TITLE:

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CITATION:

Ono, Hajime ...[et al]. Functional characterization of olfactory receptors in three Dacini fruit flies (Diptera: Tephritidae) that respond to 1-nonanol analogs as components in the rectal glands. Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology 2020, 239: 110346.

ISSUE DATE:

2020-01

URL:

<http://hdl.handle.net/2433/244592>

RIGHT:

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**Functional characterization of olfactory receptors in three Dacini fruit
flies (Diptera: Tephritidae) that respond to 1-nonanol analogs as
components in the rectal glands**

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25 ABSTRACT

26 Dacini fruit flies (Tephritidae: Diptera), including destructive pest species, are strongly
27 affected in their reproductive behaviors by semiochemicals. Notably, male lures have
28 been developed for pest management e.g., aromatic compounds for the Oriental fruit fly
29 *Bactrocera dorsalis* and the melon fruit fly *Zeugodacus cucurbitae*; terpenic α -ionone
30 analogs for the solanaceous fruit fly, *B. latifrons*. Other than those specific male
31 attractants, 1-nonanol analogs have been noticed as major aliphatic components in the
32 male rectal gland, which is considered as a secretory organ of male sex pheromones.
33 Although multiple semiochemicals associated with the life cycle of Dacini fruit flies have
34 been identified, their behavioral role(s) and chemosensory mechanisms by which the
35 perception occurs have not been fully elucidated. In this study, we conducted RNA
36 sequencing analysis of the chemosensory organs of *B. latifrons* and *Z. cucurbitae* to
37 identify the genes coding for chemosensory receptors. Because the skeletons of male
38 attractants are different among Dacini fruit fly species, we analyzed phylogenetic
39 relationships of candidate olfactory receptors (ORs) among the three species. We found
40 that the OR phylogeny reflects the taxonomic relationships of the three species. We
41 further characterized functional properties of OR74a in the three Dacini species to the 1-
42 nonanol analogs related to components in the rectal glands. The three OR74a homologs
43 responded to 1-nonanol, but their sensitivities differed from each other. The OR74a
44 homologs identified from *B. dorsalis* and *Z. cucurbitae* responded significantly to 6-oxo-
45 1-nonanol, but not to 1,3-nonanediol and nonyl acetate, indicating similar binding
46 properties of the homologous ORs.

47
48 Keywords: Chemosensory receptor; Tephritidae; 1-nonanol analogs; Functional analysis;

49 *Xenopus* oocyte

50

51 **1. Introduction**

52 The tribe Dacini (Tephritidae: Diptera) contains various fruit fly species in which
53 approximately 770 species have been described (Drew and Hancock, 2000). Among them,
54 the genera *Bactrocera* and *Zeugodacus* include many serious pest species that infest
55 cultivated fruits and vegetables in both tropical and subtropical regions. It is noteworthy
56 that some of the species are closely associated with plant-derived semiochemicals in their
57 life cycles (Tan et al., 2014). Males have a glandular complex in the rectum, known as
58 the rectal gland, which is considered to be a secretory organ of male sex pheromones in
59 many dacine tephritid fly species (Fletcher, 1968). Various compounds, including
60 aromatic and aliphatic volatiles, have been identified as components in the rectal glands
61 (Fletcher and Kitching, 1995). For example, males of the Oriental fruit fly, *B. dorsalis*,
62 and the melon fruit fly, *Z. cucurbitae*, are strongly attracted to specific aromatic
63 compounds, methyl eugenol (ME) and raspberry ketone (RK), respectively, and
64 subsequently ingest the attractants to sequester in their rectal glands as sex pheromone
65 precursor or sex pheromone itself (Nishida et al., 1988; Shelly, 2010). On the other hand,
66 males of the solanaceous fruit fly, *B. latifrons*, are attracted to non-aromatic terpenoid α -
67 ionone derivatives and sequester them in their rectal glands (Ishida et al., 2008; Nishida
68 et al., 2009; Enomoto et al., 2010). Other than those characteristic male attractants, a
69 series of 1-nonanol analogs have also been identified as specific rectal gland components
70 in several Dacini fruit flies. For example, 6-oxo-1-nonanol is produced in the male rectal
71 glands in *B. carambolae*, a species closely related to *B. dorsalis*, together with a minor

component, 1,6-nonanediol (Wee and Tan, 2005; Wee et al., 2007). The production of this compound increases concomitant with sexual maturity and triggers a chemotactic behavior known as zigzag flight in conspecific females. In *Z. cucurbitae*, 1,3-nonanediol is produced as one of the major components in the male rectal glands at a similar level to accumulated RK ingested from floral components (Nishida et al., 1993). Although a defensive role of 1,3-nonanediol against a natural enemy has been demonstrated, its pheromonal role is so far unknown (Tan, 2000). While 1-nonanol analogs are components in the male rectal glands, these compounds have also been identified in host fruits and a vegetable (Light and Jang, 1987; Siderhurst and Jang, 2010). Notably, 1-nonanol elicits electroantennogram detection (EAD) responses in *B. dorsalis* (Light and Jang, 1987). Furthermore, 1-nonanol and its analogs not only elicit EAD responses but also attract females of *Z. cucurbitae* (Siderhurst and Jang, 2010). Therefore, roles of 1-nonanol analogs are interesting from the view of pheromonal communication as well as host recognition.

Although the perception of such glandular semiochemicals seems essential in their life cycles, the constituents required for chemo-recognition have not been well characterized. In insects, three types of chemosensory receptors, olfactory receptors (ORs), gustatory receptors (GRs), and ionotropic receptors (IRs), are necessary to detect various odors and taste substances at the peripheral neurons in chemosensory organs (Fleischer et al., 2017). Recently, draft genome sequences of several Dacini fruit flies including *B. dorsalis*, *B. latifrons*, and *Z. cucurbitae* have been deciphered and reference sequences of them are now available at the NCBI web sites (*B. dorsalis*: <https://www.ncbi.nlm.nih.gov/genome/10754>; *B. latifrons*: <https://www.ncbi.nlm.nih.gov/genome/43857>; *Z. cucurbitae* :

<https://www.ncbi.nlm.nih.gov/genome/11807>). The genetic information enables us to identify sequences coding candidate chemosensory receptors. Most recently, several ORs of *B. dorsalis* that respond to semiochemicals have been characterized, i.e., BdorOR13a and BdorOR82a for plant volatiles (Miyazaki et al., 2018) and BdorOR88a for ME (Liu et al., 2018). However, there is no functional information about chemosensory receptors in tephritid fruit flies except for the abovementioned studies, despite the importance of roles of chemoreception in insect physiology as well as pest management.

In the present study, we identified an entire repertoire of insect chemosensory receptors expressed in chemosensory organs of the fruit fly species in two genera, *B. latifrons* and *Z. cucurbitae* by RNA sequencing (RNA-seq) analyses. Because the skeletons of specific male attractants are different from each other, i.e., α -ionone analogs for *B. latifrons* and aromatic compounds for *B. dorsalis* and *Z. cucurbitae*, we compared phylogenetic relationships of ORs among the three species. Furthermore, we focused on OR74a homologs among the identified chemosensory receptors, because OR74a of *Drosophila melanogaster* responds to 1-nonanol (Kreher et al., 2005). We analyzed functional properties of OR74a homologs in the three Dacini species to see if these ORs respond to analogous components of 1-nonanol found in the rectal glands of Dacini species.

2. Materials and methods

2.1. Insects

For preparation of total RNA, we used strains of *B. latifrons* and *Z. cucurbitae* cultured in Okinawa Prefectural Agricultural Research Center and Okinawa Prefectural Plant Protection Center in Japan, respectively. The strain of *B. latifrons* originally

collected in Yonaguni Island was kept at 26–27 °C under a photoperiod of 14 h light/10 h dark. The strain of *Z. cucurbitae* originating from Taiwan was kept at 25 °C and 60 to 70% relative humidity under a photoperiod of 14 h light/10 h dark, with a dawn and dusk twilight. The adult flies of both species were provided with water and a diet of four parts sucrose and one part dry yeast AY-65 (Asahi Food & HealthCare, Ltd., Tokyo, Japan).

2.2. RNA sequencing and assembly

Adult flies of *B. latifrons* and *Z. cucurbitae* were staged at 0–1, 1–3, 4–6, and 7–9 days and 0–2, 2–4, and 6–8 days, respectively. Approximately two hundred males and females were equally collected from each adult stage. Total RNAs were extracted from male and female antennae and proboscises using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD, USA), and purified by NucleoSpin RNA (Macherey-Nagel, Germany). Sequence libraries were constructed using the TruSeq RNA sample Preparation Kit v2 (Illumina Inc., San Diego, CA, USA). RNA sequencing was performed on an Illumina MiSeq system using the Miseq Reagent Kit v3 600 cycle (Illumina Inc., San Diego, CA, USA). The reads were preprocessed with Trimmomatic v0.33 (Bolger et al., 2014) for quality trimming using the parameters as described previously (Miyazaki et al., 2018). The resulting data from clean reads were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession numbers PRJDB7958. The pass-through reads were subjected to *de novo* assembly using the Trinity, Bowtie, eXpress, and DEGseq (PE) programs implemented in the maser pipeline of the Cell Innovation Program at the National Institute of Genetics (https://cell-innovation.nig.ac.jp/maser/index_en.html). Fragments per kilobase of exon per million (FPKM) values were calculated to estimate the expression levels of the transcripts. Summary of sequence data was analyzed using

the Seqkit program (<https://bioinf.shenwei.me/seqkit>).

2.3. Screening and characterization of sequences of candidate chemosensory receptors

We identified candidate chemosensory receptor genes from Trinity contigs using Pfam domains and amino acid sequences of chemosensory receptors in *D. melanogaster*. We obtained the following Pfam domains of *D. melanogaster* from the Pfam database (<https://pfam.xfam.org/>): 7tm odorant receptor (PF02949), 7tm chemosensory receptor (PF08395), trehalose receptor (PF06151), and ligand gated ion channel (PF00060). We also obtained the following amino acid sequence data of chemosensory receptors in *D. melanogaster* from the InterPro database (<https://www.ebi.ac.uk/interpro/>): Olfactory receptor (IPR004117), 7TM chemoreceptor (IPR013604), and ionotropic glutamate receptor (IPR001320). We screened the Trinity contigs by similarity to these amino acid sequences using a BLASTX search at an Expect value (E-value) threshold of $1e^{-5}$. We listed the names of contigs hit by the BLASTX search in a text file and built a FASTA file exemplified in File S1 using the Biostrings package within R software. We obtained open reading frames (ORFs) of the extracted contigs using EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) and used them as queries in a BLASTP search against the NCBI non-redundant protein database. Contigs that ranked highly with ORs, GRs, or IRs were considered candidate genes coding for insect chemosensory receptors. Overlapping variants with identical ORFs were merged at this step by selecting the longest as the representative transcript of a variant group. We also performed RT-PCR to merge the partial sequences of *BlatOR13a*, *BlatOR74a*, *ZcucOR35a*, and *ZcucOR59a* using a pair of primers (Table S1). We named the candidate chemosensory receptors according to gene names of the top blast hits in most cases. In some exceptions, we named

ORs by their homologs in *D. melanogaster* or *B. dorsalis* to match phylogenetic relationships. For homologous chemosensory receptors with amino acid similarities of less than 80%, the names of the homologs were differentiated with a numerical postscript, e.g., BlatOR7a-1 and BlatOR7a-2. In cases where the amino acid similarities were 80% or more, version numbers were assigned to the receptors, e.g., BlatOR49b-v1 and BlatOR49b-v2. In cases where multiple partial sequences of a candidate chemosensory receptor were identified, each sequence was labeled -part1, -part2, etc., e.g., ZcucOR13a-part1 and ZcucOR13a-part2.

2.4. Sequence analysis

Deduced amino acid sequences of candidate ORs were aligned using the Clustal W 2.1. program (Thompson et al., 1994). Prior to this process, we merged the partial sequences of ORs in which multiple sequences were partially identified. We selected candidate ORs with sequences of more than 200 amino acid for phylogenetic analysis and constructed a phylogenetic tree from the aligned sequences. We applied the maximum likelihood method with the Jones–Taylor–Thornton (JTT) model, with among-site rate heterogeneity according to gamma distribution with invariant sites (G + I) using MEGA5 software (Tamura et al., 2011). Putative transmembrane domains were predicted using the PHDhtm algorithm (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_htm.html) (Rost et al., 1995).

2.5. Expression analyses of the candidate receptors by quantitative RT-PCR (qPCR)

Total RNAs were prepared from male and female antennae of the staged adults within 2 days after eclosion. Reverse transcription was performed using the ReverTra Ace qPCR

RT Master Mix (TOYOBO, Tsuruga, Japan). The generated cDNAs were used as a template for qPCR using the THUNDERBIRD SYBR qPCR Mix (TOYOBO, Tsuruga, Japan) on a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan). We investigated four or five independent biological samples to quantify the levels of transcription. The transcription levels were normalized with *rpS3* or *rpL23* transcription levels in the same samples of *B. latifrons* or *Z. cucurbitae*, respectively. The primers used for qPCR are listed in Table S1.

2.6. Expression of ORs in *Xenopus* oocytes and two-electrode voltage-clamp recording

Full-length coding sequences of candidate ORs were cloned into pCS2P+ vectors (<https://www.addgene.org/17095/>) for heterologous expression. Full- or partial-ORFs of *ORCO* and *OR74a* homologs of the three Dacini fruit flies were PCR amplified from cDNA prepared from male antennae using primers designed from predicted ORFs based on the assembled contigs. The PCR products were cloned into pCS2+ using restriction enzymes—BamHI and XbaI—or an In-Fusion HD cloning Kit (Takara, Otsu, Japan). The primers used for construction are listed in Table S1. Complementary RNAs (cRNAs) were synthesized from linearized pCS2 vectors by mMESSAGE mMACHINE (Thermo Fisher Scientific, Waltham, MA, USA). Preparation of *Xenopus laevis* oocytes, microinjection of receptor gene RNA, and recording of whole-cell currents were performed as described previously (Miyazaki et al., 2018). In brief, Stage V to VII *Xenopus* oocytes treated with collagenase in Ca²⁺-free saline solution were microinjected with a mixture comprising OR and ORCO cRNAs (25 ng each). Using a two-electrode voltage clamp (OC-725, Warner, Hamden, CT, USA), we recorded whole cell currents from injected oocytes after incubation for 4–5 days at 20 °C in an assay buffer. The inward current was monitored at

a holding potential of -80 mV. Each ligand was diluted with the assay buffer to a specific concentration containing 0.1% dimethyl sulfoxide (DMSO). The assay buffer containing 0.1% DMSO was used as a negative control. Data acquisition and analyses were carried out using Digidata 1322A and pCLAMP software (Axon Instruments, Foster City, CA, USA).

2.7. Chemicals

Chemicals used for functional analyses are listed in Table S2 and their structures are shown in Fig. 4A and S1. Stock solution of chemicals were prepared in DMSO and stored at -20 °C.

3. Results

3.1. RNA sequencing and identification of chemosensory receptors

Using the Illumina MiSeq system, we obtained 279,730 and 89,818 assembled contigs from the transcriptomes of the male and female antennae and proboscises of *B. latifrons* and *Z. cucurbitae*, respectively (File S2 for *B. latifrons*; File S3 for *Z. cucurbitae*). The summary of the assembly is shown in Table 1 and 2. We identified chemosensory receptors—namely, ORs, GRs, and IRs—by a BLASTX search of the contigs against Pfam domains and amino acid sequences of chemosensory receptors in *D. melanogaster* (Table 3 and File S4 for *B. latifrons*; Table 4 and File S5 for *Z. cucurbitae*).

A homology search based on the Pfam domains of the 7tm odorant receptor and the amino acid sequences of the *Drosophila* ORs revealed approximately 50 candidate ORs both in *B. latifrons* and *Z. cucurbitae*. Among them, we merged the partial sequences

of *ZcucOR13a* and *ZcucOR59a* by RT-PCR. In total, full-length coding sequence of 30 and 27 ORs were determined in *B. latifrons* and *Z. cucurbitae*, respectively. To assign names to the ORs, we used these sequences as queries in a BLASTP search against the NCBI non-redundant protein database. Top blast hits were mostly obtained from predicted sequences by automated computational analysis based on reference genome sequences. Others were obtained from databases of several Tephritid fruit flies, *Bactrocera* and *Rhagoletis* species. We named the candidate ORs according to gene names of the top blast hits with some exceptions, i.e., *BlatOR7a-8*, *ZcucOR7a-5*, *ZcucOR7a-7*, *ZcucOR7a-8*, *ZcucOR7a-9*, *ZcucOR35a*, *ZcucOR43a-2*, and *ZcucOR43a-3* were named according to their homologs in *D. melanogaster* or *B. dorsalis*.

We identified several homologs of sugar receptors such as the *GR5a* and *GR64* subfamilies (Freeman and Dahanukar, 2015) by a homology search based on Pfam domains of *Drosophila* trehalose receptors (PF06151). Using Pfam domains of *Drosophila* 7tm chemosensory receptors (PF08395), we also identified 25 and 12 GRs, including homologs of carbon dioxide receptors such as the *GR21a* and *GR63a* subfamilies (Jones et al., 2007; Kwon et al., 2007), from *B. latifrons* and *Z. cucurbitae*, respectively. A single gene of *GR21a* has been identified in *D. melanogaster*, and here, we identified multiple homologs of *GR21a* from *B. latifrons* and *Z. cucurbitae*, as multiple homologs were also found in *B. dorsalis* (Miyazaki et al., 2018). Among GRs, except for *GR21a* and *GR63a* genes coding carbon dioxide receptors, a full-length coding sequence was identified only in *BlatGR64b* from the transcriptomes, likely due to relatively low transcriptional levels of GR genes in the chemosensory organs.

We identified ligand gated ion channels by homology search based on Pfam domains of the *Drosophila* ligand gated ion channel (PF00060) and ligated ion channel

L-glutamate- and glycine-binding site (PF10613). Among them, 19 and 17 candidate IRs were identified by a BLASTP search based on translated protein sequences from *B. latifrons* and *Z. cucurbitae*, respectively. We determined full-length coding sequences of several IRs including *IR8a* and *IR25a* subfamilies which have been characterized as co-receptors (Benton et al., 2009; Rytz et al., 2013). On the other hand, multiple partial fragments were identified in several IRs owing to their large coding region. We found that high FPKM values were sex-specifically shown in several transcripts of both *B. latifrons* and *Z. cucurbitae* (Table S3 and S4). We used qPCR to quantitatively compare the transcription levels of these genes—*BlatIR21a*, *BlatIR40a*, *BlatIR64a-2*, *BlatIR92a-1*, *ZcucIR40a-1*, *ZcucIR40a-2* and *ZcucIR64a*, and *ZcucIR92a*—in male and female antennae. We found that all the IRs tested expressed in both sexes, whereas a significant difference in the transcriptional level of *ZcucIR40a* was observed between the male and female antennae (Fig. 1).

3.2. Phylogenetic relationship of ORs among three *Dacini* fruit flies

We have identified ORs expressed in chemosensory organs of three *Dacini* fruit flies in this and previous studies (Miyazaki et al., 2018). To see a relationship between them, we constructed a phylogenetic tree of ORs with sequences of more than 200 amino acids in the three *Dacini* fruit flies (Fig. 2). The tree reveals remarkable divergence of several ORs with multiple homologous genes such as OR7a, OR63a, and OR67d subfamilies in the three related species. Although common OR subfamilies of the three species mostly converged on the same lineages, ORs of *Z. cucurbitae* were slightly apart from those of *B. dorsalis* and *B. latifrons* in most cases. Because *B. dorsalis* and *B. latifrons* belong to the same genus but *Z. cucurbitae* is taxonomically apart from them

(Krosch et al., 2012), the phylogenetic relationship of OR families corresponds to the taxonomic relationship.

3.3. Transcriptional profiles of *OR74a* homologs

Among the identified candidate ORs, we focused on *OR74a* homologs, because *Drosophila* *OR74a* is a receptor for 1-nonanol (Kreher et al., 2005) which is an analog of rectal gland components in several Dacini fruit flies. Since only male flies possess rectal glands, chemical factors in the tissue likely function as sexual cues. Therefore, we compared transcription levels of the *OR74a* homologs between male and female chemosensory organs by qPCR to see if sexually biased expression could be observed in these receptors. However, there were no significant differences in the transcription levels of *BlatOR74a* and *ZcucOR74a* between male and female antennae (Fig. 1). We also compared the deduced amino acid sequences of the three *OR74a* receptors. A 96 % similarity was noted between the amino acid identities of *BdorOR74a* and *BlatOR74a*. *ZcucOR74a* revealed an 89 % and 87 % similarity between amino acid identities with *BdorOR74a* and *BlatOR74a*, respectively (Fig. 3).

3.4. Functional characterization of *OR74a* homologs by two-electrode voltage-clamp recording

We co-expressed each of the *OR74a* homolog proteins with the co-receptor ORCO in *Xenopus* oocytes to analyze responses to the candidate 1-nonanol analog ligands. We tested four analogs including rectal gland components as shown in Fig. 4A. We found that the three *OR74a* homologs robustly responded to 1-nonanol at a concentration of 100 μ M (Fig. 4B). While *BdorOR74a* revealed an average current of

more than 0.2 μ A at this concentration, BlatOR74a and ZcucOR74a exhibited smaller currents (Table 5). The oocyte expressing BdorOR74a with BdorORCO also responded to 6-oxo-1-nonanol, which is found in the related species *B. carambolae* (Wee and Tan, 2005). The current value induced by 6-oxo-1-nonanol was significantly higher than that by the control (DMSO) (Fig. 4C). We occasionally observed weak responses of the BdorOR74a to nonyl acetate which showed an attractive activity to *B. dorsalis* females as a volatile contained in a host fruit (Siderhurst and Jang, 2006), but there were no significant differences between the current values induced by this compound and those by the control. We also observed a significant response by 6-oxo-1-nonanol, but not by 1,3-nonanediol, known as a rectal component of *Z. cucurbitae* (Nishida et al., 1993), in the oocytes expressing ZcucOR74a with ZcucORCO. The attractants and sex pheromones of the Dacini fruit flies did not elicit any response of BlatOR74a and ZcucOR74a (Fig. S2). While 1-nonanol evoked the responses of BdorOR74a and ZcucOR74a in a dose-dependent manner, the response of BlatOR74a failed to reach a plateau up to 1 mM, probably due to a low responsiveness (Fig. 4D, E).

Discussion

The specific and different attractiveness to plant-derived semiochemicals among Dacini fruit flies have been well characterized (Tan et al., 2014), but it is unclear how the related species have acquired a chemosensory system to respond to specific volatiles to gain sex-pheromone sources. Based on the different affinity to the attractants among the three Dacini fruit flies, i.e., aromatic compounds for *B. dorsalis* and *Z. cucurbitae* and α -ionone analogs for *B. latifrons*, we speculated that *B. dorsalis* and *Z. cucurbitae* share more similar chemosensory receptors in the chemo-recognition system at peripheral neurons

than those in *B. latifrons*, although *B. dorsalis* and *B. latifrons* are closely related species divergent from *Z. cucurbitae* (Krosch et al., 2012). However, the phylogenetic analysis of ORs shows that *B. dorsalis* and *B. latifrons* share more similar homologous genes than *Z. cucurbitae*. With regards to the ME receptor OR88a (Liu et al., 2018), the amino acid sequence of BdorOR88a is more similar to that of BlatOR88a (92% similarity) than that of ZcucOR88a (69% similarity). It is intriguing whether BlatOR88a and ZcucuOR88a respond to their attractants. If so, it is also interesting how the different responsiveness to the attractants occurs among the similar receptors. We should note that a few substitutions of critical amino acids possibly change a ligand-binding property. We also found divergent homologs and variants in several ORs including OR7a and OR67d subfamilies in the three species, suggesting that these repertoires are necessary for detection of semiochemicals which are commonly recognized by Dacini fruit flies.

We identified candidate GRs and IRs of *B. dorsalis* and *Z. cucurbitae*, although their full-length coding sequences could not be determined in most cases due to low transcriptional levels and/or long coding regions. With regards to GRs, we found 25 and 12 uncharacterized candidate GRs, except for sugar and carbon dioxide receptors, in *B. latifrons* and *Z. cucurbitae*, respectively. Because the attractants also show phagostimulant activities, it is intriguing whether GR is involved in the recognition of the attractants in gustatory organs. We also found the 19 and 17 candidate IRs from *B. latifrons* and *Z. cucurbitae*, respectively. Because IRs function as both olfactory and gustatory receptors, IRs are possibly associated with detection of the attractants in olfactory and gustatory organs. We noticed that FPKM values of several transcripts coding IRs were sex-specifically observed. However, all the IRs analyzed by qPCR were expressed in both sexes, although the transcriptional level of *ZcucIR40a-2* was

significantly higher in male antennae than that in female antennae. Because we observed indistinguishable transcriptional levels between male and female in ORs of *B. dorsalis* in a previous study (Miyazaki et al., 2018), sexually dimorphic behavior such as the male specific responsiveness to attractants is probably triggered by information processing in the central nervous system, based on inputs from peripheral neurons via chemosensory receptors, regardless of receptor type. Further, because IRs are thought to form heteromeric ion channels in which a co-receptor, namely IR8a or IR25a, partners one or multiple ligand-specific IRs (Abuin et al., 2011; Rytz et al., 2013), knockout of the co-receptor(s) using CRISPR/Cas9 editing will give us a clue to clarify the molecular system underlying chemo-recognition.

Because 1-nonanol analogs have been identified as the major components, other than aromatic compounds, in the male rectal glands of several Dacini fruit flies, we characterized OR74a homologs of the three fruit flies. The three OR74a homologs co-expressed with their cognate ORCO robustly responded to 1-nonanol, as reported in DmOR74a, suggesting a conservation of olfactory system between Dacini and *Drosophila* at the molecular level. Among the Dacini OR74a homologs, the sensitivities to 1-nonanol differed from each other. Despite the very high similarities of amino acid sequences between BdorOR74a and BlatOR74a, BdorOR74a exhibited the highest sensitivity to 1-nonanol, but BlatOR74a showed the weakest responsiveness, as the average current value of BlatOR74a was about one-fifth of that of BdorOR74a (Table 5). It is possible that an efficiency of expression in the heterologous expression system using *Xenopus* oocyte is different among the three OR74a homologs owing to their amino acid or nucleotide sequences. Because 1-nonanol is a repellent to larvae of *D. melanogaster* (Cobb, 1992), OR74a homologs are possibly necessary to recognize unfavorable odorants

in Dacini fruit flies. We also found that BdorOR74a significantly responded to 6-oxo-1-nonanol which is contained in male rectal glands in the closely related species, *B. carambolae*, but this was not the case in *B. dorsalis*. It is interesting to note that reproductive interference, i.e., antagonistic sexual interaction, among these species has been reported (Kitano et al., 2018). Considering this interspecific sexual interaction, BdorOR74a may detect 6-oxo-1-nonanol to avoid the competitive species, thereby reproductive success could be elevated. To examine this possibility, it is necessary to observe a behavior of *B. dorsalis* to 6-oxo-1-nonanol, and to characterize an OR74a homolog of *B. carambolae*. Besides the predicted repellent roles in dipteran species, 1-nonanol analogs may play as cues for seeking host fruits and vegetables. While roles of 1-nonanol analogs have not been well elucidated, characterization of OR74a homologs of the Dacini fruit flies gives us clues to find the roles of these compounds.

In the present study, we characterized functional properties of ORs that respond to rectal gland components following the previous identification of ORs responding to plant volatiles. Although semiochemicals play critical roles in the life cycles of Tephritid fruit flies, only a few studies have attempted functional characterization of ORs. Furthermore, according to our knowledge, there is no study that identifies a ligand for other chemosensory receptors, namely GRs and IRs. Comprehensive elucidation of properties of chemosensory receptors, including those for attractants and pheromones, will provide insights into the mechanisms underlying the chemosensory abilities that Dacini fruit flies have acquired to favorably utilize semiochemicals in their life cycles. It will also give us clues to develop effective attractants for pest control of these destructive pest fruit flies.

Funding

This work was supported by JSPS KAKENHI [grant number 26450466]; and Matsushima Horticultural Development Foundation.

Acknowledgements

We thank Rie Ukuda-Hosokawa, Tsunaki Andou and Dai Haraguchi of Okinawa Prefectural Agricultural Research Center for providing *B. latifrons*. We thank Aiko Ota and Rei Kakazu of Okinawa Prefectural Plant Protection Center for providing *Z. cucurbitae*. We also thank Yurie Hirosaki for technical assistance.

Declarations of interest

none

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Figure captions

Fig. 1. Transcriptional levels of candidate ionotropic receptors (IRs) and olfactory receptors (ORs) identified in *Bactrocera latifrons* and *Zeugodacus cucurbitae*. MA: male antennae; FA: female antennae. Each value is plotted as a dot ($n = 4-5$). The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Student's t -test: $*p < 0.05$.

Fig. 2. Phylogenetic tree of candidate olfactory receptors (ORs) identified in the three Dacini fruit flies, *Bactrocera dorsalis*, *B. latifrons* and *Zeugodacus cucurbitae*. Branch length is proportional to genetic distance estimated by the maximum likelihood method.

Fig. 3. Comparison of deduced amino acid sequences of olfactory receptors (ORs). Alignments of BdorOR74a, BlatOR74a and ZcucOR74a are shown. Predicted seven transmembrane domains (TM1–TM7) are indicated by solid lines.

Fig. 4. Responses of *Xenopus* oocytes expressing OR74a with its cognate ORCO in the three Dacini fruit flies, *Bactrocera dorsalis*, *B. latifrons* and *Zeugodacus cucurbitae* to 1-nonanol analogs. (A) Structures of 1-nonanol analogs tested as candidate ligands. (B) Current traces of an oocyte upon successive exposures to 1-nonanol analogs including DMSO (the control). Each chemical at 100 μ M was applied at the time, indicated by the arrowhead. (C) Current values measured in oocytes responding to 1-nonanol analogs at 100 μ M. Each value is plotted as a dot ($n = 9$ for BdorOR74a; $n = 6$ for ZcucOR74a). The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Significant differences between current values responding to a ligand and those

responding to DMSO (control) were shown by Student's *t*-test: **p < 0.001, ***p < 0.0001.

(D) Responses of each oocyte to 1-nonanol at various concentrations. (E) Dose-response curves of oocytes responding to 1-nonanol. Each point represents the mean current value. Error bars indicate SE (*n* = 6 for BdorOR74a; *n* = 4 for BlatOR74a; *n* = 7–8 for ZcucOR74a)

Supplementary materials

Table S1. The primers used for qPCR experiments.

Table S2. Chemicals used for functional analysis of ORs.

Table S3. Transcripts coding for candidate chemosensory receptors.

Table S4. Transcripts coding for candidate chemosensory receptors.

Fig. S1. Chemical structures of tested compounds for the functional analysis of candidate olfactory receptors.

Fig. S2. Responses of *Xenopus* oocytes expressing BlatOR74a or ZcucOR74a with its cognate ORCO to attractants, sex pheromones and/or their related compounds. Only 1-nonanol elicited the responses of BlatOR74a and ZcucOR74a as shown in Fig. 3. The structure of each compound and its corresponding abbreviation are shown in Fig. S1.

573   **File S1.** An example of a command line to build a fasta file of contigs hit by the BLASTX
574   search using Biostrings package within R software.
575
576   **File S2.** A Trinity file of *Bactrocera latifrons*.
577
578   **File S3.** A Trinity file of *Zeugodacus cucurbitae*.
579
580   **File S4.** A fasta file of chemosensory receptors of *Bactrocera latifrons*.
581
582   **File S5.** A fasta file of chemosensory receptors of *Zeugodacus cucurbitae*.

Table 1. Summary of sequence data analysis in *Bactrocera latifrons*.

	Male		Female	
	Antenna	Proboscis	Antenna	Proboscis
Raw reads	1,991,913	2,972,051	1,934,849	2,692,567
Clean reads	1,390,771	2,004,475	910,187	1,794,188
Assembled contigs	279,730			
Mean length of contigs (bp)	367			

Table 2. Summary of sequence data analysis in *Zeugodacus cucurbitae*.

	Male		Female	
	Antenna	Proboscis	Antenna	Proboscis
Raw reads	869,802	467,912	1,574,403	2,022,842
Clean reads	607,077	322,850	1,101,598	1,393,230
Assembled contigs		89,818		
Mean length of contigs (bp)		474		

Table 3. Candidate chemosensory receptors of *Bactrocera latifrons* identified from transcriptome.

Gene name	Length (AA)	CDS	E-value	BLASTP best hit (Accession number; Name; Species)
ORs				
<i>BlatORCO</i>	473	Full	0	ADK97803.1; Or83b (ORCO) <i>Zeugodacus cucurbitae</i>
<i>BlatOR2a-v1</i>	393	Full	0	XP_018802940.1; OR2a-like; <i>Bactrocera latifrons</i>
<i>BlatOR2a-v2</i>	379	Full	0	XP_018802940.1; OR2a-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-1</i>	396	Full	0	XP_018783745.1; OR7a-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-2</i>	221	Partial	4e-154	XP_018804184.1; OR42a-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-3</i>	396	Full	0	XP_018798593.1; OR43b-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-4</i>	394	Full	0	XP_018798558.1; OR7a-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-5</i>	400	Full	0	XP_018798592.1; OR43b-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-6</i>	399	Full	2e-171	XP_011178079.1; OR7a-like; <i>Zeugodacus cucurbitae</i>
<i>BlatOR7a-7</i>	392	Full	0	XP_019846037.1; OR7a-like; <i>Bactrocera dorsalis</i>
<i>BlatOR7a-8-v1</i>	430	Full	0	XP_018798519.1; OR59b-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-8-v2</i>	460	Partial	0	XP_018798519.1; OR59b-like; <i>Bactrocera latifrons</i>
<i>BlatOR7a-8-v3</i>	139	Partial	7e-90	XP_018798519.1; OR59b-like; <i>Bactrocera latifrons</i>
<i>BlatOR10a</i>	400	Full	0	XP_018791769.1; OR10a; <i>Bactrocera latifrons</i>
<i>BlatOR13a</i>	441	Full	0	XP_018784751.1; OR13a; <i>Bactrocera latifrons</i>
<i>BlatOR30a</i>	389	Partial	0	XP_019847671.1; OR30a-like; <i>Bactrocera dorsalis</i>
<i>BlatOR33b</i>	70	Partial	9e-28	XP_017488833.1; OR33b-like; <i>Rhagoletis zephyria</i>
<i>BlatOR35a</i>	320	Partial	0	XP_018789506.1; OR35a-like; <i>Bactrocera latifrons</i>
<i>BlatOR42a</i>	139	Partial	3e-93	XP_018804184.1; OR42a-like; <i>Bactrocera latifrons</i>

<i>BlatOR42b</i>	394	Full	0	XP_018786776.1; OR42b-like; <i>Bactrocera latifrons</i>
<i>BlatOR43a-1-v1</i>	378	Full	0	XP_018793811.1; OR43a; <i>Bactrocera latifrons</i>
<i>BlatOR43a-1-v2</i>	304	Full	1e-173	XP_018793811.1; OR43a; <i>Bactrocera latifrons</i>
<i>BlatOR43a-2</i>	381	Full	0	XP_018798370.1; Or2-like; <i>Bactrocera latifrons</i>
<i>BlatOR43a-3</i>	284	Partial	2e-117	XP_019847596.1; OR30a-like; <i>Bactrocera dorsalis</i>
<i>BlatOR43a-4</i>	209	Partial	7e-145	XP_018798369.1; Or43-like; <i>Bactrocera latifrons</i>
<i>BlatOR45a</i>	402	Partial	0	XP_018794773.1; OR45a-like; <i>Bactrocera latifrons</i>
<i>BlatOR47b</i>	329	Partial	0	XP_018795844.1; OR47b; <i>Bactrocera latifrons</i>
<i>BlatOR49a</i>	291	Partial	0	XP_011212431.1; OR49a-like; <i>Bactrocera dorsalis</i>
<i>BlatOR49b-v1</i>	386	Partial	0	XP_018796898.1; OR49b; <i>Bactrocera latifrons</i>
<i>BlatOR49b-v2</i>	358	Partial	0	XP_018796898.1; OR49b; <i>Bactrocera latifrons</i>
<i>BlatOR59a</i>	388	Full	0	XP_018805034.1; OR59a-like; <i>Bactrocera latifrons</i>
<i>BlatOR63a-1-v1</i>	415	Full	0	XP_018792788.1; OR63a-like; <i>Bactrocera latifrons</i>
<i>BlatOR63a-1-v2</i>	348	Full	0	XP_018783180.1; OR63a; <i>Bactrocera latifrons</i>
<i>BlatOR63a-2</i>	417	Full	0	XP_018792788.1; OR63a-like; <i>Bactrocera latifrons</i>
<i>BlatOR63a-3</i>	414	Full	0	XP_018783180.1; OR63a; <i>Bactrocera latifrons</i>
<i>BlatOR67c-v1</i>	404	Full	0	AKI29044.1; OR67c; <i>Bactrocera dorsalis</i>
<i>BlatOR67c-v2</i>	373	Partial	0	XP_018795012.1; OR67c-like; <i>Bactrocera latifrons</i>
<i>BlatOR67d-1</i>	388	Full	0	XP_018803798.1; OR67d-like; <i>Bactrocera latifrons</i>
<i>BlatOR67d-2</i>	401	Partial	1e-176	XP_017473047.1; OR67d-like; <i>Rhagoletis zephyria</i>
<i>BlatOR67d-3-v1</i>	387	Full	0	XP_017473047.1; OR67d-like; <i>Rhagoletis zephyria</i>
<i>BlatOR67d-3-v2</i>	71	Partial	2e-31	XP_017483930.1; OR67d-like; <i>Bactrocera zephyria</i>
<i>BlatOR67d-4</i>	388	Full	0	XP_018803784.1; OR67d-like; <i>Bactrocera latifrons</i>

<i>BlatOR69a-1</i>	414	Full	0	XP_018788172.1; OR69a; <i>Bactrocera latifrons</i>
<i>BlatOR69a-2</i>	423	Full	8e-179	XP_019847605.1; OR69a; <i>Bactrocera dorsalis</i>
<i>BlatOR74a-1</i>	414	Full	0	XP_011201924.2; OR74a-like; <i>Bactrocera dorsalis</i>
<i>BlatOR74a-2</i>	181	Partial	2e-125	XP_018796426.1; OR74a; <i>Bactrocera latifrons</i>
<i>BlatOR82a</i>	401	Full	0	XP_018783712.1; OR82a; <i>Bactrocera latifrons</i>
<i>BlatOR85d</i>	203	Partial	1e-138	XP_018801739.1; OR85d; <i>Bactrocera latifrons</i>
<i>BlatOR88a</i>	411	Full	0	XP_018790415.1; OR88a; <i>Bactrocera latifrons</i>
<i>BlatOR92a</i>	384	Full	0	XP_011208819.1; OR92a; <i>Bactrocera dorsalis</i>
<i>BlatOR94b</i>	379	Partial	0	XP_018801531.1; OR94b-like; <i>Bactrocera latifrons</i>
GRs				
<i>BlatGR5a-1</i>	61; 189	Partial	2e-35; 3e-128	XP_018783851.1; GR5a; <i>Bactrocera latifrons</i>
<i>BlatGR5a-2</i>	193	Partial	2e-135	XP_018783883.1; GR5a; <i>Bactrocera latifrons</i>
<i>BlatGR5a-3</i>	82	Partial	6e-51	XP_011213356.2; GR5a; <i>Bactrocera dorsalis</i>
<i>BlatGR8a-1</i>	83; 126	Partial	1e-48; 7e-79	XP_018792278.1; GR8a; <i>Bactrocera latifrons</i>
<i>BlatGR8a-2</i>	76	Partial	1e-42	XP_011185249.1; GR8a-like; <i>Zeugodacus cucurbitae</i>
<i>BlatGR21a-1</i>	456	Full	0	XP_018802085.1; GR21a-like; <i>Bactrocera latifrons</i>
<i>BlatGR21a-2-v1</i>	428	Partial	0	AOE48126.1; GR6; <i>Scaeva pyrastris</i>
<i>BlatGR21a-2-v2</i>	425	Partial	0	AOE48126.1; GR6; <i>Scaeva pyrastris</i>
<i>BlatGR21a-2-v3</i>	422	Partial	0	AOE48126.1; GR6; <i>Scaeva pyrastris</i>
<i>BlatGR28b-1</i>	452	Partial	0	XP_018796745.1; putative GR28b; <i>Bactrocera latifrons</i>
<i>BlatGR28b-2</i>	420	Partial	5e-166	XP_018796745.1; putative GR28b; <i>Bactrocera latifrons</i>
<i>BlatGR28b-3</i>	99	Partial	1e-61	XP_011180327.1; putative GR28b; <i>Zeugodacus cucurbitae</i>
<i>BlatGR32a-1</i>	267	Partial	3e-96	XP_011183921.1; uncharacterized protein LOC105213073; <i>Zeugodacus cucurbitae</i>

<i>BlatGR32a-2</i>	112	Partial	2e-40	XP_018792133.1; uncharacterized protein LOC108970891; <i>Bactrocera latifrons</i>
<i>BlatGR32a-3</i>	75	Partial	1e-44	XP_018787660.1; GR32a; <i>Bactrocera latifrons</i>
<i>BlatGR33a</i>	84	Partial	4e-52	XP_018794131.1; GR33a; <i>Bactrocera latifrons</i>
<i>BlatGR39b</i>	76	Partial	5e-44	XP_018794457.1; putative GR39b; <i>Bactrocera latifrons</i>
<i>BlatGR43a-1</i>	101; 80	Partial	2e-62; 6e-48	XP_018786267.1; GR43a-like; <i>Bactrocera latifrons</i>
<i>BlatGR43a-2</i>	126	Partial	1e-32	XP_020712594.1; GR43a-like; <i>Ceratitis capitata</i>
<i>BlatGR63a</i>	485	Full	0	XP_018793345.1; GR63a; <i>Bactrocera latifrons</i>
<i>BlatGR64a</i>	87	Partial	3e-53	XP_018783856.1; GR64a; <i>Bactrocera latifrons</i>
<i>BlatGR64b</i>	425	Full	0	XP_011181425.1; GR64b; <i>Zeugodacus cucurbitae</i>
<i>BlatGR64c</i>	60	Partial	2e-32	XP_018783854.1; GR64c-like; <i>Bactrocera latifrons</i>
<i>BlatGR64e</i>	76; 368; 89	Partial	2e-41; 0; 1e-49	XP_018783853.1; uncharacterized protein LOC108965721; <i>Bactrocera latifrons</i>
<i>BlatGR64f</i>	307	Partial	0	XP_018783853.1; uncharacterized protein LOC108965721; <i>Bactrocera latifrons</i>
<i>BlatGR66a</i>	105; 119	Partial	3e-68; 1e-77	XP_018803775.1; GR66a; <i>Bactrocera latifrons</i>
<i>BlatGR68a</i>	95	Partial	1e-57	AKI28984.1; GR68a; <i>Bactrocera dorsalis</i>
<i>BlatGR93a</i>	47	Partial	2e-21	XP_014095798.1; GR93a; <i>Bactrocera oleae</i>
<i>BlatGR94a</i>	122	Partial	3e-81	XP_018792091.1; GR94a; <i>Bactrocera latifrons</i>
<i>BlatGR98b-1-v1</i>	210; 187	Partial	9e-130; 6e-94	XP_018799113.1; putative GR98b; <i>Bactrocera latifrons</i>
<i>BlatGR98b-1-v2</i>	86	Partial	5e-51	XP_014093197.1; putative GR98b; <i>Bactrocera oleae</i>
<i>BlatGR98b-2</i>	111	Partial	2e-67	XP_019846334.1; putative GR98b; <i>Bactrocera dorsalis</i>
<i>BlatGR98b-3</i>	104	Partial	1e-60	XP_011205406.1; putative GR98b; <i>Bactrocera dorsalis</i>

IRs

<i>BlatIR8a</i>	944	Full	0	XP_011211753.1; glutamate receptor ionotropic, kainate 2; <i>Bactrocera dorsalis</i>
<i>BlatIR21a</i>	106; 79; 225	Partial	4e-65; 6e-49; 2e-154	XP_018792132.1; IR21a; <i>Bactrocera latifrons</i>

<i>BlatIR25a</i>	940	Full	0	XP_018787692.1; IR25a; <i>Bactrocera latifrons</i>
<i>BlatIR40a</i>	119; 223	Partial	1e-77; 2e-159	AKI28985.1; IR40a; <i>Bactrocera dorsalis</i>
<i>BlatIR41a-v1</i>	677	Full	0	P_018789519.1; uncharacterized protein LOC108969330; <i>Bactrocera latifrons</i>
<i>BlatIR41a-v2</i>	505	Full	0	P_018789519.1; uncharacterized protein LOC108969330; <i>Bactrocera latifrons</i>
<i>BlatIR48b</i>	318	Partial	0	P_014089212.1; uncharacterized protein LOC106616838; <i>Bactrocera oleae</i>
<i>BlatIR64a-1</i>	365	Partial	0	XP_018785740.1; uncharacterized protein LOC108966998; <i>Bactrocera latifrons</i>
<i>BlatIR64a-2</i>	112	Partial	9e-53	XP_019845172.1; uncharacterized protein LOC105224490; <i>Bactrocera dorsalis</i>
<i>BlatIR75a</i>	85; 549	Partial	4e-43; 0	XP_019845037.1; glutamate receptor; <i>Bactrocera dorsalis</i>
<i>BlatIR75b</i>	635	Partial	0	XP_014088428.1; uncharacterized protein LOC106616338; <i>Bactrocera oleae</i>
<i>BlatIR75d</i>	558	Partial	0	XP_018803411.1; uncharacterized protein LOC108977898; <i>Bactrocera latifrons</i>
<i>BlatIR76a</i>	657	Partial	0	XP_014086277.1; uncharacterized protein LOC106614874; <i>Bactrocera oleae</i>
<i>BlatIR76b</i>	660	Full	0	XP_018785025.1; glutamate receptor ionotropic, delta-1; <i>Bactrocera latifrons</i>
<i>BlatIR84a</i>	702	Partial	0	XP_011193628.1; glutamate receptor 1; <i>Zeugodacus cucurbitae</i>
<i>BlatIR92a-1</i>	173; 156	Partial	9e-117; 3e-105	XP_018789816.1; uncharacterized protein LOC108969515; <i>Bactrocera latifrons</i>
<i>BlatIR92a-2</i>	207; 70	Partial	8e-98; 2e-40	XP_019845610.1; uncharacterized protein LOC105225754 <i>Bactrocera dorsalis</i>
<i>BlatIR93a-1</i>	98; 79; 389	Partial	1e-56; 1e-44; 0	XP_018791489.1; glutamate receptor ionotropic, kainate 5; <i>Bactrocera latifrons</i>
<i>BlatIR93a-2</i>	56	Partial	6e-27	XP_018791489.1; glutamate receptor ionotropic, kainate 5; <i>Bactrocera latifrons</i>

Table 4. Candidate chemosensory receptors of *Zeugodacus cucurbitae* identified from transcriptome.

Gene name	Length (AA)	CDS	E-value	BLASTP best hit (Accession number; Name; Species)
ORs				
<i>ZcucORCO</i>	473	Full	0	XP_011183998.1; Or83b (ORCO) <i>Zeugodacus cucurbitae</i>
<i>ZcucOR2-1</i>	245	Partial	0	XP_011187627.1; OR2a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR2-2</i>	139	Partial	3e-86	XP_011198390.1; OR2a-like; <i>Bactrocera dorsalis</i>
<i>ZcucOR7a-1</i>	396	Full	0	XP_019847175.1; OR7a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR7a-2</i>	375	Partial	0	XP_019845111.1; OR7a-like; <i>Bactrocera dorsalis</i>
<i>ZcucOR7a-3</i>	72	Partial	1e-28	XP_011178079.1; OR7a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR7a-4</i>	394	Full	0	XP_011182064.1; OR7a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR7a-5</i>	82	Partial	4e-49	XP_018798592.1; OR43b-like; <i>Bactrocera latifrons</i>
<i>ZcucOR7a-6</i>	399	Full	0	XP_011178079.1; OR7a-like; <i>Bactrocera dorsalis</i>
<i>ZcucOR7a-7</i>	400	Full	0	XP_014086206.1; OR43b-like; <i>Bactrocera oleae</i>
<i>ZcucOR7a-8</i>	162	Partial	2e-72	XP_019845105.1; OR43b-like; <i>Bactrocera dorsalis</i>
<i>ZcucOR7a-9</i>	129	Partial	4e-67	XP_018798592.1; OR43b-like; <i>Bactrocera latifrons</i>
<i>ZcucOR10a-1</i>	402	Full	0	XP_011184667.1; OR10a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR10a-2</i>	138	Partial	4e-36	XP_011184667.1; OR10a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR13a</i>	104; 257	Partial	9e-70; 0	XP_011177369.1; OR13a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR24a</i>	91	Partial	3e-54	XP_011190505.1; OR24a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR30a</i>	178	Partial	4e-126	XP_011187028.1; OR30a-like; <i>Bactrocera cucurbitae</i>
<i>ZcucOR33b</i>	104	Partial	7e-27	XP_017488833.1; OR33b-like; <i>Rhagoletis zephyria</i>

<i>ZcucOR35a</i>	416	Full	0	XP_011185366.1; OR13a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR42a</i>	440	Partial	0	XP_011183261.1; OR42a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR42b</i>	394	Full	0	XP_011184786.1; OR42b-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR43a-1</i>	380	Full	0	XP_011178893.1; OR43a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR43a-2</i>	375	Full	0	XP_011194820.1; OR2a-like; <i>Bactrocera cucurbitae</i>
<i>ZcucOR43a-3</i>	375	Full	5e-168	XP_014097484.1; OR2a-like; <i>Bactrocera oleae</i>
<i>ZcucOR45a</i>	394	Full	0	XP_011181253.1; OR45a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR47b-v1</i>	443	Full	0	XP_011196690.1; OR47b isoform X1; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR47b-v2</i>	440	Full	0	XP_011196690.1; OR47b isoform X1; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR47b-v3</i>	383	Full	0	XP_011196690.1; OR47b isoform X1; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR49a</i>	154; 108	Partial	9e-100; 1e-68	XP_011181266.1; OR49a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR49b-v1</i>	371	Full	0	XP_011196302.1; OR49b; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR49b-v2</i>	284	Partial	0	XP_011196302.1; OR49b; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR59a</i>	136; 141	Partial	5e-86; 3e-95	XP_011188666.1; OR59a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR63a-1</i>	415	Full	0	AKI29042.1; OR63a-1; <i>Bactrocera dorsalis</i>
<i>ZcucOR63a-2</i>	415	Full	0	XP_011195821.1; OR63a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67c-1</i>	253	Partial	2e-177	XP_011191013.1; uncharacterized protein LOC105217623; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67c-2-v1</i>	236	Partial	1e-171	XP_011191024.1; OR67c-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67c-2-v2</i>	167	Partial	4e-112	XP_011191013.1; uncharacterized protein LOC105217623; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67d-1-v1</i>	388	Full	0	XP_011186895.1; OR67d-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67d-1-v2</i>	388	Full	0	XP_011186895.1; OR67d-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67d-2</i>	386	Full	0	XP_017473047.1; OR67d-like; <i>Rhagoletis zephyria</i>

<i>ZcucOR67d-3</i>	388	Full	0	XP_017473047.1; OR67d-like; <i>Rhagoletis zephyria</i>
<i>ZcucOR67d-4</i>	243	Partial	6e-175	XP_011186909.1; OR67d-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67d-5</i>	144	Partial	2e-83	XP_011186909.1; OR67d-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR67d-6</i>	86	Partial	9e-38	XP_011186895.1; OR67d-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR69a-1</i>	413	Full	0	XP_011191101.1; OR69a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR69a-2</i>	98	Partial	7e-65	XP_011191113.1; OR69a isoformA; <i>Bactrocera cucurbitae</i>
<i>ZcucOR74a</i>	414	Full	0	XP_011201924.2; OR74a-like; <i>Bactrocera dorsalis</i>
<i>ZcucOR82a</i>	241; 79	Partial	2e-173; 7e-48	XP_011181975.1; OR82a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR83a</i>	66	Partial	2e-38	XP_011184140.1; OR83a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR85c</i>	98	Partial	2e-62	XP_011192524.1; OR85c; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR88a</i>	407	Full	0	XP_011183038.1; OR88a; <i>Zeugodacus cucurbitae</i>
<i>ZcucOR92a</i>	384	Full	0	XP_011208819.1; OR92a; <i>Bactrocera dorsalis</i>
<i>ZcucOR94a</i>	377	Partial	0	XP_011179733.1; OR94a-like; <i>Zeugodacus cucurbita</i>
<i>ZcucOR94b</i>	414	Full	0	XP_014094554.1; OR94a-like; <i>Bactrocera oleae</i>
GRs				
<i>ZcucGR5a</i>	68	Partial	1e-33	XP_014092165.1; GR5a; <i>Bactrocera oleae</i>
<i>ZcucGR8a</i>	68	Partial	8e-13	XP_012161678.1; GR8a; <i>Ceratitis capitata</i>
<i>ZcucGR21a-1-v1</i>	457	Full	0	XP_011194684.1; GR21a-like; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR21a-1-v2</i>	79	Partial	1e-48	XP_014101212.1; GR21a-like; <i>Bactrocera oleae</i>
<i>ZcucGR21a-2</i>	426	Partial	1e-85	AID61262.1; GR; <i>Calliphora stygia</i>
<i>ZcucGR22d</i>	374	Partial	0	XP_018800490.1; GR22d; <i>Bactrocera latifrons</i>
<i>ZcucGR23a</i>	41	Partial	7e-11	XP_011194021.1; uncharacterized protein LOC105219521; <i>Zeugodacus cucurbitae</i>

<i>ZcucGR28a</i>	68	Partial	5e-32	XP_011180394.1; GR28a; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR28b</i>	149	Partial	4e-98	XP_018796745.1; GR28b; <i>Bactrocera latifrons</i>
<i>ZcucGR32a</i>	85; 158	Partial	3e-53; 1e-108	XP_011178366.1; GR32a; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR63a</i>	485	Full	0	XP_011187146.1; GR63a; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR64b</i>	104; 67	Partial	2e-66; 6e-39	XP_011181425.1; GR64b; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR64e</i>	22	Partial	9e-15	XP_011181429.1; uncharacterized protein LOC105211608; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR64f</i>	72	Partial	1e-40	XP_011181429.1; uncharacterized protein LOC105211608; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR66a</i>	195	Partial	8e-137	XP_011196225.1; GR66a; <i>Zeugodacus cucurbitae</i>
<i>ZcucGR94a</i>	91	Partial	2e-56	XP_011178240.1; GR94a; <i>Zeugodacus cucurbitae</i>
IRs				
<i>ZcucIR8a</i>	950	Full	0	XP_011186380.1; uncharacterized protein LOC105214572; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR21a</i>	932	Full	0	XP_011183925.1; uncharacterized protein LOC105213077; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR25a</i>	940	Full	0	XP_011178452.1; glutamate receptor 3; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR31a</i>	97	Partial	2e-54	XP_011194781.1, uncharacterized protein LOC105220077; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR40a-1</i>	141; 166	Partial	2e-30; 6e-108	XP_011182177.1; uncharacterized protein LOC105212100; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR40a-2</i>	282	Partial	0	XP_011212457.2; uncharacterized protein LOC105232474; <i>Bactrocera dorsalis</i>
<i>ZcucIR41a</i>	336; 302	Partial	0; 0	AKI28986.1; IR41a; <i>Bactrocera dorsalis</i>
<i>ZcucIR64a</i>	131; 641	Partial	2e-79; 0	XP_011180795.1; uncharacterized protein LOC105211160; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR75a-1</i>	166; 481	Partial	4e-87; 0	XP_011180343.1; uncharacterized protein LOC105210861; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR75a-2</i>	73; 205; 206	Partial	5e-32; 4e-143; 5e-143	XP_011180358.1; uncharacterized protein LOC105210866; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR75d</i>	751	Partial	0	XP_011186430.1; uncharacterized protein LOC105214604; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR76a</i>	660	Full	0	XP_011180078.1; uncharacterized protein LOC105210679; <i>Zeugodacus cucurbitae</i>

<i>ZcucIR76b</i>	661	Full	0	XP_011180180.1;glutamate receptor ionotropic, delta-1; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR84a</i>	703	Partial	0	XP_011193628.1; glutamate receptor 1; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR85a</i>	77	Partial	6e-37	XP_017489388.1; uncharacterized protein LOC108377629; <i>Rhagoletis zephyria</i>
<i>ZcucIR92a</i>	111; 101; 57; 244	Partial	1e-41; 2e-61; 8e-31; 2e-175	XP_011184367.1; uncharacterized protein LOC105213333; <i>Zeugodacus cucurbitae</i>
<i>ZcucIR93a</i>	49; 100; 177; 68; 129	Partial	8e-25; 1e-60; 1e-113; 2e-37; 1e-83	XP_011177762.1; uncharacterized protein LOC105209182; <i>Zeugodacus cucurbitae</i>

Table 5. Response properties of OR74a homologs to 1-nonanol.

OR	Response at 100 μ M (Mean $\pm$ SD)
BdorOR74a	$0.202 \pm 0.0277 \mu\text{A}$
BlatOR74a	$0.0424 \pm 0.0145 \mu\text{A}$
ZcucOR74a	$0.0704 \pm 0.0051 \mu\text{A}$

BdorOR74a E E N K K L M K L I A L A I H L N S N P F R L T G L N F S V V N Y E T V V A I L R G A G S Y F T V I Y A Y R
BlatOR74a E E N K K L M K L I A L A I H L N S N P F R L T G L N F S V V N Y E T V V T I L R G A G S Y F T V I Y A Y R
ZcucOR74a E E N K K L M K L I A L A V H L N S N P F R L T G L N F S V V N Y E T V V S I L R G A G S Y F T V I Y A Y R

* * * * *

Fig. 4

B

C

D

E

Fig. S1

1-Nonanol (1-N)

1,3-Nonanediol (1,3-ND)

6-Oxo-1-nonanol (6-ON)

Nonyl acetate (N-OAc)

Methyl eugenol (ME)

Raspberry ketone (RK)

Cue-lure (CL)

Zingerone (ZN)

2-Allyl-4,5-dimethoxyphenol (DMP)

(*E*)-Coniferyl alcohol (CF)

(*Z*)-Coniferyl alcohol (Z-CF)

(*Z*)-3,4-Dimethoxycinnamyl alcohol (Z-DMC)

Eugenol (EU)

(±)-3-Oxo-7,8-dihydro- α -ionone (P3)

(±)-4-Propionyloxyisophorone (E0P)

Fig. S2