Identification of odorant-binding proteins in the reproductive system of *Athetis dissimilis* using transcriptome analysis

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Abstract

Odorant-binding proteins (OBPs) are prevalent in the antennal transcriptomes of different orders of insects. Studies on OBPs have focused on their role in the insect chemosensory system, but knowledge of their functions in the insect testis is limited. We sequenced the transcriptomes of the *Athetis dissimilis* reproductive organs and analyzed the expressive of OBPs in different tissues. We identified a total of 54 OBPs including 23 OBPs in the transcriptomes of testis and ovaries, and 31 OBPs in antennal transcriptomes. Through fluorescence qPCR, the 23 identified OBPs were found to be highly expressed in both female and male antennae compared to the reproductive organs. Of the identified OBPs, 5/23 showed comparable expression in female and male antennae; 3/23 were more highly expressed in males compared to females; and 15/23 OBPs were more highly expressed in females compared to males. A total of 24 OBPs were highly expressed in the testis of *A. dissimilis* whilst expression in the ovaries was low. These findings highlight the functional diversity of OBPs in insects and can facilitate further studies on the OBPs in *A. dissimilis* and lepidopteran species.

Background

The olfactory system in insects regulates their intersex communication, host-plant interactions, oviposition, foraging, escape from predators and reproduction [1–5]. Insects have a complex chemosensory system in which pheromones and plant odors are initially recognized by odorant-binding proteins (OBPs) expressed in the antennal sensilla lymph that transfer the odorants to membrane-bound olfactory receptors (ORs) to activate olfactory receptor neurons (ORNs) and stimulate behavioral responses [6–11].

OBPs are small water soluble proteins that have six positionally conserved cysteines to form three interlocking disulphide bridges that stabilize the protein's three-dimensional structure [12–17]. OBPs were first discovered in the antenna of *Antheraea polyphemus* that distinguish [12] and bind to lipophilic odorant compounds [18–23]. However, emerging data suggests that OBPs are not restricted to the sensory organs of insect and show expression in non-sensory organs including reproductive organs. Li et al. showed that OBPs22 was highly expressed in the male reproductive organs of *Aedes aegypti* and transfers to females during mating as a carrier for the urine and saliva of vertebrate [24]. Sun et al. also found that OBP10 is highly abundant in seminal fluid of *Helicoverpa armigera* and *H. assulta* and transfers to female during mating. OBP10 also binds 1-dodecene, a known insect repellent [25].

Athetis dissimilis Hampson (Lepidoptera: Noctuidae) is an important agricultural pest. Li et al. distinguished *A. dissimilis* from *A. lepigone* [26] that mainly distributes to Asian countries including China, Japan, Philippines, Korea, Indonesia and India causing serious damages to maize, wheat, peanut, soybean and sweet potato [27–28]. Because of the fact that larvae of *A. dissimilis* live under plant residues, it is difficult to control the spread of the pest with chemical pesticides. Therefore, novel control managements are urgently needed to mitigate crop damage. We first sequenced the antennal transcriptomes of *A. dissimilis* [29] and characterized 5 OBPs that showed tissue-specific expression patterns [30]. Of note, *AdisOBP6* was highly expressed in the testes of *A. dissimilis* [30]. We reasoned that the testis of insects possess a defined set of OBPs in a manner comparable to the antenna. In this study, we sequenced the transcriptomes of the *A. dissimilis* reproductive organs and studied the expression of the OBPs in the antenna of female and males and testis and ovaries, and provide new targets for pest management in the future.

Results

Illumina sequencing and assembly

A total of 34,565,866, 32,154,799, and 26,952,526 clean reads containing 10.35, 9.63, and 8.07 giga base (Gb) pairs of clean nucleotides respectively, were obtained from the three replicates of the *A. dissimilis* ovaries. A total of 27,752,168, 28,900,040, and 30,838,686 clean reads containing 8.29, 8.65 and 9.23 giga base (Gb) pairs of clean nucleotides respectively, were obtained from the three replicates of *A. dissimilis* testes. The quality of the transcriptome sequences was high, with Q30 percentages of 94.03%, 94.36%, 94.21%, 94.42%, 94.27% and 94.01% for the three replicates of *A. dissimilis* ovaries and testes, with a GC content were ~ 50% (Table 1). Then 221,074 transcripts and 82,016 unigenes with N50 length of 1,350 and 1,243, were obtained from assembled using Trinity (Table 2).

Table 1
Summary of the sequence assemblies according to the RNA-seq data of the *A. dissimilis*.

Sample name		Clean reads	Clean bases	GC Content (%)	Q30 (%)
Ovaries	Repeat 1	34,565,866	10.35 G	48.00	94.03
	Repeat 2	32,154,799	9.63 G	48.35	94.36
	Repeat 3	26,952,526	8.07 G	48.27	94.21
Testis	Repeat 1	27,752,168	8.29 G	48.85	94.42
	Repeat 2	28,900,040	8.65 G	47.20	94.27
	Repeat 3	30,838,686	9.23 G	46.65	94.01

Table 2
Summary of de novo assembly of the *A. dissimilis* transcriptomes.

Length Range	Transcript	Rate%	Unigene	Rate%
< 300	0	0	0	0
300–500	83,670	37.85	37104	45.24
500–1000	70,088	31.70	24792	30.23
1000–2000	44,935	20.33	12864	15.68
> 2000	22,381	10.12	7256	8.85
Total Number	221,074		82,016	
Total Length	216,261,287		73,549,396	
N50 Length	1,350		1,243	
Mean Length	978.23		896.77	

Functional annotation

Significant matches of 33,587 unigenes (96.91%) in the NR; 29,936 (86.38%) in the eggnog; 20,134 (58.09%) in the Pfam; 15,174 (43.78%) in the Swissprot database; 14,775 (42.63%) in the KEGG; 7,797 (22.50%) in the GO; and 6,712 (19.37%) in the COG were observed. As a result, up to 34,658 putative coding sequences were identified (Table 3). NR database queries revealed a high percentage of *A. dissimilis* sequences that closely matched to sequences of *Helicoverpa armigera* (19072, 56.87%), *Amyelois transitella* (1936, 5.77%), *Bombyx mori* (1543, 4.60%), *Papilio machaon* (1155, 3.44%), *Papilio xuthus* (868, 2.59%), *Plutella xylostella* (844, 2.52%), *Danaus plexippus* (634, 1.89%), *Branchiostoma belcheri* (473, 1.41%), and *Papilio polytes* (368, 1.10%) (Fig. 1).

Table 3
Functional annotation of the *A. dissimilis* transcriptomes.

Database	Number	Rate (%)	$300 \leq \text{Length} < 1000$	$\text{Length} \geq 1000$
COG	6,712	19.37	2,638	4,074
GO	7,797	22.50	4,453	3,344
KEGG	14,775	42.63	8,205	6,570
Pfam	20,134	58.09	8,577	11,557
Swissprot	15,174	43.78	6,987	8,187
eggNOG	29,936	86.38	16,283	13,653
NR	33,587	96.91	18,939	14,648
All	34,658		19,914	14,744

For GO analysis, 7,797 unigenes (22.50%) could be assigned to three GO terms including: cellular components (886 unigenes, 11.36%), molecular functions (5,683 unigenes, 72.89%) and biological process (1,228 unigenes, 15.75%) (Fig. 2). The “molecular functions” were highest represented (72.89% transcripts). For the “molecular functions” ontology, catalytic activity and binding were most prevalent.

Identification of putative odorant-binding proteins

In the *A. dissimilis* antennal and reproductive organ transcriptome, we identified 54 candidate OBPs (Genbank accession number: KR780027-KR780030, MH900289-MH900338), 31 of which were from the antennae (through the analysis of previous *A. dissimilis* antennal transcriptomes) and 23 from the testis and ovaries (Table 4). A total of 44 AdisOBP sequences had full-length ORFs. Their cDNAs encoded protein of 131–293 amino acids with molecular weights of 11.6–33.2 kDa and isoelectric points of 4.44–9.74. Excluding the 7 AdisOBPs (AdisOBP28, 30, 31, 35, 36, 41, 42, 52, 53 and 54) signal peptides were predicted at the N-terminus. AdisOBPs had 39–99% sequence homology with previously identified OBPs from other insect species, displaying a high level of sequence similarity. For example, AdisOBP13 has a 95% identity with *Spodoptera exigua* OBP9 (Table 1). *A. dissimilis* OBPs had only 11.87% identity.

Table 4
The characteristic of candidate OBP genes in the antennae and reproductive organs of *A. dissimilis*.

Order	Gene name	GenBank accession no.	ORF (aa)	Molecular weight(kD)	Isoelectric point	Signal peptide	Full length	Homology search with the known proteins		
								Gene annotation	Species	Protein ID
c69042	AdisPBP1	KR780029	166	17.32	5.19	Yes	yes	PBP1	Mamestra brassicae	AAC05702
c65047	AdisPBP2	KR780030	162	18.08	5.30	Yes	Yes	PBP2	Mamestra brassicae	AAC05701
c65143	AdisPBP3	MH900289	164	18.71	5.25	Yes	yes	PBP3	Agrotis ipsilon	AFM36758
c47645	AdisGOBP1	KR780027	163	18.89	5.19	Yes	yes	GOBP1	Sesamia inferens	AGS36742
c60029	AdisGOBP2	KR780028	161	18.09	5.09	Yes	yes	GOBP2	Agrotis ipsilon	AFM36760
c68783	AdisOBP1	MH900290	293	33.20	5.76	Yes	yes	OBP	Bombyx mori	NP_001153663
c69959	AdisOBP2	MH900291	246	27.36	5.40	Yes	yes	OBP10	Ostrinia furnacalis	BAV56797
c60098	AdisOBP3	MH900292	145	16.22	8.37	Yes	yes	OBP	Spodoptera exigua	ADY17886
c65852	AdisOBP5	MH900293	242	26.78	6.33	Yes	yes	OBP35	Dendrolimus punctatus	ARO70194
c72710	AdisOBP8	MH900294	240	27.01	6.53	Yes	Yes	OBP25	Spodoptera exigua	AKT26502
c61153	AdisOBP9	MH900295	167	18.50	4.51	Yes	Yes	OBP10	Sesamia inferens	AGS36751
c60049	AdisOBP11	MH900296	141	16.38	4.47	Yes	Yes	OBP8	Spodoptera exigua	AGH70104
c65401	AdisOBP13	MH900297	133	15.14	9.01	Yes	yes	OBP9	Spodoptera exigua	AGH70105
c58306	AdisOBP14	MH900298	185	20.13	6.04	Yes	Yes	OBP1	Agrotis ipsilon	AGR39564
c64058	AdisOBP15	MH900299	146	16.43	6.29	Yes	yes	OBP6	Agrotis ipsilon	AGR39569
c53621	AdisOBP16	MH900300	118	-	-	-	internal	OBP18	Spodoptera exigua	AKT26496
c68160	AdisOBP17	MH900301	252	28.95	6.19	Yes	Yes	OBP23	Spodoptera exigua	AKT26500
c67912	AdisOBP18	MH900302	203	22.50	5.69	Yes	Yes	OBP19	Helicoverpa assulta	AGC92793
c60881	AdisOBP19	MH900303	139	14.55	8.58	Yes	yes	OBP5	Agrotis ipsilon	AGR39568
c71719	AdisOBP20	MH900304	139	15.69	7.52	Yes	Yes	OBP8	Spodoptera litura	AKI87969
c65033	AdisOBP21	MH900305	147	15.65	4.90	Yes	Yes	OBP5	Helicoverpa armigera	AEB54581
c63129	AdisOBP22	MH900306	146	15.92	7.53	Yes	yes	OBP23	Spodoptera litura	XP_022826767
c57331	AdisOBP23	MH900307	149	15.96	5.03	Yes	yes	OBP26	Spodoptera exigua	AKT26503
c64709	AdisOBP24	MH900308	148	16.77	5.45	Yes	yes	OBP7	Helicoverpa armigera	AEB54591
c81048	AdisOBP25	MH900309	71	-	-	-	Internal	OBP22	Spodoptera exigua	AKT26499
c53707	AdisOBP26	MH900310	134	14.28	4.51	Yes	yes	OBP34	Helicoverpa assulta	ASA40070

Order	Gene name	GenBank accession no.	ORF (aa)	Molecular weight(kD)	Isoelectric point	Signal peptide	Full length	Homology search with the known proteins		
								Gene annotation	Species	Protein ID
c28876	AdisOBP27	MH900311	124	-	-	-	internal	OBP11	Spodoptera exigua	AGP03457.1
c67118	AdisOBP28	MH900312	236	27.80	4.90	No	yes	OBP9	Spodoptera litura	ALD65883
c57589	AdisOBP29	MH900313	129	-	-	-	5' lose	OBP33	Helicoverpa assulta	ASA40072
c62521	AdisOBP30	MH900314	180	20.26	4.84	No	yes	OBP9	Helicoverpa armigera	AEB54592
c63839	AdisOBP31	MH900315	116	12.77	6.12	No	yes	OBP14	Spodoptera exigua	AGP03460
Gene.53346	AdisOBP32	MH900316	184	20.65	6.32	Yes	Yes	GOBP70	Helicoverpa armigera	XP_021188671
Gene.77161	AdisOBP33	MH900317	207	23.94	9.19	Yes	Yes	OBP19	Helicoverpa assulta	AGC92793
Gene.60926	AdisOBP34	MH900318	193	22.42	5.48	Yes	Yes	OBP9	Cnaphalocrocis medinalis	ALT31639
Gene.32069	AdisOBP35	MH900319	137	15.34	8.85	No	Yes	OBP	Helicoverpa armigera	AEX07279
Gene.44893	AdisOBP36	MH900320	143	15.92	5.57	No	Yes	OBP19	Helicoverpa assulta	AGC92793
Gene.35132	AdisOBP37	MH900321	102	-	-	-	5' lose	OBP24	Cnaphalocrocis medinalis	ALT31654
Gene.54044	AdisOBP38	MH900322	141	15.05	8.77	Yes	Yes	OBP5	Agrotis ipsilon	AGR39568
Gene.7082	AdisOBP39	MH900323	156	17.94	4.86	Yes	Yes	PBP1	Helicoverpa armigera	XP_021192649
Gene.113597	AdisOBP40	MH900324	166	19.09	8.61	Yes	Yes	OBP38	Dendrolimus punctatus	ARO70197
Gene.77158	AdisOBP41	MH900325	141	16.29	9.12	No	Yes	OBP19	Helicoverpa assulta	AGC92793
Gene.14505	AdisOBP42	MH900326	102	11.15	5.44	No	Yes	OBP23	Spodoptera litura	ALD65897
Gene.54039	AdisOBP43	MH900327	76	-	-	-	5' lose	OBP	Helicoverpa armigera	AEX07280
Gene.58201	AdisOBP44	MH900328	76	-	-	-	5' lose	OBP23	Spodoptera litura	ALD65897
Gene.32531	AdisOBP45	MH900329	150	16.43	4.77	Yes	Yes	OBP2	Agrotis ipsilon	AGR39565
Gene.5319	AdisOBP46	MH900330	70	-	-	-	5' lose	OBP14	Spodoptera exigua	AGP03460
Gene.86678	AdisOBP47	MH900331	120	-	-	-	5' lose	OBP13	Sesamia inferens	AGS36753
Gene.141496	AdisOBP48	MH900332	106	12.10	6.95	No	Yes	OBP39	Dendrolimus punctatus	ARO70198
Gene.142856	AdisOBP49	MH900333	157	17.96	9.74	Yes	Yes	OBP18	Dendrolimus punctatus	ARO70177
Gene.17592	AdisOBP50	MH900334	144	16.21	4.44	Yes	Yes	OBP9	Helicoverpa armigera	AEB54592
Gene.54647	AdisOBP51	MH900335	84	-	-	-	5' lose	OBP39	Dendrolimus punctatus	ARO70198
Gene.76032	AdisOBP52	MH900336	105	11.60	4.71	No	Yes	OBP	Spodoptera litura	ALD65897
Gene.111996	AdisOBP53	MH900337	105	12.28	8.21	No	yes	OBP	Operophtera brumata	KOB73304

Order	Gene name	GenBank accession no.	ORF (aa)	Molecular weight(kD)	Isoelectric point	Signal peptide	Full length	Homology search with the known proteins		
								Gene annotation	Species	Protein ID
Gene.158529	AdisOBP54	MH900338	131	14.34	4.86	No	yes	OBP11	Spodoptera exigua	AGP03457

Multiple sequence alignments of the *A. dissimilis* OBPs revealed the presence of expected conserved cysteines (Fig. 3). The phylogenetic tree of *A. dissimilis* and other lepidopteran OBPs constructed using the neighbor-joining method, indicated five clades that contained four possible protein subfamilies (Fig. 4). In addition, the tree showed low levels of clustering highlighting the diversity of the lepidopteran OBPs. Five (AdisPBP1-3, GOBP1-2) AdisOBPs belonged to PBP/GOBP. A total of 35 OBPs (AdisOBP1, 3–5, 6–10, 12–17, 19–22) were ‘Classic’ OBPs that contained six positionally-conserved cysteine residues. Seven OBPs (AdisOBP14-16, 18, 33, 36 and 41) belonged to ‘Plus-C’ subclass OBP genes. Nine OBPs belonged to ‘Minus-C’ subclass OBP genes. Interestingly, AdisOBP1, AdisOBP17 and AdisOBP40 did not belong to any of the four protein subfamilies (Fig. 4). The transcription abundance of *A. dissimilis* OBPs in antennae of female and males, ovary and testis are profiled in Fig. 5.

Expression of the OBPs in the antennae, ovaries and testis of *A. dissimilis*

To understand the functions of the identified OBPs in *A. dissimilis*, we measured the relative expression levels of OBPs in different tissues of *A. dissimilis* via fluorescence qRT-PCR (Fig. 6). A total of 23 OBPs (AdisGOBP1-2, PBP1-3, OBP1-2, 8–9, 11, 17, 20–22, 24, 26–31, 50 and 54) were highly expressed in both female and male antennae compared to the reproductive organs of females and males. A total of 5/23 OBPs (Adis GOBP1, OBP8-9, 24 and 29) showed comparable expression in the male and female antennae of *A. dissimilis*; 3/23 (AdisPBP1, OBP17 and OBP26) were higher in males; and 15/23 were higher in females.

A total of 24 OBPs (AdisOBP3, 5, 15, 18–19, 23, 25, 33–41, 44–45, 47–49 and 51–53) were highly expressed in the testis of *A. dissimilis* compared to other tissues. The expressive of the OBPs were low in the ovaries of *A. dissimilis*.

Discussion

Insects rely on peripheral sensilla on the antennae to distinguish plant odorants and pheromones [31],³⁷ a knowledge of the molecular mechanisms of olfaction is essential for better using olfactory-based pest management strategies and the development of novel strategies. OBPs are more accessible targets for research, considering they are small, soluble, stable and easier to manipulate and modify. About exact functions of the OBPs are unclear, but it is widely believed that their function is to capture and transfer outside odorants to ORs located on the membranes of ORNs [8, 11, 32].^{8,11,38} Insect OBPs are present on the antennae where they execute odorant functions [11, 33–36].^{11,39–42} In this study, we identified 31 novel OBPs through the analysis of *A. dissimilis* antennal transcriptomes, expressing five previously reported AdisOBPs [30].³⁰ The number of OBPs in *A. dissimilis* antennae were similar to the antennal transcriptomes of *S. litura* (33) [37]⁴³ and *S. littoralis* (36) [38]⁴⁴ but more abundant than *S. exigua* (11) [39]⁴⁵, *M. sexta* (18) [40]⁴⁶ and *H. armigera* (26) [41].⁴⁷ We additionally sequenced the transcriptomes of *A. dissimilis* ovaries and testis. The alignments against the Nr database showed that 56.87% of the *A. dissimilis* unigenes were comparable to *Helicoverpa armigera* sequences. A total of 24 OBPs were identified in the transcriptomes of *A. dissimilis* reproduction organs.

Based on the cluster analysis of the phylogenetic trees, five AdisOBPs belonged to PBP/GOBP; 35 AdisOBPs belonged to ‘Classic’ OBPs; 7 AdisOBPs belonged to ‘Plus-C’ OBPs; and 9 AdisOBPs belonged to ‘Minus-C’ OBPs. These results were similar to the classifications of most insect OBPs [25, 37, 42].^{25,43,48} Interestingly, AdisOBP1, AdisOBP17 and AdisOBP40 did not cluster into these 4 protein subfamilies, but multiple sequence alignments of the *A. dissimilis* OBPs revealed that 3 of the OBPs contain no conserved cysteines. Their construction requires further to verification.

Insect OBPs are expressed in the sensory organs [22, 25, 37, 43–45].^{22,25,43,49–51} Our result showed that 23 AdisOBPs were significantly expressed in both female and male antennae compared to other tissues. Only the expression of 3 AdisOBPs were significantly higher in the antennae’s of males compared to females, suggesting that females require more abundant OBPs for spawning. OBPs are also expressed in the non-olfactory organs, such as those required for reproduction [24-25.46-48].^{24–25,52–54} In this study, 24 AdisOBPs showed significant expression in the testis of *A. dissimilis* compared to other tissues, but the expression of AdisOBPs in the ovaries were low. It was previously speculated that OBPs expressed in the testis deliver compounds to the females during mating [24–25].^{24–25} Hence, it is understandable to presume that such stable proteins could be used in the testis of insect where there is need for transportation of hydrophobic molecules in aqueous media or protection of chemicals from degradation, as well as to assure a gradual release of semiochemicals in the environment. So these proteins have been named for “encapsulins”, to imply the common role of encapsulating small ligands [49].⁵⁵

Conclusions

In summary, we demonstrate that the *A. dissimilis* chemosensory genes show functional diversity. These findings enhance our knowledge of the roles of OBPs in *A. dissimilis* and lepidopteran species and provide a base for studying OBPs novel targets of pest management strategies.

Methods

Insect rearing and sample preparation

The *A. dissimilis* strain was collected from Luoyang (province of Henan, China) corn fields (112°26' E, 34°43' N) in 2014 and maintained at the Henan Science and Technology University. Colonies were reared on an artificial diet at 25 ± 1 °C, 80 ± 5% relative humidity and a 16-h/8-h light/dark cycle.

Based on preliminary data, we found that the *A. dissimilis* sperm and eggs began to mature 3 days after emergence. We respectively collected the ovaries and testes of 3-day old virgin females and male adults (n = 40 per treatment) from three biological replications. Dissections were performed in sterile PBS-DEPC and immediately frozen in liquid nitrogen until RNA isolation.

cDNA library preparation and sequencing

Total RNA from the *A. dissimilis* ovaries and testis tissues were extracted using RNAiso Plus kit (TaKaRa, Dalian, China) and treated with DNase I (TaKaRa, Dalian, China) as per the manufacturer's protocols. RNA was assessed through 1% agarose gel electrophoresis and Nanodrop 2000 ® (Thermo Scientific, Waltham, MA, USA), Qubit 2.0 (Life Technologies, Carlsbad, CA, USA) and Agilent 2100 (Agilent, Santa Clara, CA, USA) analysis.

Following the TruSeq RNA Sample Preparation Guide v2 (Illumina, San Diego, CA, USA), mRNA was enriched using magnetic beads crosslinked with Oligo (dT). Enriched RNA was then fragmented using fragmentation buffer and first-strand cDNA synthesis was used to produce small mRNA fragments, random primers, reverse transcriptase, and second-strand cDNA synthesis through the addition of dNTPs, DNA polymerase I, and RNase H. Double-stranded cDNA was purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA) and treated to repair ends, remove poly(-A) tails, and link sequencing adapters. Fragment sizes were selected using AMPure XP beads and cDNA libraries were constructed through PCR amplification (Veriti™ 96-Well Thermal Cycle, Applied Biosystems, Foster City, USA). The concentration and insert size of the cDNA libraries were detected using Qubit 2.0 and Agilent 2100 and quantified via q-PCR (CFX-96, Bio-Rad, Hercules, CA, USA).

Finally, sequencing was performed using the Illumina HiSeq™ 4000 platform to generate 150-bp paired-end reads. Sequencing analyses were performed by the Genomics Services of the Beijing Biomarker Technologies Co., Ltd. (Beijing, China). Raw data processing and base calling were performed using Illumina software.

Assembly and Functional annotation

Raw data (raw reads) in the FASTQ format were first modified into clean data (clean reads) through Perl scripts. This was performed through the removal of reads containing adapter sequences, > 10% unknown nucleotides and quality values ≤ 20. The Q20, Q30, and GC content were then calculated using high-quality data.

Transcriptomes were assembled using Trinity (version trinityrnaseq_r20131110) with default settings, except for min_kmer_cov set to 2 [50].³¹ Unigene functions were annotated based on NCBI non-redundant protein sequences (NR, NCBI blast 2.2.28+, e-value = 1e-5), NCBI nucleotide sequences (NT, NCBI blast 2.2.28+, e-value = 1e-5), Protein family (Pfam, HMMER 3.0 package, hmmscan, e-value = 0.01), eukaryotic Ortholog Groups (KOG, NCBI blast 2.2.28+, e-value = 1e-3), SwissProt (NCBI blast 2.2.28+, e-value = 1e-5), the Kyoto Encyclopedia of Genes and Genomes (KEGG; KEGG Automatic Annotation Server [KASS], e-value = 1e-10) and Gene Ontology (GO, Blast2GO v2.5, e-value = 1e-6). Coding sequences (CDS) were predicted through aligning transcriptome sequences to the Nr and Swiss-Prot database or using estscan 3.0.3 [51].³²

Sequence and phylogenetic analysis

Sequence similarities were assessed using the NCBI-Blast network server (<http://blast.ncbi.nlm.nih.gov/>). The signal peptides of OBPs were predicted using SignalP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) [52].³³ Multiple sequence alignments were assessed using DNAMAN 6.0. Sequence alignments of the candidate OBPs were performed using ClustalX 2.1 [53]³⁴ and used to construct phylogenetic trees with PhyML in Seaview v.4 based on the Jones–Taylor–Thornton (JTT) model with nearest-neighbor interchanges. Trees were viewed and edited using FigTree v.1.3.1.

Transcript expression profiling

Sequencing Reads were compared to the Unigene libraries using Bowtie, and expression levels were estimated by combining RSEM. FPKM values are used to represent the expression abundance of the corresponding Unigenes [54].³⁵ Averages of three biological replicates as the actual expression values for each transcript were obtained. Based on the RNA-seq data, tissue-specific expression of the OBPs were profiled.

Expression analysis through quantitative real-time polymerase chain reaction

Male antennae, female antennae, ovaries and testes tissue from adults at 3 post-eclosion were excised and frozen in liquid nitrogen. Total RNA was extracted using RNAiso Plus kits (TaKaRa, Dalian, China) and isolated RNA was transcribed to first-strand cDNA using PrimeScript™ RT reagent with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's protocols. Real-time quantitative PCR (RT-qPCR) was performed with SYBR® Premix Ex Taq™ II (TaKaRa). The *A. dissimilis* GADPH gene was used as an endogenous control to correct for sample-to-sample variations. A 200 ng/μL cDNA sample was used for per tissue. Primers were designed using Primer Premier 5.0 software and are listed in supportment Table 1. RT-qPCR reactions contained: 10 μL of SYBR Premix Ex Taq II, 20 ng of cDNA template, 0.2 μM of each primer and nuclease-free water. The cycling conditions were 1 cycle of 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 55 °C for 30 s. Melt curve conditions were 95 °C for 10 s and 65 °C for 30 s. No-template controls (NTC) were included to detect possible contamination. Three biological replicates were analyzed and the relative expression of the OBP genes across the samples were measured using the 2^{-ΔΔCT} method [55].³⁶ Expression was calculated relative to levels in the female antennae, which were arbitrarily set to 1. Differences in the expression of AdisOBP genes between the different tissues were compared using a one-way nested analysis of variance (ANOVA), followed by a Tukey's honestly significance difference (HSD) test using SPSS (SPSS Institute 17.0, SPSS Inc, Chicago, IL, USA).

Abbreviations

CO:Carbon dioxide; FPKM:Fragments per kb per million fragments; GO:Gene ontology; GR:Glutamate receptor; iGluR:Ionotropic glutamate receptor; IR:Ionotropic receptor; JTT:Jones-Taylor-Thornton amino acid substitution model; OR:Odorant receptor; ORF:Open reading frame; TMD:Transmembrane domain.

Declarations

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Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors' contributions

All authors contributed to research design and manuscript preparation. Conceived and designed the experiments: YS, HS, JZ. Performed the experiments: YS, HS, JZ. Analyzed the data: YS, HS, JZ. Contributed reagents/materials/analysis tools: YS, JZ. Wrote the paper: YS, HS. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

The Blastx results of *A. dissimilis* reproductive organs unigenes in NR database.

Figure 2

Gene Ontology (GO) classifications of *A. dissimilis* reproductive organs unigenes according to their involvement in biological processes, cellular component and molecular function.

Figure 3

Sequence alignments of *A. dissimilis* OBPs.

Figure 4
Phylogenetic relationships of candidate OBP proteins from *A. dissimilis* and Lepidoptera species.

Figure 5

Heat map showing the abundance of unigenes encoding OBPs in the *A. dissimilis* different tissues transcriptomes presented as normalized reads in reads per kilobase per million mapped reads (RPKM). In the figure each column represents 1 samples; each line represents 1 OBP gene. The color depth represents the number of reads contained in OBPs; red means more; blue means less. FA: female antennae; MA: male antennae; Ov: ovaries; Te: testis.

Figure 6

Expression profiles of the candidate OBPs in different tissues of *A. dissimilis*. FA: female antennae; MA: male antennae; Ov: ovaries; Te: testis. The standard errors are represented by the error bars; different lowercase letters (a, b, c) above the bars denote significant differences at $p < 0.05$.

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