger et al., 1996). These small globular proteins in the antennal sensillum fluid were suggested to act as solubilizers and carriers of the lipophilic odorants (Pelosi and Maida, 1995; Steinbrecht, 1998; Pelosi et al., 2006; Zhang et al., 2013; Suh et al., 2014). Membrane receptors mainly refers to olfactory receptors (ORs) and co-receptor (Orco, formerly called Or83b), which are trans-membrane proteins and form a ligand-gated ion channel located in the dendrite membrane of receptor neurons (Clyne et al., 1999; Sato et al., 2008; Touhara and Vosshall, 2009). Besides these chemosensory proteins, the olfactory system also includes gustatory receptors (GRs), sensory neuron membrane proteins (SNMPs), and ionotropic receptors (IRs), which participate in odorant perception (Scott et al., 2001; Vogt et al., 2009; Croset et al., 2010; Leal, 2013).

The oriental armyworm *Mythimna separata* (Lepidoptera: Noctuidae) is one of the most serious pests of cereals in Asia which endanger 33 species of eight plant families (Zou, 1956; Sharma and Davies, 1983; Ashfaq et al., 1999; Jiang et al., 2011; Zeng et al., 2013). Chemical sensing mediates key behavior in seeking host plants, finding mating partners and selecting oviposition sites for *M. separata* (Brigaud et al., 2009). According to previous researches, the sex pheromone produced by female *M. seprata* was identified as a blend of (Z)-11-hexadecenyl acetate (Z11-16:Ac) and (Z)-11 -hexadecenol (Z11-16:OH) (Takahashi et al., 1979), but Zhu et al. (1987) reported the male *M. Seprata* was more attracted to (Z)-11-hexadecenal (Z11-16:Ald). However, little was known about the olfactory mechanisms of *M. Seprata*

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Table 1
Summary of RNA-Seq data.

Total raw reads	82,290,798
Total clean reads	77,734,418
Total clean nucleotides (nt)	6,996,097,620
Q20 percentage	98.29%
N percentage	0.00%
GC percentage	43.68%
Total number of contig	123,094
Total length of contig (bp)	33,860,271
Mean length of contig (bp)	275
N50 of contig	379
Total number of unigene	62,779
Total length of unigene (bp)	31,579,378
Mean length of unigene (bp)	503
N50 of unigene	734
Distinct clusters	15,727
Distinct singletons	47,052

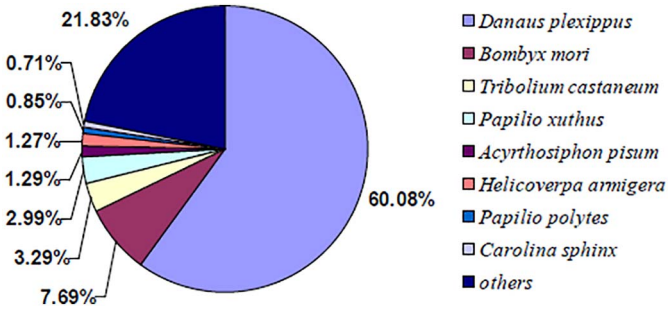

Fig. 1. Proportional homology distribution among other species based on the best BLAST hits against the NR database of *M. separata* antennal transcriptome.

recognizing different pheromone ingredients for linmited genetic information of *M. Seprata* chemosensory system (Mitsuno et al., 2008). To better understand the mechanisms of olfactory related behaviors and identify new attractant of adults for developing environment-friendly control strategies, in this study, RNA-Seq (Ansorge, 2009) was applied to obtain abundant olfactory-related genes from antennal transcriptome of *M. separate* without full genome, and further we analyzed temporal expression profiles of seven OBPs from different tissues of *M. separata* qPCR.

2. Materials and methods

2.1. Insects and tissues collection

M. separata larvae were collected from fields in Yicheng (111°57′E; 31 N°26′), P. R. China in May 2013, reared on wheat shoots (Huamai 2152) at 24 ± 1 °C under a 12 h dark:12 h light cycle. After emergence, about 3000 pooled antenna of female and male adults of different ages were dissected and stored at − 70 °C for *de novo* analysis of transcriptome. Meanwhile, newly emerged male or female adults of *M. separata* were placed individually into a Petri dish (9 cm diameter) to avoid any contact of the two sexes. Both were provided with a 10% (w/v) sucrose solution during experiments. Antennae, heads (excluding antennae), thoraxes, abdomens, wings and legs of 0-, 1-, 3-, 5-day-old adults were dissected and stored at − 70 °C for qPCR. All experiments were performed in triplicate.

2.2. Extraction of total RNA

Frozen tissues were transferred and homogenized with a liquid nitrogen-cooled pestle and mortar containing RNAiso (TaKaRa Bio Inc., Shiga, Japan), then total RNA was extracted following the manufacturer's instructions. The RNA quality and quantity were determined with a Nanodrop ND-2000 spectrophotometer (NanoDrop products, Wilmington, DE, USA).

2.3. Illumina sequencing and sequence assembly

Illumina sequencing was done at the Beijing Genomic Institute (Shenzhen, Guangdong, China). First, mRNA was purified from total RNA using magnetic beads with oligo (dT), and then fragmented into short fragments. First-strand cDNA was synthesized using the mRNA fragments as templates, followed the second-strand cDNA. After the adapters had been connected, the fragments were used as templates for PCR amplification for constructing the cDNA library. Quality control steps used an Agilent 2100 Bioanalyzer and an ABI Step-One-Plus Real-Time PCR system. Finally, the cDNA library was sequenced using an Illumina HiSeq 2000 system. The raw reads were filtered and assembled

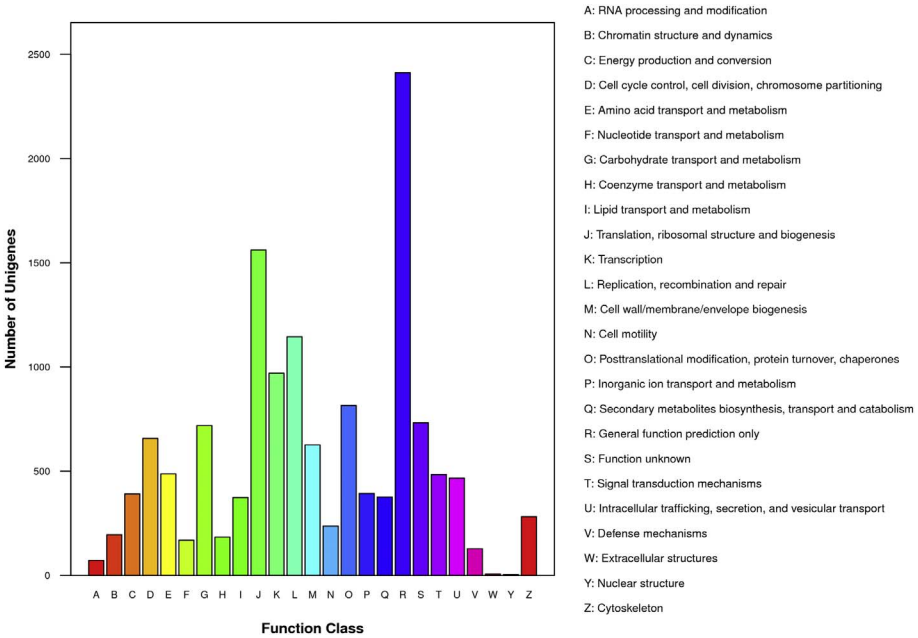

Fig. 2. COG functional classification of *M. separata* antennal transcriptome.

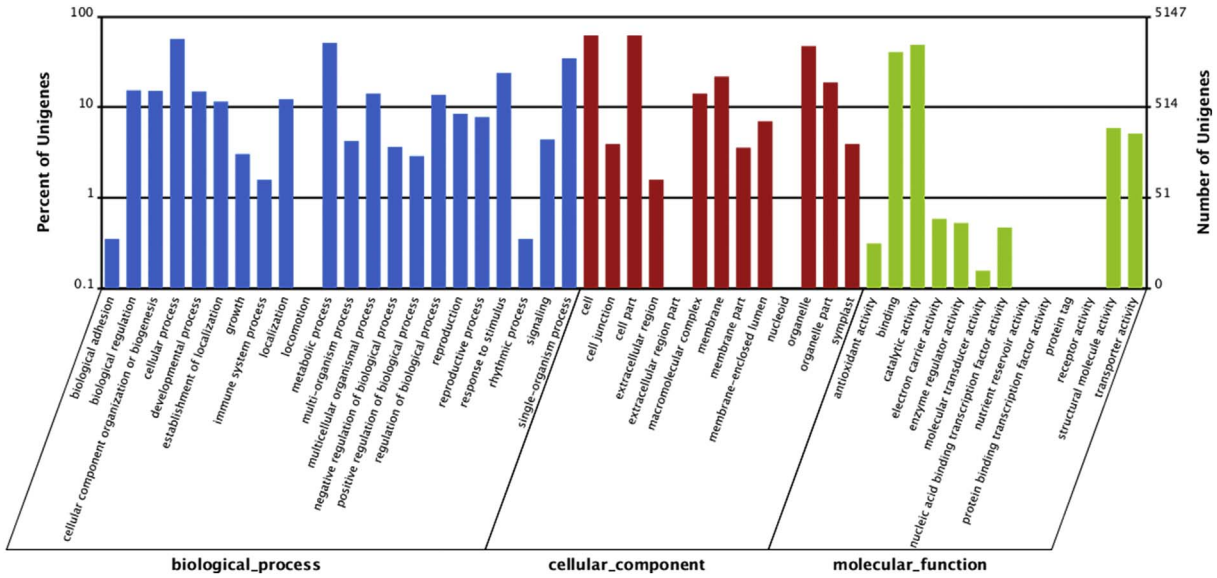

Fig. 3. Gene Ontology (GO) classification analysis of *M. separata* antennal transcriptome. Unigenes were classified into three categories: biological process, cellular component, and molecular function. GO functions is showed in the x-axis. The right y-axis shows the number of genes which have the GO function, and the left y-axis shows the percentage.

using the Trinity with default parameters (Grabherr et al., 2011). Contigs representing significant parts of individual isoforms were clustered on the basis of gene sequence homology, and the contig clusters were assembled into unigenes. The unigenes were adjusted for sequence splicing, and redundant sequences were removed to obtain non-redundant unigenes.

2.4. Unigene annotation and classification

Unigenes were aligned to the database, including NCBI non-redundant protein database (NR), Swiss-Prot, Kyoto encyclopedia of genes and genomes (KEGG), cluster of orthologous groups (COG) and gene ontology (GO) databases using BlastX with a criterion of *e*-value $< 10^{-5}$. The Blast2GO GO program (Conesa et al., 2005) was used to assign GO annotations, including molecular function, cellular component and biological process, based on the NR annotations. The WEGO software was used to assign GO functional classifications and evaluate the distribution of GO annotations (Ye et al., 2006). Unigene functions were predicted on the basis of alignment with sequences in the COG database. The KEGG database was performed to predict relationship among the unigenes and construct pathways (Kanehisa et al., 2008).

2.5. Gene identifications and phylogenetic analysis

The unigenes annotated as OBPs, CSPs, ORs, IRs, GRs and SNMPs were selected manually and reconfirmed using the BlastX network server in NCBI. The protein sequences were obtained using the open reading frame (ORF) Finder in NCBI. Putative N-terminal signal peptides of ligand-binding proteins were predicted by Signal IP 4.1 (http://www.cbs.dtu.dk/services/SignalP/). The transmembrane domains (TMDs) of ORs were predicted using TMHMM Server version2.0 (http://www.cbs.dtu.dk/services/TMHMM).

Phylogenetic trees were reconstructed for the analyses of OBPs, CSPs and ORs. MEGA5.2.2 software (Tamura et al., 2011) was used to construct the maximum likelihood trees, the bootstrap procedure based on 1000 replicates to assess node support and the node support values < 50% are not shown. In this study, the best model of evolution for the maximum likelihood trees was the Dayhoff model, which had the lowest Bayesian information criterion score.

2.6. Temporal and spatial expression profiles

Seven putative *OBPs* (*MsepOBP5*, *-7*, *-19*, *-20*, *-22*, *-24* and *-26*) were checked in temporal (antenna, head (antenna excluded), thorax, abdomen, leg and wing) and spatial (age 0, age 1, age 3 and age 5) expression profiles of female and male moths by qPCR. The primer sequences used in the qPCR analysis were designed online (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers used to amplify OBPs and actin genes are given in Table S1. The β-actin gene of *M. separata* (GQ856238) was used as an internal control. The qPCR used a Realplex[4] (Eppendorf AG, Hamburg, Germany) and a mixture of 10 μl SYBR II Master Mix (Takara-Bio, Shiga, Japan), 0.8 μl each primer, two μl sample cDNA and 6.4 μl sterilized ultrapure water. The qPCR primers designed using negative controls were non-template reactions (replacing cDNA with water). The reaction protocols were 2 min at 95 °C, 10 s at 95 °C, followed by 40 cycles at 95 °C for 5 s and 60 °C for 15 s.

Biological triplicate replications were used for each sample and each biological replication including three technique replications. Relative quantification was analyzed using the comparative $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001). One-way analysis of variance was used for calculation of the logarithmically transformed mean values of gene expression levels with the Data Processing System software v9.5 (Tang and Zhang, 2013). The level of statistical significance was set at $p \leq 0.05$.

3. Results

3.1. M. separata antennal cDNA sequencing

To obtain an overview of the transcriptome of the oriental armyworm, one cDNA library was constructed and sequenced. After removing adaptor sequences, ambiguous reads and low-quality reads, the clean reads were assembled into 123,094 contigs with an average length of 275 bp (Table 1). The contigs were clustered based on gene sequence homology and assembled into 62,779 unigenes with an average length of 503 bp.

Unigenes were annotated with the NR, Swiss-prot, KEGG, COG and GO databases. In total, 12,281 unigenes were annotated. For coding region prediction analysis, the number of CDS annotated by the protein database was 11,600, and 10,571 other CDS were predicted. There were 22,171 CDS in total.

Table 2
Sequences information of OBPs of *M. separata*.

	Gene name	ORF (aa)	Com-plete ORF	SP (aa)	Homology search with known proteins					
					Species	Source	Name	Acc. number	*E*-value	Identity (%)
MsepOBP1	CL645.contig1	154	Y	N	Heliothinae, *Helicoverpa armigera*	-	OBP	AEX07280.1	2e-48	77
MsepOBP2	CL1687.contig2	132	Y	N	Amphipyrinae, *Sesamia inferens*	-	OBP6	AGS36748.1	2e-72	89
MsepOBP3	CL1836.contig1	165	Y	23	Hadeninae, *Mamestra brassicae*	Male antenna	PBP1 precursor	AAC05702.2	3e-95	81
MsepOBP4	CL2797.contig2	139	N	N	Heliothinae, *Agrotis segetum*	-	GOBP1	ABI24159.1	7e-52	95
MsepOBP5	CL4701.contig1	148	Y	21	Heliothinae, *Heliothis virescens*	Female antenna	ABP	CAC33574.1	2e-65	69
MsepOBP6	CL5731.contig1	237	Y	19	Amphipyrinae, *Spodoptera exigua*	-	OBP25	AKT26502.1	4e-96	62
MsepOBP7	CL7088.contig1	248	Y	22	Amphipyrinae, *Spodoptera litura*	-	OBP1	AKI87962.1	3e-98	84
MsepOBP8	CL7646.contig1	149	Y	21	*Helicoverpa armigera*	-	OBP5	AEB54581.1	4e-58	75
MsepOBP9	CL7646.contig2	146	Y	21	*Mamestra brassicae*	-	PBP4	AAL66739.1	3e-82	84
MsepOBP10	CL7647.contig1	166	Y	23	Heliothinae, *Helicoverpa armigera*	Antenna	OBP9	AEB54592.1	1e-41	48
MsepOBP11	Unigene308	139	Y	18	*Helicoverpa armigera*	Antenna	OBP8	AEB54589.1	9e-85	88
MsepOBP12	Unigene2752	129	N	17	Saturniidae, *Antheraea yamamai*	-	ABP7	ADO95155.1	1e-08	36
MsepOBP13	Unigene2871	333	Y	20	Bombycidae, *Bombyx mori*	Male	GOBP71	XP_004927370.1	4e-64	64
MsepOBP14	Unigene3718	197	N	17	Heliothinae, *Helicoverpa assulta*	-	OBP19	AGC92793.1	1e-76	60
MsepOBP15	Unigene19982	101	N	N	Amphipyrinae, *Spodoptera exigua*	-	OBP26	AKT26503.1	6e-42	75
MsepOBP16	Unigene21183	87	Y	N	*Spodoptera exigua*	-	OBP13	AGP03459.1	3e-16	42
MsepOBP17	Unigene28320	140	N	19	*Spodoptera exigua*	-	OBP10	AGP03456.1	2e-69	74
MsepOBP18	Unigene28508	141	Y	18	*Spodoptera exigua*	-	OBP8	AGH70104.1	5e-80	86
MsepOBP19	Unigene29008	145	Y	17	*Spodoptera exigua*	larva	OBP4	ADY17886.1	3e-80	80
MsepOBP20	Unigene29069	147	Y	15	*Spodoptera exigua*	Antenna	OBP6	AFM77984.1	4e-58	60
MsepOBP21	Unigene31160	142	Y	21	*Helicoverpa armigera*	Antenna	OBP2	AEB54586.1	3e-86	86
MsepOBP22	Unigene31770	145	Y	24	*Spodoptera exigua*	-	OBP12	AGP03458.1	2e-70	80
MsepOBP23	Unigene32401	154	N	27	Hadeninae, *Mythimna separata*	-	PBP	BAG71416.1	3e-97	98
MsepOBP24	Unigene32404	162	Y	21	Heliothinae, *Heliothis viriplaca*	-	GOBP2	AFI25168.1	3e-95	91
MsepOBP25	Unigene32426	164	Y	20	*Spodoptera exigua*	-	OBP24	AKT26501.1	8e-118	98
MsepOBP26	Unigene32708	141	N	20	Noctuinae, *Agrotis ipsilon*	-	PBP3	AFM36758.1	2e-84	86
MsepOBP27	Unigene33562	146	Y	25	Crambidae, *Cnaphalocrocis medinalis*	-	OBP1	AFG72998.1	5e-76	74
MsepOBP28	Unigene33672	100	N	N	Amphipyrinae, *Sesamia inferens*	-	OBP4	AGS36746.1	4e-30	71
MsepOBP29	Unigene34049	133	Y	16	*Spodoptera exigua*	-	OBP9	AGH70105.1	2e-81	90
MsepOBP30	Unigene34083	137	Y	20	*Heliothis virescens*	Antenna	ABPX	CAA05508.1	6e-57	89
MsepOBP31	Unigene34667	68	N	17	Noctuinae, *Xestia cnigrum*	-	GOBP1	AGS41498.1	2e-28	100
MsepOBP32	Unigene42513	71	N	N	*Spodoptera exigua*	-	OBP11	AGP03457.1	3e-35	79

Note: ORF, open reading frame; SP, signal peptides; aa, amino acid. GOBP: general odorant-binding protein; ABP: antennal binding protein; PBP: pheromone-binding protein. "-": no source notation.

3.2. Comparative analysis

Predicted proteins based on *M. separata* antenna RNA-Seq data were compared with protein sequences derived from the draft genomes of *Danaus plexippus*, *Bombyx mori*, *Tribolium castaneum*, *Papilio xuthus*, *Acyrthosiphon pisum*, *Helicoverpa armigera* and other insects using the BlastP algorithm (*e*-values $\leq 10^{-5}$). In all, 23,309 unigenes were annotated with the databases of NR based upon similarity to protein sequences in other insect species. The analysis showed that most *M. separata* protein sequences were orthologues of proteins in *D. plexippus* (60.1%) and *B. mori* (7.7%). On the other hand, *M. separata* shares little similarity of protein sequences with Noctuidae moths *H. armigera* (1.27%) and *S. frugiperda* (0.68%) (Fig. 1).

3.3. Classification of clusters of orthologous groups

We annotated the unigenes to the COG database and predicted the possible functions to help us understand the gene function distribution characteristics of the species (Fig. 2). From the 25 COG categories, the cluster for "general function prediction" was the largest group (2411, 17.36%), followed by the group for "translation, ribosomal structure and biogenesis" (1561, 11.24%) and the groups of "extracellular structures" (7, 0.05%) and "nuclear structure" (4, 0.03%) were the smallest classes.

3.4. Unigene GO classification

We obtained GO functional annotation using the Blast2GO program.

The WEBGO were used to classify GO functional annotations into different categories for all unigenes to understand the distribution of gene functions of the species at the macro level (Fig. 3). The genes expressed in the antenna annotated as molecular function were related primarily to catalytic activity (47.57%) and binding activity (39.56%), following 4.96% of unigenes involved in transporter activity.

3.5. Identification of putative chemosensory genes

We identified a total of 32 OBPs, 16 CSPs, 71 ORs, 8 IRs, 1 GR and 2

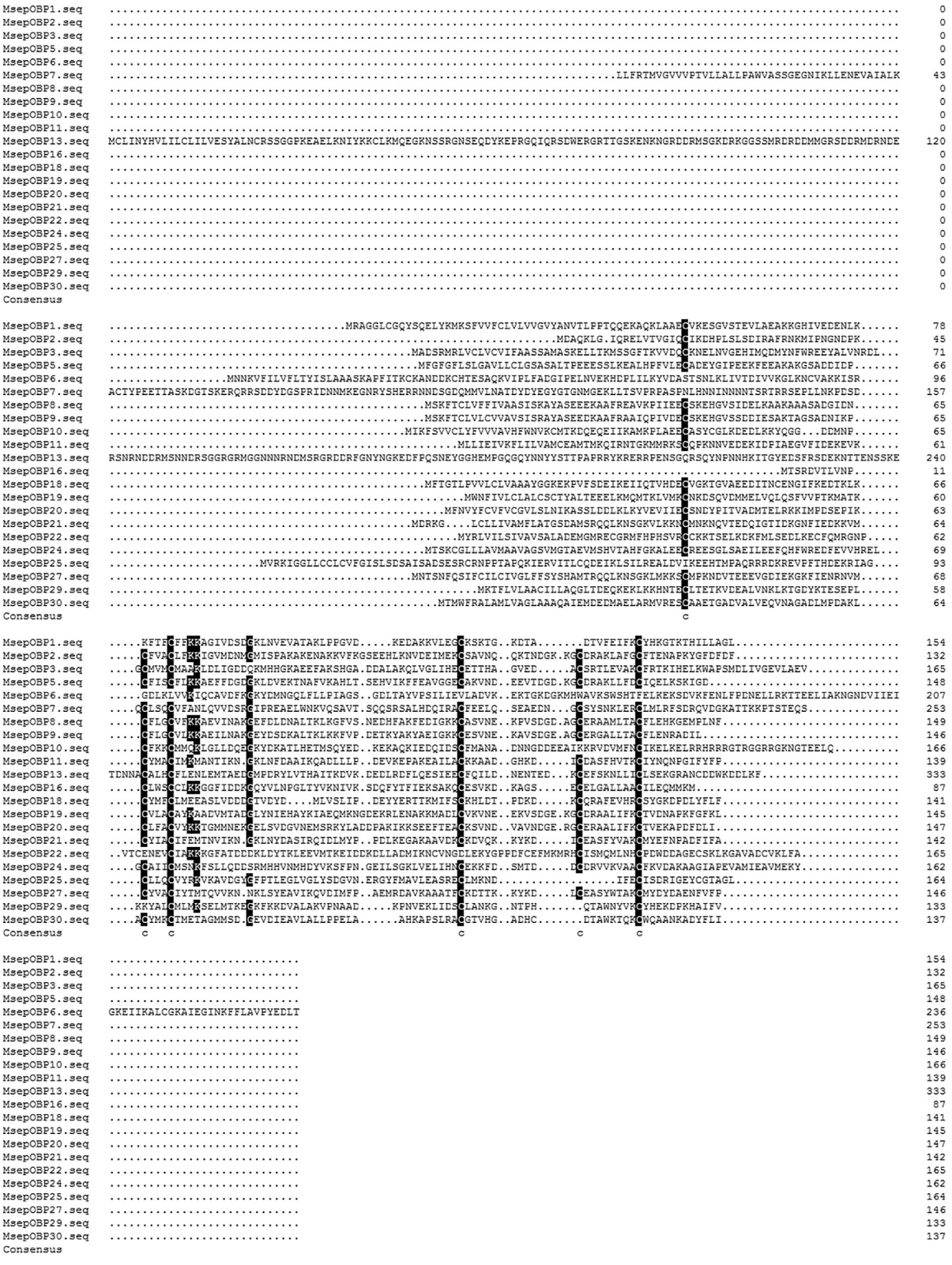

Fig. 4. Alignment of amino acid sequences of putative OBPs of *M. separata*. Six Conserved residues are highlighted.

SNMPs from RNA-Seq data. The OBPs family can be classified into classic OBPs that containing 6 cysteine residues (Cys), and atypical OBPs, such as minus-C OBPs (4 Cys), plus-C OBPs (8 Cys and 1 Pro), and dimers (Gong et al., 2009; Hekmat-Scafe et al., 2002). Of 32 identified *M. separata* OBPs, 22 had an intact ORF (Table 2). A total of 25 OBPs had signal peptides at the hydrophobic N-terminus. An alignment of 22 genes with a complete ORF in DNAMAN 6 (Lynnon Corp., Quebec, Canada) showed that 12 (MsepOBP2, -3, -5, -8, -9, -11, -18, -19, -20, -21, -24 and -27) belonged to classic OBPs and the other ten (MsepOBP1, -6, -7, -10, -13, -16, -22, -25, -29 and -30) were minus-C OBPs (Fig. 4); no plus-C, dimers, and atypical types of OBPs were found in this study.

The OBPs phylogenetic tree was constructed after removing the highly divergent signal peptide sequences. Among the 32 OBPs, 12 classic OBPs and 10 minus-C OBPs were spread across several branches. All putative OBPs were clustered with at least one ortholog in Lepidoptera except for MsepOBP4, -15, -28 and 32, which occurred in one small branch (Fig. 5). Accession numbers for amino acid sequences of OBPs used in phylogenetic analysis are given in Table S2.

All of 16 putative CSPs identified contained an intact ORF (Table S3). Alignment of the amino acid sequences revealed four conserved cysteine residues of these CSPs except MsepCSP1, -10 and -12, which had fewer cysteine residues and were clustered in the same branch of the phylogenetic tree (Figs. 6 and 7). Accession numbers for amino acid sequences of the CSPs used in phylogenetic analysis are given in the Table S4.

A total of 70 different genes were annotated as putative ORs and named from MsepOR1.1 – MsepOR71 (Table S5); 24 of these genes had an intact ORF, whereas only one protein (MsepOR38) had seven TMD and 13 proteins had six TMD. In our study, MsepOR1.1, -3.1 and -71 were almost identical at the amino acid level with MsepOR1 (BAG71414.1), MsepOR3 (BAG71423.2) and MsepOR2 (BAG71415.1) respectively, which had been identified as *M. separata* ORs and ORco (Mitsuno et al., 2008).

The phylogenetic tree of the ORs showed MsepOR4 were clustered with BmorOR3, MsepOR1, AsegOR4, SinfOR21 and SinfOR29, which were recognized as pheromone receptors (Zhang and Löfstedt, 2015; Zhang et al., 2014) and ORco (MsepOR71, HvirOR2, SinfOR2 and BmorOR2) were clustered in one small branch (Fig. 8). Accession numbers for amino acid sequences of ORs used in the phylogenetic analysis are shown in Table S6.

Eight putative IRs, one GR and two SNMPs were identified (Tables S7 and S8). 3 of 8 IRs had complete ORF and 7 of 8 IRs shared high amino acid identities (73–97%) with homologs of other moths.

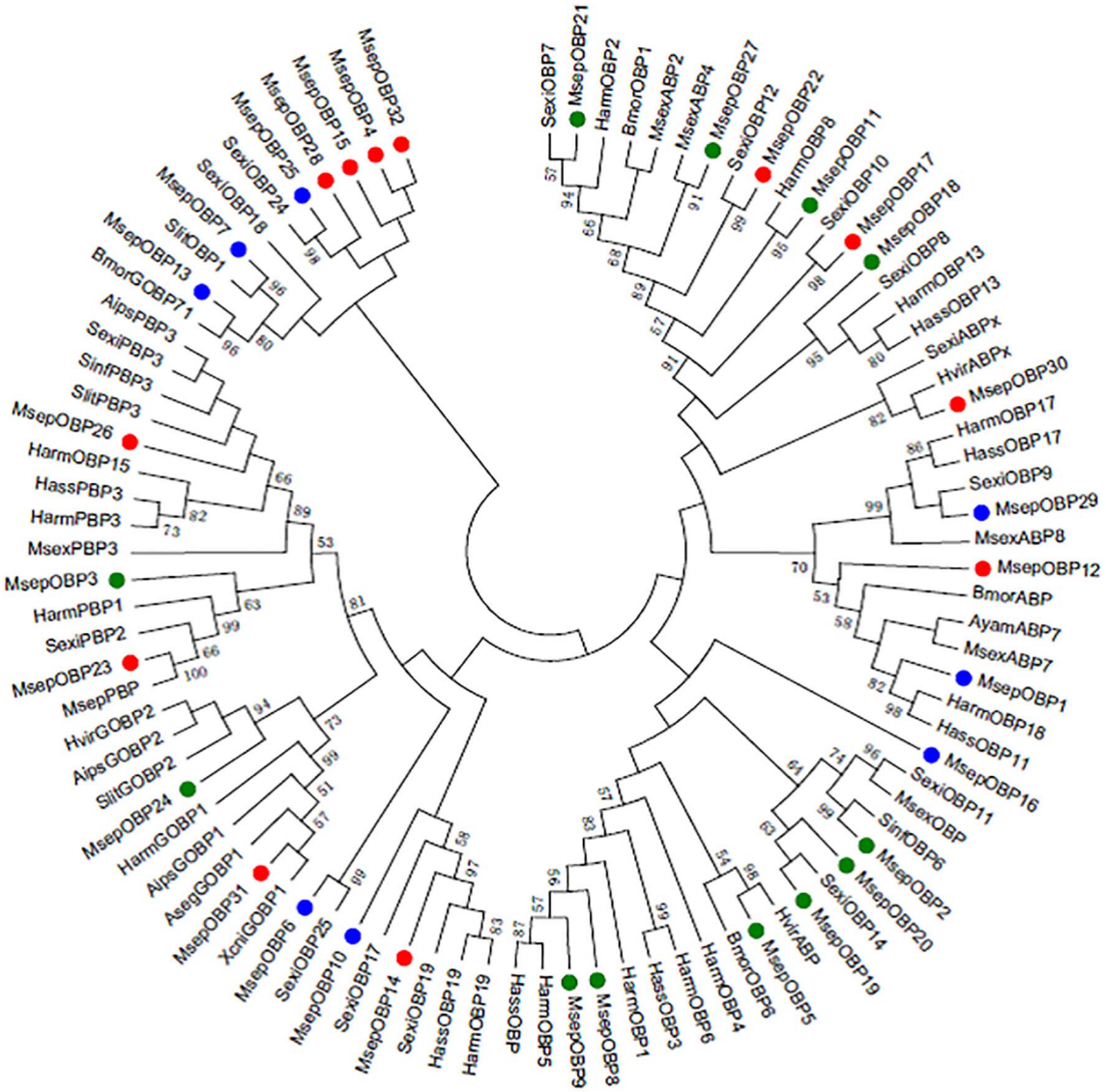

Fig. 5. Phylogenetic tree of putative OBPs from *M. separata* and other moths. Blue and green bold circles represented Minus-C OBPs and classic OBPs of *M. separata* respectively, and red bold circles represented other putative OBPs of *M. separata*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

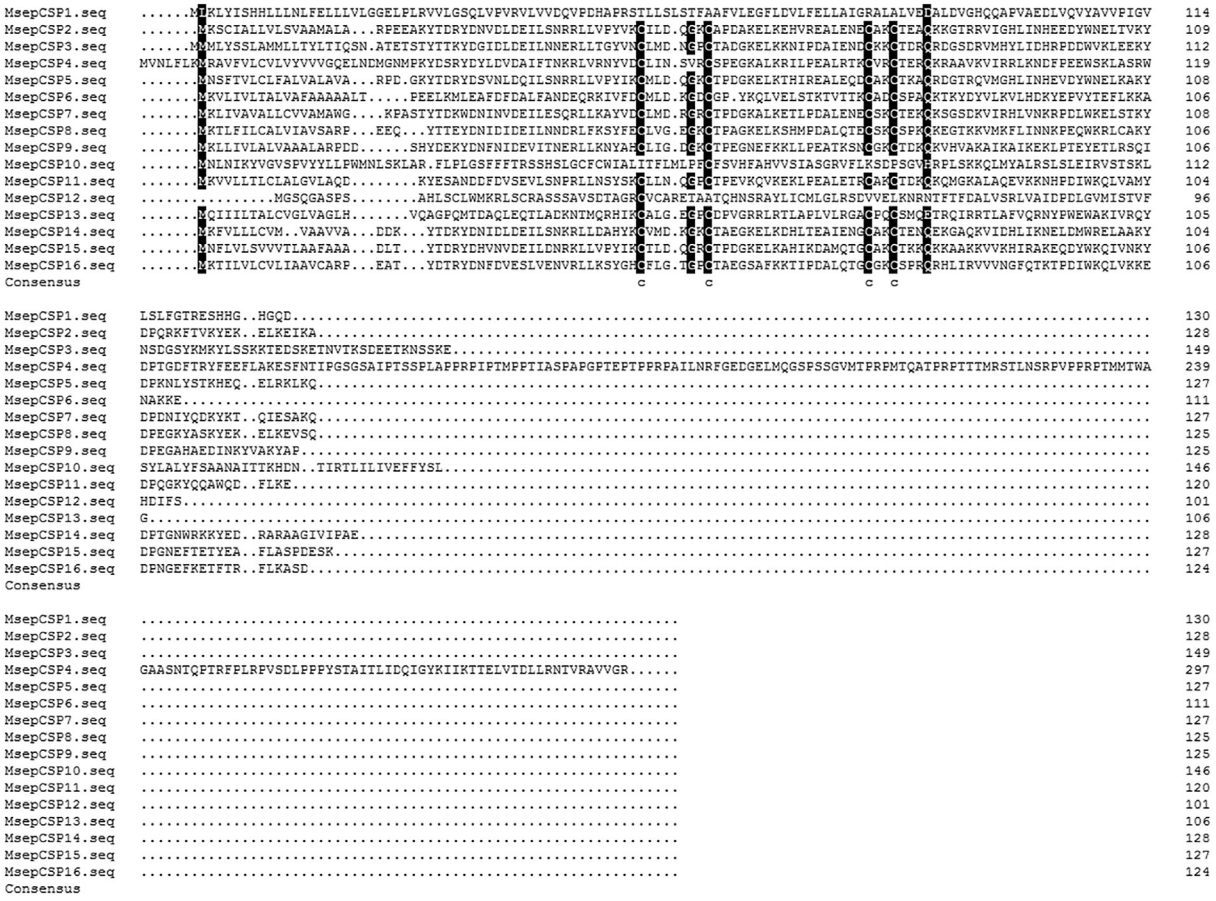

Fig. 6. Alignment of amino acid sequences of putative CSPs of *M. separata*. Four Conserved residues are highlighted.

MsepGR1 and SNMP1 of *M. seprata* also showed as high as 93 and 98% identities with homologs of Noctuidae moths.

3.6. Expression patterns

The relative expression levels of *MsepOBP5*, *-7*, *-22*, *-24* and *-26* were significantly higher in the antenna than in the head (antenna excluded), thorax, abdomen, leg and wing. *MsepOBP20* expressed very low in the antenna, and *MsepOBP19* expressed ubiquitously, with a particularly high-level expression in the wing of 0-day-old adult (Fig. 9; Fig. S1).

Except for *MsepOBP24*, the expression levels of *MsepOBP*s varied in different day-old adults. For the female antenna, the expression level of *MsepOBP5* and *-19* were significantly higher in 0- or 5-day-old adult respectively ($P < 0.05$), *MsepOBP26* were significantly lower in 1-day-old adult ($P < 0.05$). For males, the expression level of *MsepOBP19* was significantly higher in 0-day-old adult ($P < 0.05$), while *MsepOBP5*, *-22*, *-7* and *-26* were significantly higher in 1- or 3-day-old adult or both of them, respectively ($P < 0.05$) (Fig. 9). For *MsepOBP19*, the expression level was significantly higher on 0-day-old adult compared with other days in the head (antenna excluded), thorax, abdomen, leg and wing of male (Fig. S1).

Within the antenna, *MsepOBP5* exhibited female-biased expression in 0- and 5-day-old adult, while no gender bias in 1- and 3-day-old adult, the similar expression profiles with *MsepOBP7*, *20*, *24* and *26*. While *MsepOBP22* was female biased expression in 0- and 5-day-old adult, but male-biased in the 3-day-old adult.

4. Discussion

A total of 130 transcripts were identified, including 32 OBPs, 16 CSPs, 71 ORs, 8 IRs, 1 GR and 2 SNMPs. Except for OBP23, OR1.1, OR3.1 and MsepOR71, the remaining 126 transcripts were novel for *M. separata*. All these information supplied the basis for elucidating molecular mechanisms of olfactory-related behaviors of *M. separata*. The identified genes in this study were comparable to other Noctuidae moths chemosensory genes of *Spodoptera littoralis* with 26 OBPs, 36 ORs, 5 GRs (Jacquin-Joly et al., 2012), *H. armigera* with 26 OBP, 12 CSPs, 47 ORs, 12 IRs and 2 SNMPs (Liu et al., 2012), *Sesamia inferens* with 24 OBPs, 24 CSPs, 39 ORs, 3 IRs and 2 SNMPs (Zhang et al., 2013) and *Athetis dissimilis* with 60 ORs and 12 IRs (Dong et al., 2016). The number of identified chemosensory genes of *M. separata* might still be not complete, for some OBPs expressed at the development stages and some gene paralogs with highly sequence similarity could be missing from the current analysis, because they are difficult to separate by polymorphism without the genome sequence (Kaori et al., 2006; Liu et al., 2012).

Although *M. separata* belongs to the subfamily Hadeninae in taxonomy, most putative OBPs of *M. separata* homologies belongs to the subfamilies Heliothinae and Amphipyrinae of Noctuidae (Table 2). This indicated that olfactory genes evolution had a weak relation with the taxonomy of moths (Mitsuno et al., 2008). It appears to be meaningless to discuss the gender bias of *OBPs* expression without mentioning adult age for the transcripts level changing in different day-old adult. The expression levels of some olfactory genes depending on the age of adult were also found in *Cnaphalocrocis medinalis* (Zeng et al., 2013) and *Nilaparvata lugens* (Zhou et al., 2014), although within 24 h period post eclosion, the OBPs expression levels of *S. littoralis* (Merlin et al., 2008) and *Plutella xylostella* (Zhang et al., 2009) were constant. Within antenna, *MsepOBP5* exhibited female-biased expression in 0- and 5-day-old adult, while no gender bias in 1- and 3-day-old adult, the similar expression profiles with *MsepOBP7*, *20*, *24* and *26*. *MsepOBP22*

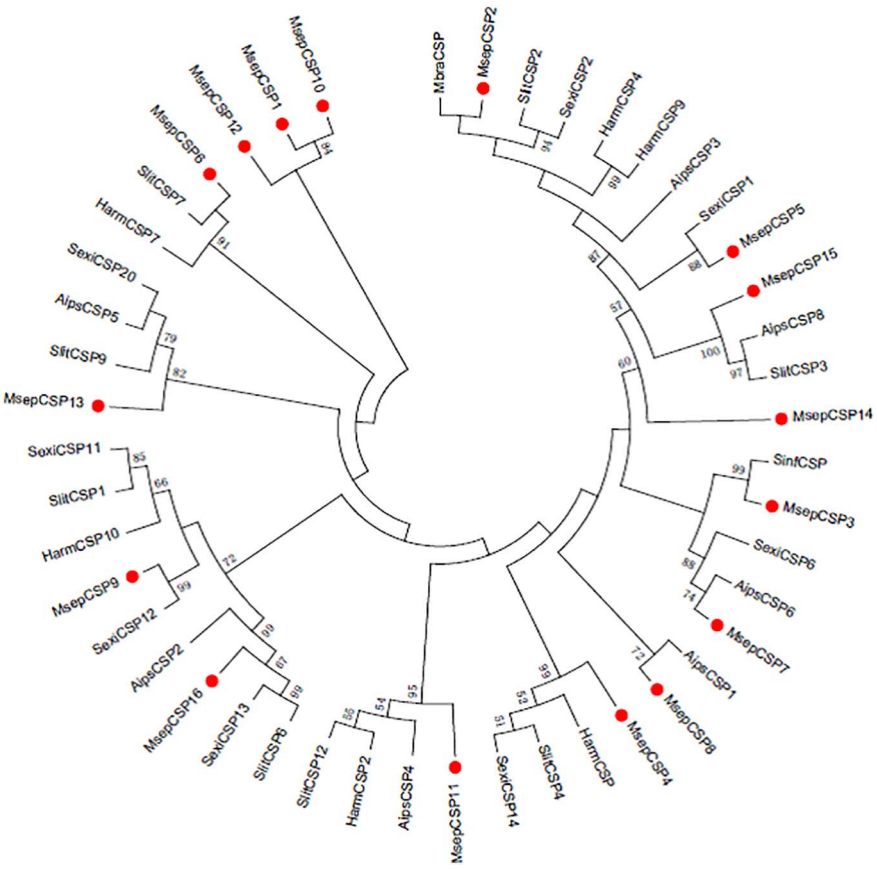

Fig. 7. Phylogenetic tree of putative CSPs of *M. separata* and other moths. Red bold circles represent putative CSPs of *M. separata*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

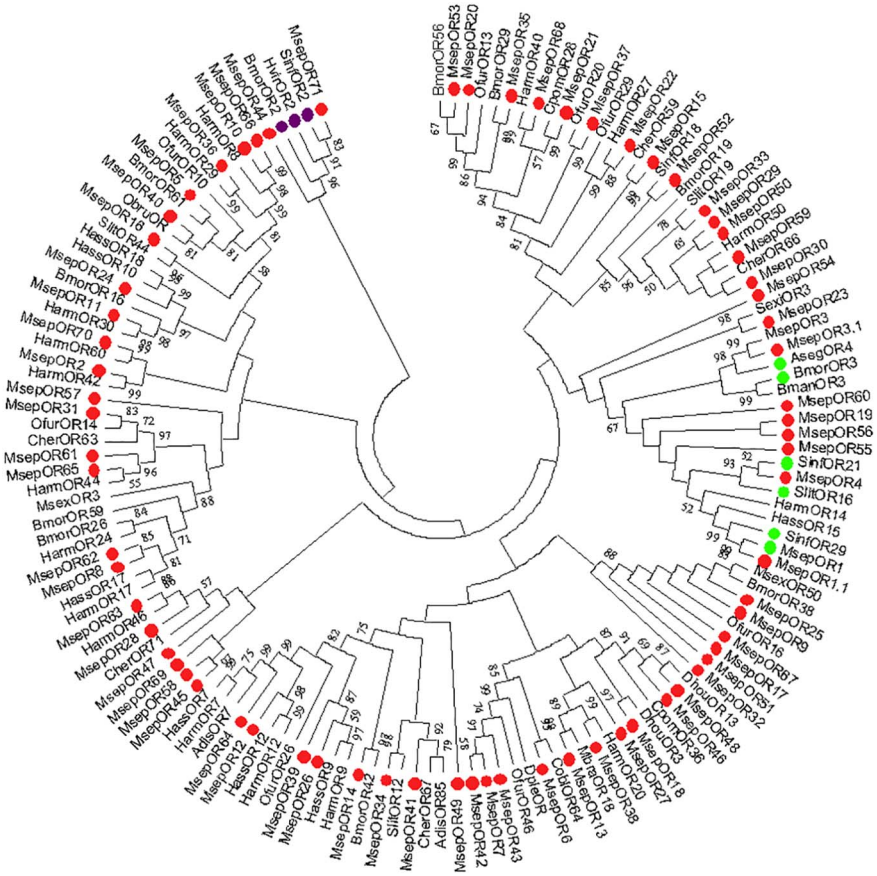

Fig. 8. Phylogenetic tree of putative ORs of *M. separata* and other moths. Red bold circles represented putative ORs of *M. separata*, bold green circles and bold purple circles represented Pheromone Receptors (PRs) and ORco of other moths respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was a female-biased expression in 0- and 5-day-old adult, but male-biased in 3-day-old adult. The phenomenon also was found in other insects. The expression of *NlugOBP6* in *N. lugens* showed male biased in 0-day-old long-wing adult, while female biased in 3-day-old long-wing adult (Zhou et al., 2014). Insects switch their olfactory response from mate-searching to oviposition-sites-searching at different days after eclosion (Saveer et al., 2012), this switch may be induced by the regulation of gene expression levels (Ji et al., 2013; Zhou et al., 2009). However, the age of different days had no effect on expression levels of ORs of *Heliothis virescens* and *H. subflexa* (Soques et al., 2010), so we need more replication and behavioral, electrophysiological response experiments to confirm the expression trend of *MsepOBPs*.

CSP gene families are not so divergent compared to those of OBPs (Vieira and Rozas, 2011). In our study, 16 MsepCSPs had similar protein lengths of 101–130 amino acid residues, excluded MsepCSP4. These CSPs had higher levels of amino acid identity (70–99%) across insect species. However, they were divergent in the phylogenetic tree, except for MsepCSP1, -10, 12 clustered in a small branch, which has less four Cys. The diversification of CSPs in the tree indicates the functional diversity of CSPs (Ozaki et al., 2008) and also illustrate that they are presumably homologous proteins, but their orthogologous/paralogous relationships are yet unclear (Jacquin-Joly et al., 2001).

71 *M. separata* ORs were identified, the number of ORs identified in our study was significantly more than other Noctuidae moths. However, 24 ORs with full length and 1 OR with seven TMDs were identified. Low rates of full-length OR sequences were found in other insects, such as 13/47 *H. armiger* ORs (Liu et al., 2012), 11/43 *Ips typographus* ORs, 27/49 *Dendroctonus ponderosae* ORs (Andersson et al., 2013) and 2/39 *S. inferens* ORs (Zhang et al., 2013) were identified as full length. In the putative ORs of *M. separata* identified in this study, MsepOR1.1, -3.1 and -71 were identical with previously identified *M. separata* OR (Mitsuno et al., 2008), but the other ORs were all novel. In all 71 ORs, MsepOR1.1, -3.1 and -4 were clustered with PRs (MsepOR1, SinfOR29, SinfOR21, SlitOR16 and AsegOR4) in the phylogenetic tree, we infer these three ORs (MsepOR1.1, -3.1, -4) might be PRs for sex pheromone detection (Mitsuno et al., 2008; Zhang and Löfstedt, 2015). MsepOR71 (ORco) was clustered with SinfOR2 of *S. inferens* (Zhang et al., 2013), HvirOR2 of *Heliothis viriplaca* (Krieger et al., 2002), and BmorOR2 of *B. mori* (Sakurai and Kaziro, 2004), all of which are ORco in the respective insects.

Insect IRs, *e.g.* in *Cnaphalocrocis medinalis*, have three trans-membrane domains (TMDS) (Zeng et al., 2015). In eight putative IRs in this study, three had three TMDS. The identity values of putative IRs of *M. separata* with other moths showed that IRs were more highly conserved

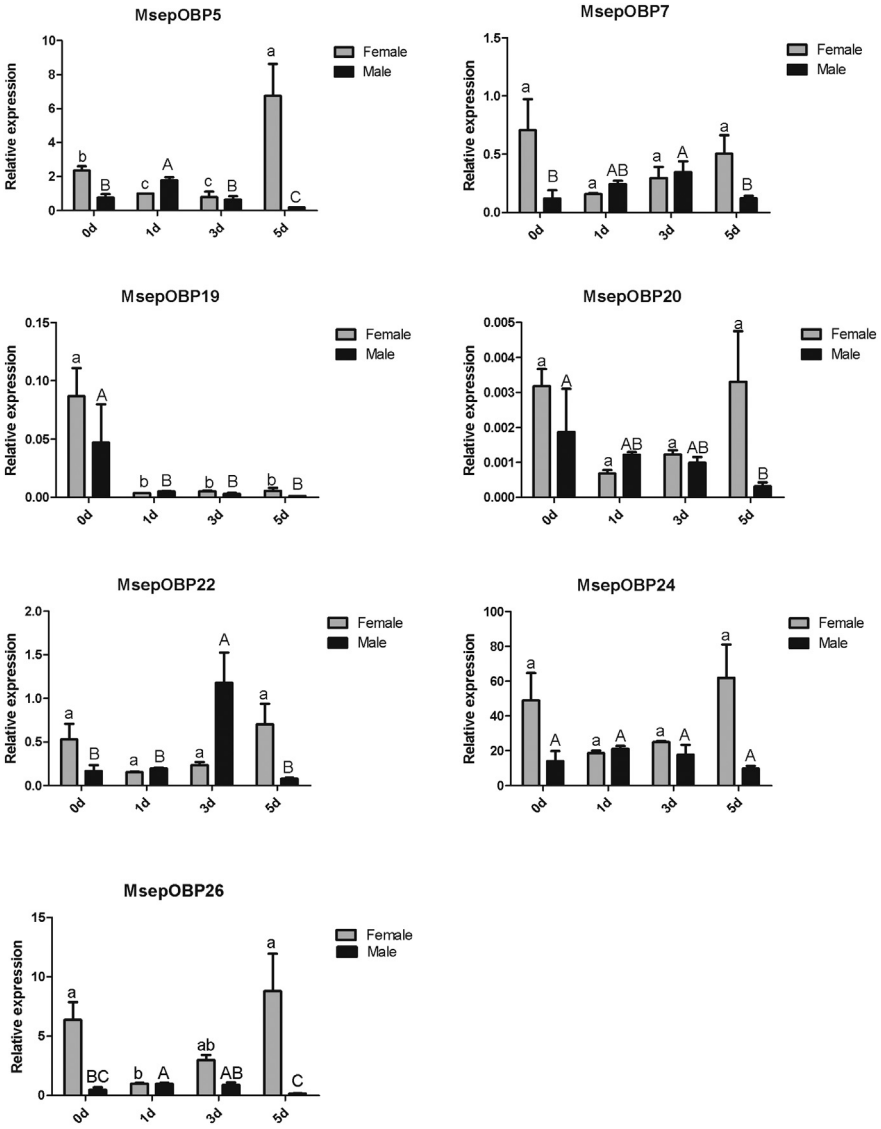

Fig. 9. Relative expression profiles of *M. separata* OBPs in antennae of different day-old adults.Note: 0d, 1d, 3d, 5d referred to the adults 0, 1, 3 and 5-day old adult on the x axis. The significant difference in female was marked on the bars with lower case letters, and capital letters for male, $P < 0.05$. The same with the Fig. S1.

across species (Croset et al., 2010; Zeng et al., 2015).

GRs recognized the taste substances and were expressed mostly in gustatory organs, such as maxilla, labium (Scott et al., 2001; Sato et al., 2011). However, recent studies suggested that GRs were also expressed in the olfactory organs, for example, antenna of *B. mori* (Sato et al., 2011), *S. littoralis* (Jacquin-Joly et al., 2012), *A. dissimilis* (Dong et al., 2016) and *Eogystia hippophaecolus* (Hu et al., 2016). We identified one GR in the antenna of *M. separata*, which confirmed the existence of GRs in moth antenna.

SNMPs, which are located in the dendritic membrane of pheromone-specific olfactory sensory neurons OSNs, can trigger ligand delivery to the receptor (Nichols and Vogt, 2008). The two identified SNMPs of *M. separata* had > 80% identity with SNMPs of other moths, which indicated a functional conservatism within these proteins (Zhang et al., 2013; Liu et al., 2015).

5. Conclusion

The antennal transcriptome dataset of *M. separata* was constructed and 130 olfactory related genes were identified for this species. The results of qPCR showed that most of the OBPs indentified in our experiments were antenna biased. These results established a foundation for future studies of the functions of olfactory proteins in *M. separata*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbd.2017.03.001.

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