Identification and Preliminary Characterization of Odorant-Binding Proteins in *Neoceratitis asiatica* (Diptera: Tephritidae)[1]

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Abstract The fruit fly *Neoceratitis asiatica* (Becker) (Diptera: Tephritidae) is a monophagous pest that damages only wolfberry, *Lycium barbarum* L. The odorant-binding proteins (OBPs) of insects are part of the initial steps of the olfactory signal transduction cascade involved in solubilizing and transporting chemical signals to the olfactory receptors. We studied the OBP genes of *N. asiatica* by using data from RNA-seq cDNA libraries of adult flies. Seventeen putative OBP sequences in *N. asiatica* were identified, corresponding to 13 OBPs of *Drosophila melanogaster* (Megen) (Diptera: Drosophilidae). Thirteen of the *N. asiatica* genes belong to the classic subfamily, four are in the minus-C subfamily, and none were members of the plus-C and dimer subfamilies. A phylogenetic tree was constructed to elucidate the evolutionary relationship between *N. asiatica* and other related species. This investigation of OBP evolution in monophagous, oligophagous, and polyphagous fruit flies revealed that the OBPs in *N. asiatica* are more oligo and conserved. These findings lay a foundation for uncovering the relationships between monophagous insects and their OBPs.

Key Words *Neoceratitis asiatica*, odorant-binding protein genes, monophagous, phylogenetic analyses, OBP subfamilies

Olfaction, a crucial biological process, is used to detect a huge diversity of distinct odors and extract vital information from volatile chemicals in the environment, which is essential for survival and reproduction of insects (Benton 2006, Sánchez-Gracia et al. 2009). Many proteins are involved in the steps of olfactory signal transduction cascade, including odorant-binding proteins (OBPs).

OBPs are small soluble globular proteins involved in the solubilization and transportation of chemical signals through the aqueous lymph of sensillae to reach the olfactory receptors (Sánchez-Gracia and Rozas 2008, Vogt and Riddiford 1981). Amino acid sequences of OBPs exhibit a conserved domain, including six cysteine residues (from C1 to C6) of all known insect OBPs. These cysteines form three disulfide bonds that stabilize their tertiary structure and help fix a hydrophobic binding cavity (Lagarde et al. 2011, Leite et al. 2009, Pelosi and Maida 1995,

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Tsitsanou et al. 2013). Relative to the six cysteine residues, the key to functional information and phylogenetic relationships, OBPs are grouped into four subfamilies: classic (six cysteines), plus-C (more than six cysteines, always present three additional cysteines before C1 and after C6), minus-C (some members with less than six cysteines), and dimer (three complete OBP domains) (Hekmat-Scafe et al. 2002, Tegoni et al. 1996, Vieira et al. 2012, Zhou et al. 2004b).

The first OBP identified in insects was from the polyphemus moth, *Antheraea polyphemus* (Cramer) (Lepidoptera: Saturniidae), as a pheromone binding protein (Vogt and Riddiford 1981). OBPs were eventually identified from representatives of Lepidoptera, Orthoptera, Isoptera, Diptera, Hymenoptera, Hemiptera, Coleoptera, Anoplura, Blattaria, and Neuroptera (Fan et al. 2011, Li et al. 2013, Pelosi and Maida 1995, Xu et al. 2009). Genome analysis revealed that the number of OBP genes varies among taxonomic orders or species (Vieira and Rozas 2011), with 51 in *Drosophila melanogaster* (Megen) (Diptera: Tephritidae) (Hekmat-Scafe et al. 2002), 21 in *Apis mellifera* (L.) (Hymenoptera: Apidae) (Forêt and Maleszka 2006), 37 in *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) (You et al. 2013), 83 in *Anopheles gambiae* (Giles) (Diptera: Culicidae), and 46 in *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Kattupalli et al. 2021). Studies with transcriptomes have revealed 15 putative OBPs in *Ips typographus* (L.) (Coleoptera: Curculionidae), 31 in *Megacyllene caryae* (Gahan) (Coleoptera: Cerambycidae) (Andersson et al. 2013), 48 in *Blattella germanica* (L.) (Blattaria: Ectobiidae) (Niu et al. 2016), 14 in *Chrysopa pallens* (Rambur) (Neuroptera: Chrysopidae) (Li et al. 2013), and 15 in *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) (Zhou et al. 2012). OBPs have also been studied in the Tephritidae, including *Anastrepha fraterculus* (Campanini and de Brito 2016), *Anastrepha obliqua* (Wiedemann) (Campanini and de Brito 2016, Nakamura et al. 2016), *Bactrocera dorsalis* (Hendel) (Chen et al. 2019, Wu et al. 2020, Zheng et al. 2013), *Bactrocera minax* (Enderlein) (Cheng et al. 2020, Wu et al. 2019), and *Ceratitis carpitata* (Wiedemann) (Siciliano et al. 2014).

OBPs vary in the insect species that respond to different combinations of odors, thus playing an active role in odorant recognition. In addition to the role that OBPs play in reproductive processes (Leal 2013), some research has indicated that a number of OBP genes could influence feeding habits, such as the search for food resources (Campanini and de Brito 2016). In *Locusta migratoria* L. nymphs, silencing the *LmigOBP1* gene through RNA interference resulted in decreased food consumption and electroantennography responses to five maize leaf volatiles (Li et al. 2016). Interference in the *BminOBP21* gene significantly affected the antennal responses to D-limonene in *B. minax,* which is a putative attractant that normally activates antennal responsiveness for oviposition or host location (Xu et al. 2019). The transcript level of OBPs from *B. doralis* also changed with diet, indicating that these OBPs may act in olfaction (Idrees 2017).

Natural selection plays an important role in the divergent evolution of OBPs (Emes et al. 2004, Willett 2000). For example, positive selection was likely involved in the functional differentiation of new copies of the OBP multigene family (Sánchez-Gracia and Rozas 2008). OBP gene expression also differed widely in the olfactory organs of *Drosophila sechellia* Tsacas & Bachli, a narrow ecological specialist that feeds on the fruit of *Morinda citrifolia* L., compared with its close

relatives *Drosophila simulans* Sturtevant and *D. melanogaster,* which feed on a wide variety of decaying plant matter (Kopp et al. 2008).

Neoceratitis asiatica (Becker) (Diptera: Tephritidae) damages only wolfberry, *Lycium barbarum* (L.). In China, this monophagous pest is mainly distributed in wolfberry production areas of Ningxia, Tibet, and Xinjiang, where the proportion of injured trees reaches nearly 70% in severely infested areas (Guo et al. 2017). Because of the unique and restricted feeding habits of *N. asiatica*, attempts to culture colonies in the laboratory have not yet been successful; therefore, research on OBPs in *N. asiatica* and other monophagous fruit flies is far less abundant than in polyphagous (e.g., *B. dorsalis* and *A. fraterculus*) or oligophagous (e.g., *B. minax*) fruit flies. Further study of OBPs of monophagous fruit flies will add to our knowledge of their function and modes of action and provide a basis for comparisons with polyphagous and oligophagous fruit flies.

Our objectives in the present study were to identify OBPs from the transcriptome of *N. asiatica,* compare them with the OBPs of *D. melanogaster,* and construct a phylogenetic tree to elucidate the evolutionary relationships among *N. asiatica* and other fruit fly species. Investigation of OBP evolution in monophagous, oligophagous, and polyphagous fruit flies may provide clues to how their host range might be related to the olfactory process.

Materials and Methods

Collection of samples and RNA extraction. *Neoceratitis asiatica* larvae in wolfberry fruit were collected from Yinchuan, Ningxia Hui Autonomous Region in China. Infested fruits were placed in plastic trays and transported to the laboratory where mature larvae were allowed to pupate in soil. After emergence, 30 10-d-old adults were collected for RNA extraction using TRIzol reagent as instructed by the supplier (Invitrogen, Carlsbad, CA).

Transcriptome libraries, sequencing, and assembly. A total of 3 μg of RNA was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA) following the manufacturer's recommendations. The library was constructed and then sequenced with an Illumina HiSeq™ 2500 (Illumina, San Diego, CA) at the Biomarker Technologies Company (Beijing, China). Trinity software first broke the sequencing reads into shorter fragments (K-mers) then extended these small fragments into contigs and used the overlap between these fragments to obtain components. By using the method of de Bruijn for diagramming and sequencing read information, the transcript sequences were identified in each component.

Functional annotation. Gene function was annotated based on the following databases: NR (National Center for Biotechnology Information [NCBI] nonredundant protein sequences), Pfam (protein family), KOG/COG/eggNOG (clusters of orthologous groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KEGG (Kyoto Encyclopedia of Genes and Genomes), and GO (gene ontology).

Alignments and phylogenetic analysis. The sequences were processed with the BLAST program from NCBI (Protein BLAST). The protein databases were searched using a protein query (nih.gov), and then *D. melanogaster* was entered in the organism selection to search the counterparts. The amino acid sequences

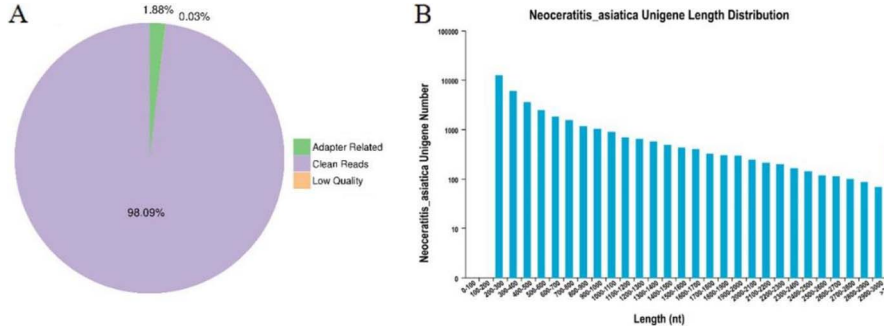

Fig. 1. Transcriptome quality. (A) Raw data distribution statistics. (B) Unigene length distribution map.

were recognized by SignalP-5.0 (Department of Health Technology, DTU, Kongens Lyngby, Denmark) to confirm the signal peptide. After removal of the signal peptides, the amino acid sequences of OBPs from *N. asiatica* were aligned with proteins from *D. melanogaster* and other dipterans using the ClustalW method in MEGA 11. The sequences of dipterans were from research (Campanini and de Brito 2016; Wu et al. 2019, 2020) and the NCBI database (nih.gov). The alignment of NasiOBPs was visualized with Geneious prime 2022.1.1. Maximum-likelihood trees were constructed in TBtools and the of IQ-TREE procedure using the best-fitting substitution model (Trifinopoulos et al. 2016). Branch support was assessed with 1,000 bootstrap replicates. Phylogenetic trees were visualized with iTOL V6 (https://itol.embl.de/). OBP sequences were from *D. melanogaster, B. dorsalis, Zeugodacus cucurbitae* (Coquillett), *B. minax, Bactrocera oleae* (Rossi)*, A. obliqua,* and *A. fraterculus.* The sequences used for constructing phylogenetic trees can be obtained by contacting the first author or corresponding authors.

Results

Assembly and annotation of transcriptome. Data quality was guaranteed by clean reads occurring 98.09% of the time (Fig. 1A). The assembly generated 36,583 unigenes with an average length of 666.6 bp and an N50 of 1,003 bp; the length distribution map is shown in Fig. 1B. The transcriptome assembly was deposited in the SRA database, which is available under accession identifier SRR17778144/PRJNA801105. There were 14,808 unigenes annotated by using Gene ontology annotation, which included the GO terms related to olfaction such as “odorant binding,” “signal transducer activity,” and “response to stimulus.”

OBP genes in *N. asiatica*. Seventeen putative OBPs were identified from the *N. asiatica* transcriptome (Table 1). The longest OBP was NasiOBP1, which included 177 amino acid residues, and the shortest was NasiOBP10 due to the absence of a signal peptide. Signal peptides were predicted at the hydrophobic N-terminal for almost all OBPs except NasiOBP6, NasiOBP10, and NasiOBP11. The differences in length of a signal peptide and in the amino acid residues cause the high diversity in the hydrophobic N-terminal.

Table 1. *Neoceratitis asiatica* assembled sequences with best-hit matches to *Drosophila melanogaster* odorant-binding protein genes.

N. asiatica OBP*	AA**	Signal Peptide	Accession Number	*D. melanogaster* gene	Subject ID	E-value†	Identity (%)
NasiOBP1	177	1-35	ON262824	84a	NP_476990.1	1*e*−34	51.79
NasiOBP2	163	1-27	ON640749	8a	ACY92870.1	2*e*−14	32.2
NasiOBP3	141	1-16	ON640750	83g	NP_731043.1	3*e*−60	58.62
NasiOBP4	142	1-15	ON640751	44a	NP_001286186.1	8*e*−18	38.02
NasiOBP5	124	1-15	ON640752	99c	ABW78556.1	2*e*−26	38.40
NasiOBP6	132	No	ON640753	99c	ACT22281.1	3*e*−44	52.80
NasiOBP7	142	1-16	ON640754	44a	NP_001286186.1	1*e*−49	62.10
NasiOBP8	142	1-16	ON640755	99b	ABW78474.1	5*e*−31	52.85
NasiOBP9	148	1-25	ON640756	19a	ACY93109.1	4*e*−46	57.03
NasiOBP10	103	No	ON640757	56h	ABW78084.1	1*e*−07	30.39
NasiOBP11	115	No	ON640758	56h	ABW78081.1	4*e*−25	44.95
NasiOBP12	164	1-24	ON640759	56a	ABW77804.1	7*e*−06	26.32
NasiOBP13	143	1-17	ON640760	19d	ACY93747.1	5*e*−23	43.24
NasiOBP14	149	1-22	ON640761	83a	NP_001287189.1	8*e*−51	57.14
NasiOBP15	158	1-32	ON640762	83a	NP_001287190.1	2*e*−71	66.24
NasiOBP16	147	1-22	ON640763	69a	NP_524039.2	2*e*−26	39.10
NasiOBP17	118	1-18	ON640764	56e	NP_611445.1	1*e*−08	38.10

* OBP = odorant-binding protein.
** AA = predicted number of amino acids.
† E-value = expected value.

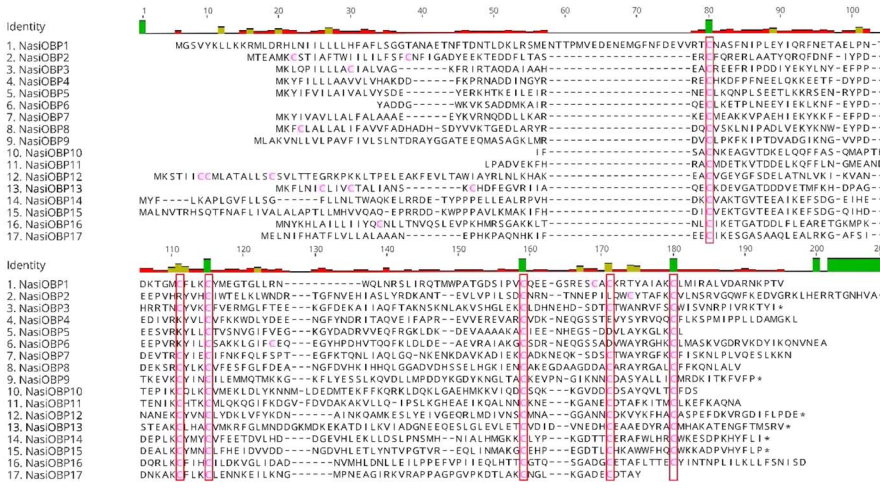

Fig. 2. Alignment of the OBP sequences of *N. asiatica*. Cysteine residues are in pink, and the conserved cysteine motifs are in the red boxes.

The OBP BLAST results revealed that within the genome of a dipteran model organism (*D. melanogaster*) all of the identified NasiOBPs shared high sequence homology with the *D. melanogaster* counterparts. A few NasiOBPs aligned with the same DmelOBPs. For instance, NasiOBP5 and NasiOBP6 were best fitted to DmelOBP99c (38.4% and 52.8%, respectively), NasiOBP10 and NasiOBP11 were best fitted to DmelOBP56h (30.39% and 44.95%, respectively), NasiOBP14 and NasiOBP15 had high identity with DmelOBP83a (57.14% and 66.24%, respectively), and NasiOBP4 and NasiOBP7 corresponded to DmelOBP44a (38.02% and 62.10%, respectively). The relative correspondence of NasiOBP1, NasiOBP3, NasiOBP8, and NasiOBP9 with DmelOBP83a, DmelOBP83g, DmelOBP99b, and Dmel19a were 51.79%, 58.62%, 52.85%, and 57.03%, respectively. The identities between NasiOBP2, NasiOBP12, NasiOBP13, NasiOBP16, NsOBP17 and their homologous counterparts of *D. melanogaster* DmelOBP8a, DmelOBP56a, DmelOBP19d, DmelOBP69a, DmelOBP56e were between $<$50% and $>$30%.

The 17 OBP proteins were further characterized by aligning them to each other (Fig. 2); the average amino acid identity among all the NasiOBPs was 17.9% (ranging from 8.24% to 48.2143%). Based on the conserved domain constituted by six cysteine residues, OBPs can be divided into four subfamilies: classic, plus-C, minus-C, and dimer. In our study, 13 OBPs belong to the classic group, including 12 of those with the six conserved cysteine residues. NasiOBP17 was the exception because its complete conserved domain was not detected at sequencing, leading to the missing C6. NasiOBP2, NasiOBP4, NasiOBP5, and NasiOBP6 were of the minus-C subfamily, lacking C2 and C5 (sites 111 and 170 in this research). None of the NasiOBPs belonged to the plus-C or the dimer subfamilies.

Phylogenetic relationships among OBP genes. The phylogenetic relationships of OBPs were determined for *N. asiatica* and *D. melanogaster*. Bootstrap values of almost every branch were ca. 90 (Fig. 3). The NasiOBPs were compared

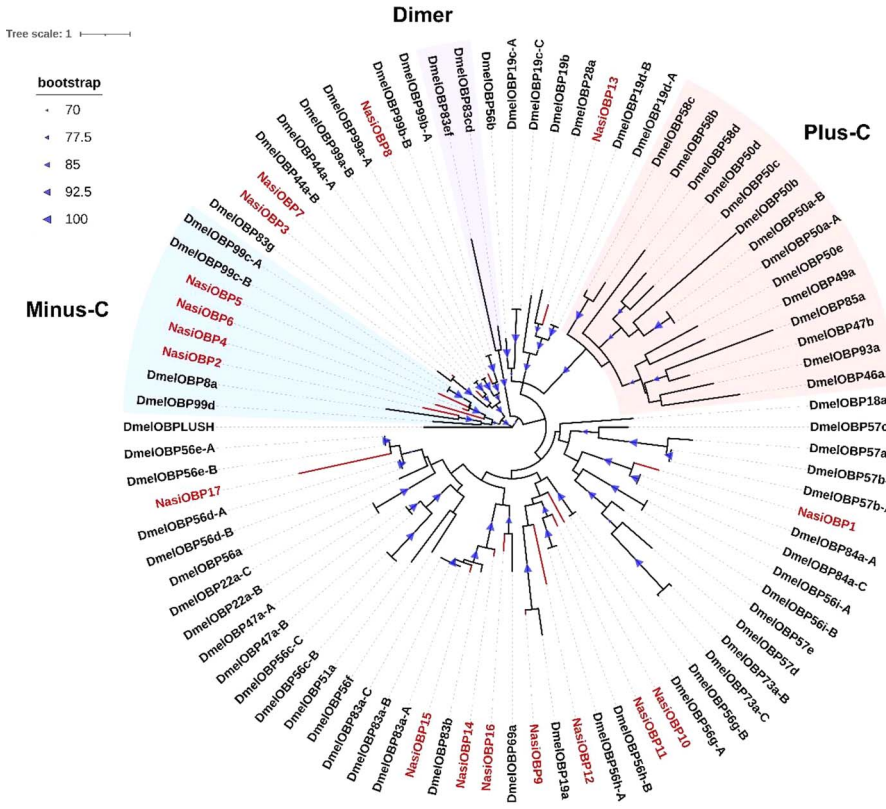

Fig. 3. Maximum likelihood phylogenetic tree of *N. asiatica* (Nasi prefix, red) and *D. melanogaster* (Dmel prefix, black) odorant-binding proteins (OBPs). The plus-C, minus-C, and dimer OBP clades are shaded. Bootstrap values >70 are shown with blue triangles (1,000 replications).

with their homologous counterparts from DmelOBPs with the same BLAST results. The OBPs from the same subfamilies were clustered together as expected except for the classic group, which has a large number of the OBPs. We focused on the minus-C subfamily because four NasiOBPs were placed in it and 10 sequences exhibited a pairwise identity of 29.2%, which is much higher than the pairwise identity of 14 members from plus-C subfamily and all the sequences separately.

Another tree also was constructed with seven fruit fly species (Fig. 4). Each terminal branch of the tephritid tree was highly credible, based on the high bootstrap values. The dimer, minus-C, and plus-C subfamilies were separated independently of each other. In the minus-C group, the identity for 23 sequences in the seven species was 33.7%, and the identity for 15 members of the plus-C subfamily and total sequences were 29.8% and 13.7%, respectively. All seven fruit fly species have the homolog sequences including OBP19a, OBP 99b, OBP56h, and OBP19d, whereas none of the OBPs appear alone in any single species of fruit fly.

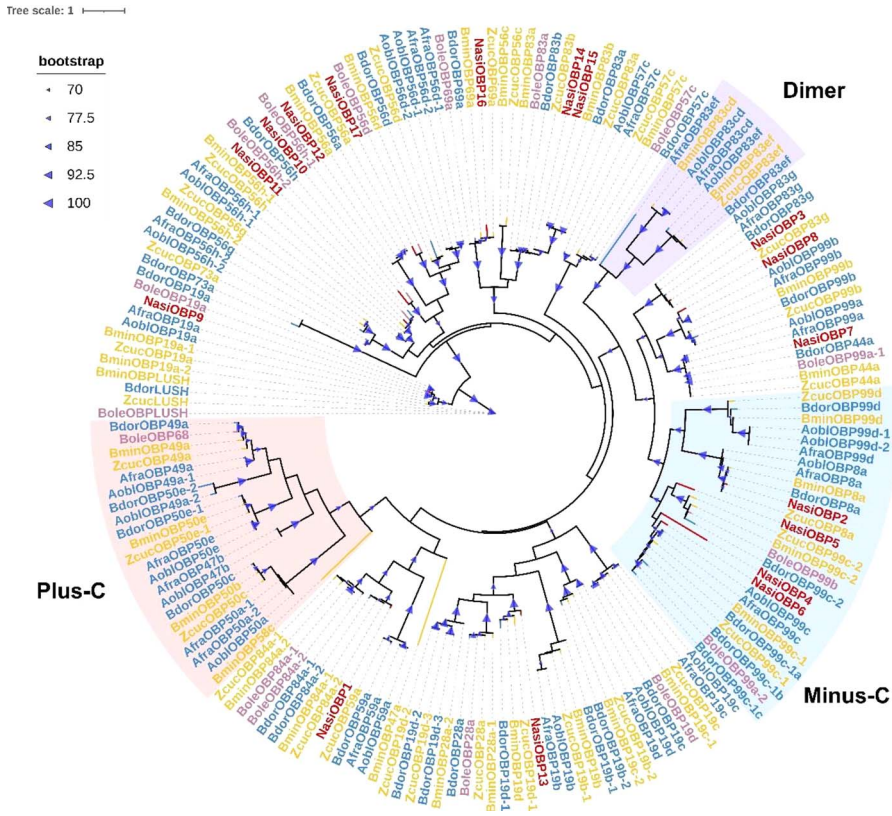

Fig. 4. Maximum likelihood phylogenetic tree of odorant-binding proteins (OBPs), including *N. asiatica* (Nasi prefix, red), *B. oleae* (Bole prefix, purple), *Z. cucurbitae* (Zcuc prefix, yellow), *B. minax* (Bmin prefix, yellow), *A. fraterculus* (Afra prefix, blue), *A. obliqua* (Aobl prefix, blue), and *B. dorsalis* (Bdor prefix, bule). The plus-C, minus-C, and dimer OBP clades are shaded. Bootstrap values >70 are shown with blue triangles (1,000 replications).

Discussion

The RNA-seq approach revealed 17 NasiOBPs that were aligned with each other in *N. asiatica* in contrast to the 23 OBPs reported from *A. fraterculus,* 24 from *A. obliqua,* 17 from *C. carpitata,* 35 from *B. dorsalis,* 34 from *Bactrocera correcta* (Bezzi), 33 from *Z. cucurbitae,* 33 from *Zeugodacus tau* (Walker), and 33 from *B. minax* (Campanini and de Brito 2016; Siciliano et al. 2014; Wu et al. 2019, 2020). With the exception of *C. carpitata* (17 OBPs), more OBPs were found in these other species of fruit flies than were identified from *N. asiatica*.

A similar situation has been reported from two lepidopteran rice pests–both in the family Pyralidae–where *Scirpophaga incertulas* (Walker) has 19 OBPs, and *Chilo suppressalis* (Walker) has 34 OBPs (Kattupalli et al. 2021, Khuhro et al.

2017). The former is monophagous, and the latter is polyphagous. Khuhro et al. (2017) also found that among some planthopper species (Hemiptera) the monophagous *Nilaparvata lugens* (Stal) has 10 OBPs and the polyphagous *Sogatella furcifera* (Horvath) and *Laodelphax striatellus* (Fallen) have 14 and 16 OBPs, respectively. Although different OBPs display distinct odorant-binding specificity (Maïbèche-Coisné et al. 1998, Prestwich et al. 1995, Zhong et al. 2012), the hypothesis that possession of lower numbers of OBPs corresponds to fewer numbers of chemicals detected by the olfactory system to support and sustain the organism could explain why the monophagous *N. asiatica* possesses only 17 OBPs and feeds and oviposits only on wolfberry. Further research is required for confirmation of this hypothesis.

Generally, OBPs are 135 to 220 amino acids in length (Sánchez-Gracia and Rozas 2008), and most of the NasiOBPs except for NasiOBP10, NasiOBP11, and NasiOBP17 were within this range. Each OBP has a hydrophobic N terminus that could serve as a signal sequence (Vonheijne 1986), which was not found in NasiOBP10, NasiOBP11, and NasiOBP6 likely because of the limit in sequencing. Thus, the sequences of NasiOBP10 and NasiOBP11 are shorter than the actual length, as occurs in the OBPs of *A. fraterculus* and *A. pisum* (Campanini and de Brito 2016, Zhou et al. 2012). Only NasiOBP17 had an incomplete conserved domain, due to the partial sequences in the 3′ terminus, resulting in the absence of the last cystine residue. However, the BLAST identity of NasiOBP17 with Dmel56e was 38.1%, a standard member of classic subfamily.

In *N. asiatica,* four OBPs were assigned to the minus-C subfamily; the remainder were considered classic OBPs. This situation has not been found in other fruit flies where at least one OBP belongs to the plus-C subfamily; however, more OBPs have been identified in the minus-C subfamily than in the plus-C subfamily in *D. melanogaster* and seven other fruit fly species. Regardless of whether the number of OBPs from the minus-C are higher or lower than those from the plus-C subfamily, the variety of the OBPs in the minus-C subfamily is lower than that of the OBPs in the plus-C subfamily; the latter rapidly evolved in each insect species studied (Zhou et al. 2004a). Hekmat-Scafe et al. (2002) deduced that the OBPs in the plus-C subfamily are most recently derived from the *Drosophila* OBP subfamily. Campanini and de Brito (2016) also reported that the plus-C OBPs possess one amino acid located in the binding cavity and may be under selection pressure to recognize and bind to a new odorant. OBPs from *N. asiatica* lack these types of sequences, which are more varied and could interact with new odors, and their OBP sequences are more conserved, corresponding to their singular host and feeding habits.

OBP19a, OBP99b, OBP56h, and OBP19d were expressed in seven fruit flies with NasOBP9, NasOBP8, NasOBP10/NasOBP11 (the same counterpart), and NasOBP13 and likely have important functions. OBP19a was identified as antennae specific in the *Bactrocera* species, and chemosensory genes in *B. dorsalis* have greater expression in the female than in the male antennae and, therefore, could play a role in odorant perception of sex pheromone or oviposition behavior. This OBP can be a potential target to attract female *B. dorsalis* flies (Wu et al. 2015, 2020). OBP56h may modulate mating behavior in *D. melanogaster,* enhancing mating behavior by reducing courtship latency (Shorter et al. 2016). OBP99b and OBP19d transcripts vary among five *Bactrocera* species, with OBP19d

exhibiting greater expression in the mature stages of *A. obliqua* (Nakamura et al. 2016). All of these OBPs are involved in adult fly reproductive processes, which are more conservative and crucial.

We discussed the OBPs of *N. asiatica* based on numbers, subfamilies, and specific types of OBPs. Compared with other feeding habit genes of fruit flies, OBPs in *N. asiatica* are more oligo and conserved. These findings provide clues for further research on the relationship between insect feeding habits and olfactory processes.

Acknowledgments

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