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Transcriptomic analyses in thirteen Tephritidae species provide insights into the ecological driving force behind odorant receptor evolution

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ABSTRACT

The insect olfactory system has evolved while guiding species to specific mating partners, different food sources, and oviposition sites. How species repertoires of odorant receptors (ORs), responsible for the detection of volatile cues, have been shaped by ecologically driven forces remains poorly understood. Due to several host switches back and forth throughout their evolutionary history, fruit flies of the Tephritidae family (Diptera) show highly diverse host preferences, making them good models to address this question. For instance, a comparative analysis of genomic and transcriptomic resources on a large variety of fruit fly species could provide statistical conclusions. Here, we used a RNAseq approach to identify the OR repertoires of thirteen Tephritidae species with different host ranges, namely *Bactrocera curvipennis*, *Bactrocera dorsalis, Bactrocera psidii, Bactrocera tryoni, Bactrocera umbrosa, Bactrocera zonata, Ceratitis capitata*, *Ceratitis catoirii*, *Ceratitis quilicii, Dacus ciliatus, Dacus demmerezi, Neoceratitis cyanescens,* and *Zeugodacus cucurbitae*. Manual curation allowed us to annotate 60–80 OR transcripts per species, including the obligatory coreceptor Orco. In total, we reported 698 new OR sequences. Differential expression analyses between antennae and maxillary palps and between the two sexes, performed in three species, revealed some organ- and sex-biased OR expression. Moreover, after adjusting for phylogenetic distance, we found significant correlations between some characteristics of the OR repertoire and species host range: sequences and relative expression level of several ORs were more conserved in polyphagous than in oligophagous species and, in addition, other ORs were found specifically in polyphagous species. Our results provide molecular insights into the ecological driving forces behind Tephritidae OR evolution.

1. Introduction

Olfaction plays a crucial role in modulating insect behaviour, including essential activities such as host seeking, mating and egg-laying (Hildebrand and Shepherd, 1997). The insect olfactory system has evolved to discriminate among different arrays of chemicals to fit various species ecology (Benton, 2006; Nei et al., 2008; Hansson and Stensmyr, 2011; Andersson et al., 2015; Ramiaranjatovo et al., 2024), and a growing body of evidence indicates that odorant receptors (ORs) have been a driving force behind insect adaptation (Cande et al., 2013; Librado and Rozas, 2016; Nouhaud et al., 2018; Auer et al., 2020).

Insect ORs have a primary role in odorant detection and discrimination. They are transmembrane proteins expressed in olfactory sensory neurons of insect chemosensory appendages and act as ligand-gated cation channels in complex with a conserved co-receptor named Orco (Sato et al., 2008; Wicher et al., 2008). They form a multigenic family that has evolved from a common ancestor through a dynamic process primarily governed by a birth-and-death model, wherein genes multiply by tandem duplication events and are removed by deletion (Robertson, 2019). The OR gene family shows significant divergence in terms of number of genes per insect species, sequences, expression level, and response spectrum (Hansson and Stensmyr, 2011; Andersson et al.,

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2015; Robertson, 2019), suggesting that each order/species evolved its own repertoire characteristics in the context of its chemical ecology. Consequently, phylogenetically close species can present OR divergence under different ecological constraints, as observed for instance in the *Drosophila* genus (Guo and Kim, 2007; McBride, 2007; McBride and Arguello, 2007; Robertson, 2009), in Lepidoptera sister species (Li et al., 2013; Glaser et al., 2015), and in pea aphid host races (Nouhaud et al., 2018). While these examples could provide enough information to examine the process of OR evolution thanks to the short time periods considered, evidence of ecological constraints on OR diversification is more difficult to address in less related species.

In this work, we took advantage of the remarkably diverse host preferences of fruit flies from the Tephritidae family (Diptera), which encompasses different species subfamilies and tribes, to search for molecular traces of ecologically-driven evolution of their OR diversity. In addition to their ecological and evolutionary relevance to address this, fruit flies are also of strong societal and economic interest (Duyck et al., 2022). With over 5000 described species, the Tephritidae family is one of the most destructive horticultural pest groups worldwide (White and Elson-Harris, 1992; Norrbom et al., 1999). Some species are highly polyphagous and infest a variety of wild and cultivated fruits, while others are more specialised and infest only one botanical family (White and Elson-Harris, 1992; Charlery de la Masselière et al., 2017a). Tephritid fruit fly species are closely associated with a diverse range of semiochemicals throughout their life cycle (Miyazaki et al., 2018; Ono et al., 2021; Scolari et al., 2021), including diet volatiles for food-seeking (Drew et al., 1983; Jacome et al., 1995; Aluja et al., 2001; Clarke et al., 2011), a variety of pheromonal compounds and specific plant metabolites, known as male attractants, for sexual communication (Benelli et al., 2014; Jacob et al., 2023; Scolari et al., 2021; Tan and Nishida, 2012), and key semiochemicals for female oviposition (Quilici et al., 2014). All these chemical signals are detected by the Tephritidae antennae and maxillary palps as their primary olfactory organs. Whereas advancements in high-throughput sequencing technology have facilitated the identification of numerous OR sequences across various insect species, complete repertoires of ORs have been annotated in only a few Tephritidae species through genome sequencing, such as in *B. dorsalis* (Jiang et al., 2022; Wang et al., 2022), *Bactrocera correcta* (Guo et al., 2023), *Bactrocera minax* (Cheng et al., 2020; Wang et al., 2022), and *C. capitata* (Papanicolaou et al., 2016), or through olfactory organ transcriptome sequencing (Miyazaki et al., 2018; Ono et al., 2020; Wu et al., 2020). Notably, the latest studies rarely distinguished antenna and maxillary palp transcriptomes.

In the current study, we first generated additional transcriptomic data, distinguishing between antennae and palps, and conducted comparative analyses of the OR repertoires of a wide range of 13 crop pest fruit fly species. These species belong to the Dacini and Ceratidini tribes within the Dacinae subfamily, and present contrasting ecologies based on their degree of specialisation (Charlery de la Masselière et al., 2017a; Moquet et al., 2021; Lauciello et al., 2024). Among them, eight species are polyphagous with overlapping host ranges encompassing many fruit species. The polyphagous species consisted of *Bactrocera curvipennis* (Froggatt, 1909), *Bactrocera dorsalis* (Hendel, 1912), *Bactrocera psidii* (Froggatt, 1899), *Bactrocera tryoni* (Froggatt, 1897), *Bactrocera zonata* (Saunders, 1841), *Ceratitis capitata* (Wiedeman, 1824), *Ceratitis quilicii* De Meyer, Mwatawala & Virgilio, 2016, and *Ceratitis catoirii* Guérin-Méneville, 1843. The other species are oligophagous, whose host ranges are restricted to the plant families Cucurbitaceae, namely *Dacus ciliatus* Loew, 1862, *Dacus demmerezi* (Bezzi, 1917), and *Zeugodacus cucurbitae* (Coquillett, 1899), Solanaceae, namely *Neoceratitis cyanescens* (Bezzi, 1923), or to the genus *Artocarpus* of the Moraceae family, namely *Bactrocera umbrosa* (Fabricius, 1805). These species were selected to demonstrate both the overlap of ecological niches among phylogenetically distant species, which is the case for many polyphagous species (e.g. *B. dorsalis* and *C. capitata*), and the different ecological requirements of phylogenetically close species, which is the case for several oligophagous species (e.g. *B. umbrosa* phylogenetically close to *B. psidii*). We questioned if any characteristics of the OR repertoire are more conserved in species with the same host range width than what would be expected according solely to their phylogenetic divergence. Following manual annotation of ORs in each species and phylogenetic analysis, we investigated the differences in OR repertoires and expression levels between antennae and maxillary palps, as well as between sexes. We also searched for significant correlations among OR characteristics, species host range, and phylogenetic position.

2. Materials and methods

2.1. Insect collection

Nine species were collected in La Réunion Island: *B. dorsalis*, *B. zonata*, *C. capitata*, *C. catoirii*, *C. quilicii*, *D. ciliatus*, *D. demmerezi*, *N. cyanescens*, and *Z. cucurbitae*. They were reared or maintained (depending on the species, see below) in CIRAD, Pôle de Protection des Plantes, Saint-Pierre, in environmental chambers (Luminincube II, Analis, Belgium; MLR-350, Sanyo, Japan). Four species were collected in New Caledonia: *B. curvipennis*, *B. psidii*, *B. tryoni*, and *B. umbrosa*. They were maintained at the Institut Agronomique néo-Calédonien (IAC), at the Station de Recherches Fruitières de Pocquereux, La Foa.

All fruit flies were maintained under laboratory conditions: L12:D12 photoperiod, at a constant temperature of 25 ± 1 °C and with 65 ± 15 % relative humidity. Larvae of *B. dorsalis*, *B. zonata*, *C. capitata*, *C. catoirii*, and *C. quilicii* were reared for 72, 190, 116, 319, and 85 generations, respectively, on the following artificial diet: a solid diet for eggs and young larvae (first instar, L1) that included dehydrated carrot powder, Bewer's yeast, dehydrated potato, water, Nipagin/sodium benzoate, citric acid, agar, and wheat germ; and a liquid diet added to a bran substrate for older larvae (second and third instar, L2-L3) (Duyck and Quilici, 2002). Larvae of *B. tryoni* were reared for 23 generations on the following artificial diet: fresh ripened banana, Torula yeast, and Nipagin. *Dacus demmerezi* and *Z. cucurbitae* were reared on zucchini (*Cucurbita pepo* L.) for three and 10 generations, respectively. *Neoceratitis cyanescens* was reared on potato (*Solanum tuberosum*) for 67 generations. For the other species, larvae were collected in the wild and maintained in the laboratory until adult emergence. Larvae of *B. curvipennis* were field collected on wild plum (*Ximenia americana*), *B. psidii* larvae on wild peach (*Prunus persica*) or guava (*Psidium guajava*), *B. umbrosa* larvae on wild jackfruit (*Artocarpus heterophyllus*) and breadfruit (*Artocarpus altilis*), and *D. ciliatus* larvae on wild bitter gourd (*Momordica charantia*). All adult fruit flies were maintained in transparent plastic cages (30 cm × 30 cm × 30 cm) aerated by meshed openings, fed with sugar and protein in the form of yeast hydrolysate (ICN Biomedical, Aurora, USA) and a wet sponge as a water source (Duyck and Quilici, 2002; Mas et al., 2020).

2.2. Tissue dissection, RNA extraction, and sequencing

Tissue dissections were performed on males and females approximatively 7–22 days after emergence for species from the Dacini tribe and 6–8 days for the species from the Ceratitidini tribe, corresponding to their average sexual maturity (Charlery de la Masselière et al., 2017b). For the three species *B. dorsalis*, *B. tryoni,* and *Z. cucurbitae*, three independent biological replicates were performed for each sex and each olfactory organ (antenna and maxillary palp) to conduct differential expression analyses. Each replicate comprised tissues collected from 100 males and 100 females randomly selected. For the other ten species (*B. curvipennis, B. psidii, B. umbrosa, B. zonata, C. capitata*, *C. catoirii, C. quilicii*, *D. ciliatus, D. demmerezi*, and *N. cyanescens*), approximately 100 males and females mixed together were used for independent dissections of antenna and maxillary palps. Ablation of antennae and maxillary palps were performed as follows: adult flies were anesthetized with CO_2, heads were removed, and antennae and palps were dissected with fine

forceps to carefully sever appendages at the most basal article under a binocular loupe on ice. Antennae and maxillary palps were immediately transferred into a 1.5 mL Eppendorf tube on ice containing 500 μL of TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −80 °C until RNA extraction. For species collected in New Caledonia, tissues were first collected in RNAlater and then transferred in TRIzol™.

For RNA extraction, tissues were ground and homogenized with a Polytron™ Pro 200 (Pro Scientific Inc. Oxford, CT, USA) for 45 s at medium speed. Total RNA was extracted using the phenol/chloroform method following the manufacturer's protocol. RNA quality (260/280 nm ratio) and quantity were measured using a NanoDrop™ ND-2000 spectrophotometer (Thermo Fisher Scientific).

The 56 RNA samples were sent in dry ice to Novogene Company Limited (Cambridge, United Kingdom) for directional library construction and sequencing. Briefly, messenger RNAs were purified from total RNAs using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using dUTP. The directional libraries were ready after end repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification, and purification. Sequencing was performed using an Illumina NovaSeq6000 instrument with a paired read length of 150 bp and an output of 18 Gb of clean data per library.

2.3. De novo transcriptome assembly

Data processing and analysis were performed on the Galaxy server hosted at the BioInformatics Platform for Agro-ecosystems Arthropods (Rennes, France). Raw reads were converted in fastqsanger format using FastQ Grommer v1.1.1 (Blankenberg et al., 2010). Quality control of the raw reads in FastQ format was performed using FastQC v0.72 (Andrews, 2010) and low-quality reads were trimmed using Trimmomatic v0.36.6 (Bolger et al., 2014). The parameters were as follows: sliding window = 4; average quality = 20; minlen = 30 bases; headcrop = 10 bases; trailing minimum quality = 20. A *de novo* assembly was conducted for each species, mixing all data (antenna and maxillary palp), using the Trinity transcriptome assembler v2.8.4 (Grabherr et al., 2011) by setting a minimum contig length of 200 and a minimum count of 1 for K-mers to be assembled. Coding sequences were extracted from the reference transcriptome using Transdecoder v5.5.0 (Haas et al., 2013) with a minimum protein length of 50 amino acids. Redundant sequences were clustered using CD-HIT EST v1.2 (Li and Godzik, 2006) with a similarity threshold of 0.9 and a word size of 8 (data metrics summarized in **Suppl. Table 1**).

The completeness of the thirteen assembled transcriptomes was assessed using BUSCO v4.1.4 (Simão et al., 2015), which tests the assembly for the presence of 1367 single-copy orthologs highly conserved in insects (insecta_odb10).

2.4. Annotation of odorant receptors

For OR annotation, we first created an OR dataset containing amino acid sequences manually annotated in genomes or transcriptomes of a variety of insect species, including the beetle *Rhantus suturalis* (Montagné et al., 2021), the moths *Bombyx mori* (Tanaka et al., 2009) and *Spodoptera littoralis* (Meslin et al., 2022), the hymenopteran *Apis mellifera* (Forêt et al., 2007), and the dipteran species *Drosophila melanogaster* (Robertson et al., 2003), *Bactrocera correcta* (Guo et al., 2023), *B. dorsalis* (Jiang et al., 2022), *Bactrocera latifrons* (Cheng et al., 2020; Ono et al., 2020), *Bactrocera minax* (Xu et al., 2019), *C. capitata* (Calla et al., 2014), *Rhagoletis pomonella* (Schwarz et al., 2009), *Z. cucurbitae* (Ono et al., 2020), and *Zeugodacus tau* (Wu et al., 2020). These amino acid sequences were used as queries to search for ORs in the 13 Tephritidae reference transcriptomes using tblastn v0.3.1 (Cock et al., 2015) with an *e-value* cutoff set at 1e−3. The open reading frames (ORFs) of these sequences were extracted using GetORFs v0.2.3 (Cock et al., 2013) and the redundant sequences were clustered using CD-HIT PROTEIN v1.2 (Li and Godzik, 2006) with a similarity threshold of 0.9 and a word size of 5. In parallel with the alignment search strategy, a protein domain analysis was performed on translated transcriptomes with rpsblast v2.10.1 (Cock et al., 2015) using the Pfam-A and CDD-NCBI databases. Results for the domains pfam02949 and 7tm_6 Odorant receptor were mined.

Candidate protein sequences were compared manually to the NCBI non-redundant (nr) database using blastp (Johnson et al., 2008). In some cases, redundant unigenes encoding the same protein but not clustered by CD-HIT PROTEIN were manually verified to rebuild a longer sequence by multialignment. The presence of transmembrane domains within sequences of candidate ORs was predicted using TOPCONS web server (Tsirigos et al., 2015). Tephritidae OR transcripts considered homologous to *B. dorsalis* sequences previously annotated (Xu et al., 2023) were given the same name, and multiple copies were given the same name followed by a dash, a number, and a letter if necessary (e.g. BzonOR7a-12, BzonOR7a-4a). Genes were considered complete when a start and a stop codon were identified and when the sequence length was greater than 350 amino acids. A description of manual OR annotation is given in **Suppl. Table 2**.

2.5. Phylogenetic inferences

The species phylogenetic tree of the 13 Tephritidae species and *D. melanogaster* was constructed on the basis of the amino acid sequences of 19 single-copy ortholog BUSCO genes (sequences available in **Suppl. Table 3**). The alignment of the concatenated amino acid sequences was performed using MAFFT web-based v7 (Katoh et al., 2019) with default parameters, resulting in 13,279 aligned positions. The phylogenetic tree was inferred with the maximum-likelihood method using IQ-TREE v2.2.0 (Nguyen et al., 2015). The best-fit model of protein evolution was selected by ModelFinder (Kalyaanamoorthy et al., 2017), using the Bayesian Information Criterion. The node support estimations were obtained using the ultrafast bootstrap method (Hoang et al., 2018), using 1000 bootstrap iterations.

To rebuild the phylogeny of ORs, amino acid sequences from *B. curvipennis*, *B. dorsalis*, *B. psidii*, *B. tryoni*, *B. umbrosa*, *B. zonata*, *C. capitata*, *C. catoirii*, *C. quilicii*, *D. ciliatus*, *D. demmerezi*, *N. cyanescens*, and *Z. cucurbitae* were aligned with the 62 amino acid sequences of *D. melanogaster* ORs using MAFFT with default parameters (sequences listed in **Suppl. Table 3**), and the phylogenetic tree was inferred with the maximum-likelihood method using IQ-TREE. The resulting species and OR trees in Newick format were visualized with FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

OrthoFinder v2.5.5 (Emms and Kelly, 2015) was employed to assign orthology groups for ORs across the 13 Tephritidae species and *D. melanogaster*, and to calculate gene duplication events.

2.6. Differential expression analyses

To measure expression levels, clean reads were mapped on the reference transcriptome with Bowtie2 (Langmead and Salzberg, 2012). Transcript abundance was then measured in each sample as transcripts per million (TPM), using the RSEM method (Li and Dewey, 2011). Transcripts with a low expression (0.1 counts per million reads) were filtered out from the analysis and the total number of filtered reads was then normalized using the trimmed mean of M values method (TMM) (Robinson and Oshlack, 2010). The TPM values were used to draw expression heatmaps using the $\log_2(\text{TPM} + 1)$ values. For the three species with biological replicates (*B. dorsalis*, *B. tryoni* and *Z. cucurbitae*), differential expression of transcripts according to sex and tissues was calculated using edgeR (Chen et al., 2014), and the false discovery rate (FDR) method was used to determine the threshold of *p*-value in multiple tests. Differences in transcript expression were judged using a threshold FDR < 0.1 and a $-1 < \text{logFC} > 1$ Raw expression data from the

RSEM and edgeR methods are presented in **Suppl. Table 4**. Heatmaps were built using RStudio v2023.12.0 (http://www.rstudio.com/), using 'circlize' (Gu et al., 2014), 'ComplexHeatmap' (Gu et al., 2016) and 'ggplot2' (Wickham, 2009) packages.

2.7. Statistical analysis

To test if characteristics of the OR repertoires depend on the degree of specialisation, the effects of phylogenetic distance between species needs to be offset. A pairwise analysis was thus performed on the 13 studied species.

First, we calculated a matrix of phylogenetic distance between species. As a robust estimation, we used the distance between the amino acid sequences of the 19 single-copy ortholog BUSCO genes used to construct the species phylogenetic tree. The phylogenetic distance was obtained as follow: first, we performed a Needleman-Wunsch alignment, then the distance was defined as the proportion of amino acid substitutions among the total sequence. A Phylogenetic Generalized Least Squares (PGLS) analysis was performed to test if the number of OR genes depended on the host range, using the phylogenetic distance as a correlation structure ('gls' function from the R package 'nlme' (Pinheiro et al., 2021)).

Then, we calculated a matrix of ecological category between species. The ecological category was set to 1 if the two species are polyphagous (28 combinations of two species) or 2, if at least one species is not (50 combinations of two species). We also calculated a matrix of the number of OR orthogroups shared between two species, by reassigning the orthogroups with OrthoFinder using only the ORs of the two species. Finally, we calculated matrices of correlation coefficient between TPM expression levels of ORs in the antennae and maxillary palp, and for each OR a matrix of species-to-species distance between its amino acid sequence.

For each of the parameters that characterises the OR repertoire, we tested if it correlates with the matrix of phylogenetic distance using a bilateral Mantel test, based on Pearson correlation coefficient (r) and 10,000 permutations (R package 'vegan', Oksanen et al., 2024). We also tested if it correlates with the matrix of ecological category, conditioned on the phylogenetic distance, using a bilateral partial Mantel test, based on partial Pearson correlation coefficient (ρ) and 10,000 permutations. A partial Mantel test corrects an eventual bias due to uneven sampling of ecological category among the phylogenetic tree.

3. Results

3.1. Sequencing and transcriptome assemblies

Illumina sequencing was conducted on a total of 56 libraries. Between 114 and 169 million pairs of raw reads were generated from each RNAseq library. After filtering low-quality raw reads, between 55 and 82 million clean reads were obtained. Clean reads from the two libraries (antennae and maxillary palps) for each of the following 10 species: *B. curvipennis*, *B. psidii*, *B. umbrosa*, *B. zonata*, *C. capitata*, *C. catoirii*, *C. quilicii*, *D. ciliatus*, *D. demmerezi* and *N. cyanescens*, were pooled and assembled together, resulting in between 123 and 286 thousand contigs per species. For the three other species, namely *B. dorsalis*, *B. tryoni* and *Z. cucurbitae*, clean reads from female antennae, female maxillary palps, male antennae and male maxillary palps libraries were assembled together, resulting in between 467 and 648 thousand contigs per species. After redundant sequence clustering, the final reference transcriptomes consisted of the following unigene counts: 63,362 (*B. curvipennis*), 135,753 (*B. dorsalis*), 52,138 (*B. psidii*), 109,897 (*B. tryoni*), 56,189 (*B. umbrosa*), 54,651 (*B. zonata*), 68,476 (*C. capitata*), 59,197 (*C. catoirii*), 61,672 (*C. quilicii*), 50,118 (*D. ciliatus*), 41,704 (*D. demmerezi*), 68,001 (*N. cyanescens*), and 183,856 (*Z. cucurbitae*) (summarized in **Suppl. Table 1**).

The BUSCO analysis conducted separately on the thirteen reference transcriptomes showed a high level of completeness and a low level of redundancy, with more than 89 % of BUSCO genes identified as complete and in a single-copy (**Suppl. Fig. 1**).

3.2. Candidate OR gene annotation

We annotated and manually curated 877 transcripts potentially coding for ORs in the 13 Tephritidae reference transcriptomes (Fig. 1), including the obligatory co-receptor Orco. We found ~87 % identity between the Orco protein sequence of *D. melanogaster* and that of the 13 Tephritidae species, as expected for this conserved co-receptor. Among the 13 Tephritidae species, Orco proteins exhibited sequence identities ranging from 96 % to 100 %, depending on evolutionary distance, and in agreement with previous studies (Zheng et al., 2012; Yi et al., 2014; Tsoumani et al., 2020). Among the ORs we annotated, 689 ORs (80 %) were predicted to have a complete sequence, ranging from 355 to 475 amino acids.

Depending on the species, 55–80 OR transcripts were identified, including Orco. These numbers are in the range of what has been observed in other dipterans including Tephritidae, in which 44 to –85 ORs have been described (Hill et al., 2002; Robertson et al., 2003; Papanicolaou et al., 2016; Olafson and Saski, 2020; Xu et al., 2020). Previous studies have identified candidate OR sequences in *B. dorsalis*, *Z. cucurbitae* and *C. capitata*. A comprehensive comparison between those sequences and the ones discovered in the current study has been conducted (**Suppl. Fig. 2**). For *B. dorsalis*, we compared the 80 ORs we identified with those previously annotated in the *B. dorsalis* genome and different transcriptomes (Xu et al., 2023, 74 ORs; Miyazaki et al., 2018, 49 ORs, **Suppl. Fig. 2a**). Our study uncovered 11 previously unidentified ORs, even in the recent high-quality *B. dorsalis* genome (Xu et al., 2023). Conversely, these former studies identified eight ORs not found in our analysis, likely due to their use of genomic data. In total, 42 ORs were common to all three studies. For *Z. cucurbitae*, we compared the 79 ORs annotated in our study with those found in previous chemosensory transcriptomes (Ono et al., 2020a, 45 ORs; Wu et al., 2020, 40 ORs, **Suppl. Fig. 2b**). We revealed 32 previously unidentified ORs in *Z. cucurbitae*. Only one OR identified in Ono's study was not found in our study. Thirty-seven ORs were common in all three studies. For *C. capitata*, we compared our set of 66 ORs to the 75 ORs identified in the genome by Papanicolaou et al. (2016a) (75 ORs, **Suppl. Fig. 2c**). We retrieved 64 ORs previously described and identified two new ORs, whereas 11 ORs were absent from our dataset. As a whole, our study reports a total of 698 new candidate OR sequences in fruit fly species.-

In the Ceratitidini tribe, the numbers of identified ORs were more homogeneous, ranging from 61 to 66 ORs per species (Fig. 1**b**). These numbers are close to the numbers of OR genes (60) and encoded transcripts (62) identified in the dipteran relative *D. melanogaster* (Robertson et al., 2003). In contrast, the OR repertoire across the Dacini tribe exhibited a larger variability, ranging from 55 to 80 ORs in, respectively, *D. ciliatus* and *B. dorsalis*.

3.3. Phylogenetic inferences

The phylogenetic reconstruction of the 13 Tephritidae species confirmed a clear subdivision of the species into two distinct tribes: Ceratitidini and Dacini (Fig. 1**a**). The Ceratitidini tribe comprised four species from two genera, *Neoceratitis* and *Ceratitis*, while the Dacini tribe is represented by nine species from three genera, *Zeugodacus*, *Dacus*, and *Bactrocera*. The tree is consistent with the known topology of Ceratitidini as monophyletic clade (Segura et al., 2006; Zhang et al., 2010; Virgilio et al., 2015). Within the Dacini tribe, and more particularly in the *Bactrocera* genus, we obtained a different topology from that observed in the Bayesian analysis of COI and 16S sequences of Virgilio et al. (2015). Their analysis placed *B. zonata* and *B. psidii* as sister species, while our analysis indicated that *B. zonata* was sister species of *B. dorsalis* and *B. psidii* was sister species of *B. umbrosa*. Our topology of *Bactrocera*

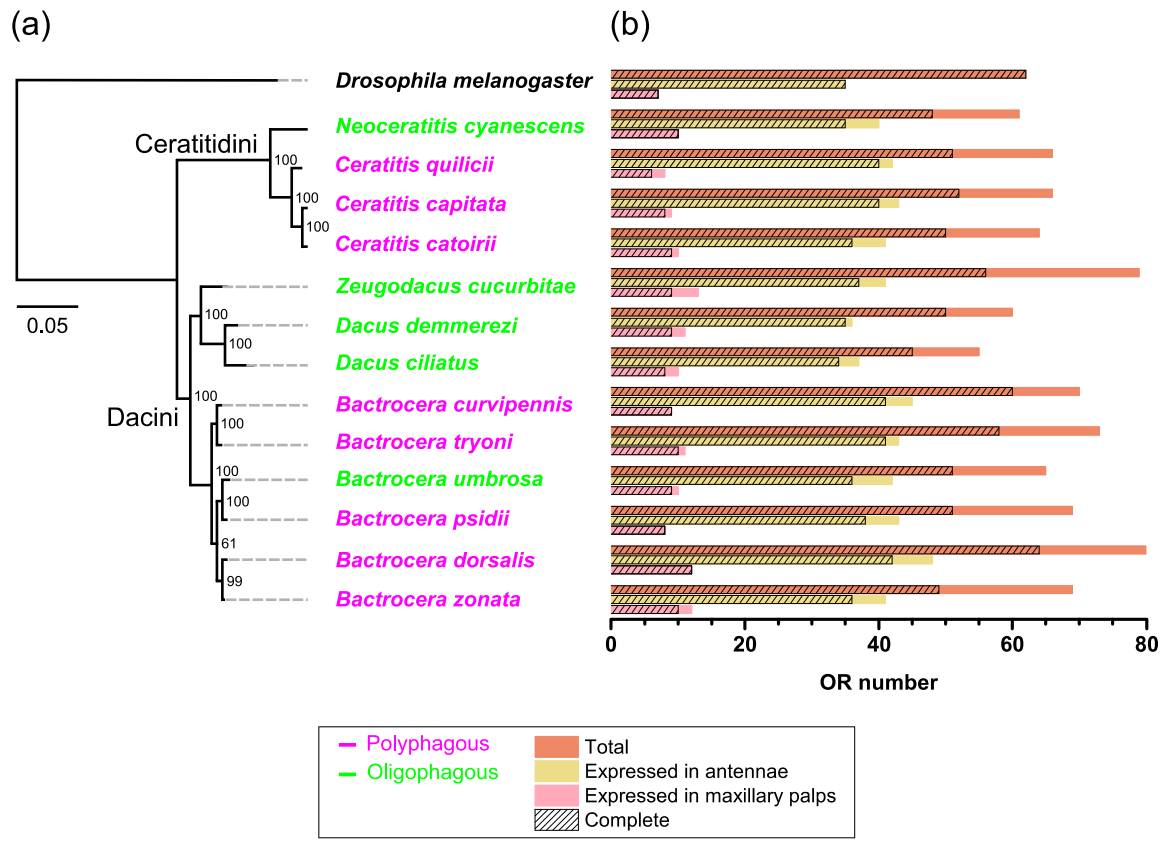

Fig. 1. Numbers of candidate OR genes annotated within the olfactory transcriptomes of 13 Tephritidae species and of ORs expressed in *D. melanogaster*. **(a)** The species tree was built from an alignment of the 19 amino acid BUSCO sequences from the 13 Tephritidae fruit fly species, and the same 19 BUSCO genes of *D. melanogaster* were used as outgroup. The best substitution model used was calculated according to the Bayesian Information Criterion and corresponded to Q.plant + F + I + R2. The branch support values are indicated (UFBoot, n = 1000). The scale bar indicates the expected number of amino acid substitutions per site. The resulting tree reveals a subdivision of the 13 Tephritidae species into two tribes. The Ceratitidini tribe comprises four species, while the Dacini tribe is represented by nine species. **(b)** Numbers and expression distribution of ORs identified in the olfactory transcriptomes of 13 Tephritidae species and in *D. melanogaster*.

genus is however consistent with the *B. zonata* and *B. dorsalis* topology from Zhang et al. (2023), although, this last analysis only includes four out of the six *Bactrocera* species used in our study. In our analysis, the bootstrap support values at the basal node for each genus were high, indicating strong robustness in the inferred relationships.

To provide a comprehensive understanding of the evolutionary dynamics of the ORs within Tephritidae and to estimate their divergence with ORs from *Drosophila*, we performed a phylogenetic analysis, as shown in Fig. 2**a**. The ultrafast bootstrap values were generally high, especially at terminal branches, attesting to the robustness of the analysis. The tree is consistent with the known topology of *Drosophila* and Tephritidae OR trees (Guo and Kim, 2007; Jacob et al., 2017; Guo et al., 2023). ORs were classified in 42 orthogroups using OrthoFinder (Fig. 2), and the species tree generated by OrthoFinder perfectly matched the species tree we generated using BUSCO (**Suppl. Fig. 3**). Many Tephritidae proteins clustered into different orthogroups with one representative per species, frequently together with a *D. melanogaster* OR, such as Orco, OR13a, and OR45a groups, and the topology of the orthogroups matched the species tree (Fig. 1**a**). In contrast, several OR groups presented Tephritidae OR expansions compared to *Drosophila*, such as the OR7a cluster (Fig. 2**b**). In this cluster, the most considerable OR7a expansions were found in the genera *Bactrocera* and *Zeugodacus*, with approximately 14 paralogs per species, whereas the genera *Dacus*, *Ceratitis,* and *Neoceratitis* presented approximately eight paralogs per species. Moderate expansions, with approximately four paralogous ORs, were observed among tephritid species in the OR67d, OR33b, and OR59a groups (Fig. 2**b**, **Suppl. Fig. 4**).

3.4. Tissue and organ differential expression

To investigate the expression pattern of the 877 identified ORs in the antennae and palps, we assessed their abundance in both tissues using the RSEM method (**Suppl. Fig. 5**). As expected, the highly conserved olfactory co-receptor Orco was expressed equally in antennae and palps in all species and presented the highest expression level across all samples (e.g. 1065 and 1238 TPM in antennae and palps of *B. psidii*, respectively) (**Suppl. Table 4**).

In all species studied, we found that the two organs presented distinct OR expression patterns. Antennae expressed more ORs than palps, with 36 to 48 ORs per species in antennae and 8 to 13 ORs in palps (Fig. 1**b**), and little overlap was observed between these two organs. Overall, the ORs that were found to have an antennae-biased expression, accounting for at least 50 % relative to the abundance of Orco, belonged to the main following clades: OR7a, OR10a, OR13a, OR35a, OR42a, OR43a, OR47b, OR59a, OR63a, OR67c, OR67d, OR69a, OR74a, OR88a and OR94b clades. ORs that were found to be maxillary palps-enriched belonged to the main following clades: OR33b, OR46a, OR59a-3, OR83a, OR85c and OR94a clades (**Suppl. Fig. 5**). In some species, ORs belonging to these clades showed contrasting expression between antennae and palps. Some were equally expressed in both organs (BcurOR7a-1, ZcucOR7a-11, and Bpsi/BzonOR59a-1), and some others were enriched in palps (Bumb/CquiOR59a-1, Bdor/BzonOR59a-1b, and BcurOR67c-3) (**Suppl. Fig. 5**).

In the three fruit fly species with biological RNAseq replicates (*B. dorsalis*, *B. tryoni,* and *Z. cucurbitae*), a statistical analysis has been conducted to investigate, tissue and sex differential expression (Fig. 3,

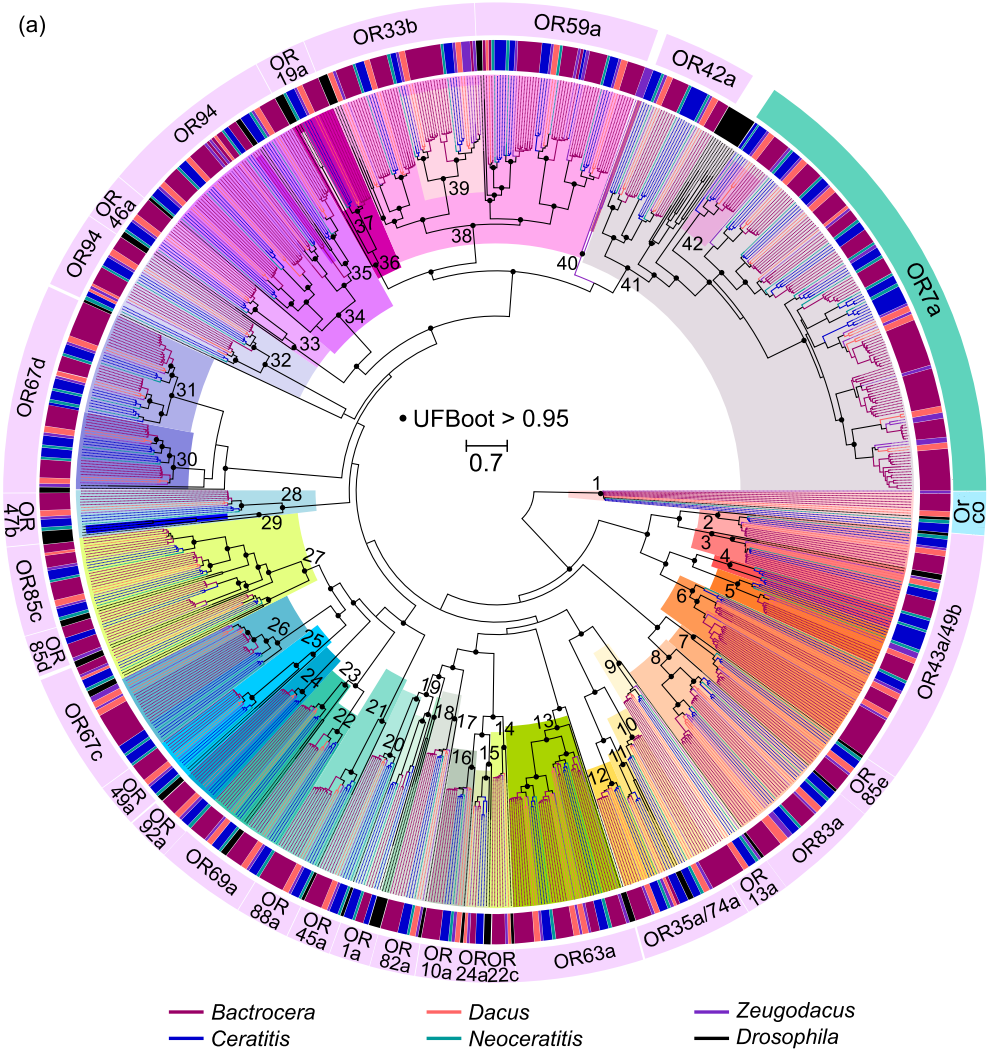

Fig. 2. Maximum likelihood phylogeny of the Tephritidae and *Drosophila melanogaster* ORs. (a) The tree was built from an alignment of 877 amino acid sequences from the 13 tephritid species and 62 from *D. melanogaster* (available in Suppl. Table 3). The odorant coreceptor Orco clade is used as an outgroup. The best substitution model used was calculated according to the Bayesian Information Criterion and corresponded to JTT + F + R8. Black circles indicate deep nodes highly supported by the ultrafast bootstrap method (UFBoot > 0.95, n = 1000). For aesthetic consideration, bootstraps are represented only at the terminal branches. The scale bar indicates the expected number of amino acid substitutions per site. ORs were classified in 42 orthogroups using OrthoFinder, with each orthogroup's basal node labeled 1 to 42 and its subtree highlighted in a different color. In the outer circle, OR clades are colour coded as follows: blue for the Orco clade, green for the OR7a clade, purple for OR clades with *D. melanogaster* members. In the inner circle, colours represent the species genus (one to six species per genus). **(b)** Phylogenetic tree showing the remarkable expansion of OR7a in the Tephritidae family. The branch support values are indicated (UFBoot, n = 1000). The scale bar indicates the expected number of amino acid substitutions per site. Bdor = *B. dorsalis*, Bzon = *B. zonata*, Bcur = *B. curvipennis*, Bpsi = *B. psidii*, Bumb = *B. umbrosa*, Btry = *B. tryoni*, Dcil = *D. ciliatus*, Ddem = *D. demmerezi*, Zcuc = *Z. cucurbitae*, Ncya = *N. cyanescens*, Ccap = *C. capitata*, Ccat = *C. catoirii*, Cqui = *C. quilicii*, Dmel = *D. melanogaster*.

raw data available in Suppl. Table 4). Consistent with our observations in the transcript abundance analysis, the current differential expression analysis highlighted important differences in the expression of ORs across tissues: 48, 42, and 39 ORs exhibited over-expression in the antennae compared to the maxillary palps, and 12, 11, and 13 ORs were found to be under-expressed in the antennae compared to the maxillary palps in *B. dorsalis*, *B. tryoni,* and *Z. cucurbitae*, respectively. According to sex, we observed six and 11 female-biased ORs in, respectively, *B. dorsalis* and *Z. cucurbitae*. For instance, OR33b-3 was expressed significantly more in the maxillary palps of *B. dorsalis* females than in males (FDR < 10^{-21}). Although not significant, this OR also appears to be more expressed in *B. tryoni* (FDR = 0.243) and *Z. cucurbitae* (FDR = 0.656) female palps than in males. ORs from the OR7a-2 clade also exhibited significantly higher expression levels in *B. dorsalis* female antennae compared to male ones (FDR < 10^{-11}), and the same tendency was observed although not statistically significant in *B. tryoni* (FDR = 0.619).

3.5. Correlation between OR repertoires and host range

We initially conducted a PGLS analysis to test whether the number of OR genes in a species depends on its host range (polyphagous *vs.* oligophagous). However, the analysis did not reveal a significant relationship (F(1,11) = 1.66, p = 0.22, PGLS). Importantly, the total OR gene count does not fully capture the dynamics of gene gain and loss, as species with identical OR gene counts may differ substantially in the proportion of orthologous genes they share. To better account for these dynamics, we examined pairwise species distances at the phylogenetic, ecological, and OR repertoire levels.

The phylogenetic distance between each combination of two species

Fig. 2. (*continued*).

was estimated using 19 BUSCO reference sequences. Between any two species in our dataset, less than 12 % amino acid substitutions in these sequences was observed, and this proportion was even less than 7 % for any two species of the same tribe and less than 4 % for any two species of the same genus. The 78 combinations of two species were categorized according to their host range. No obvious correlation was observed

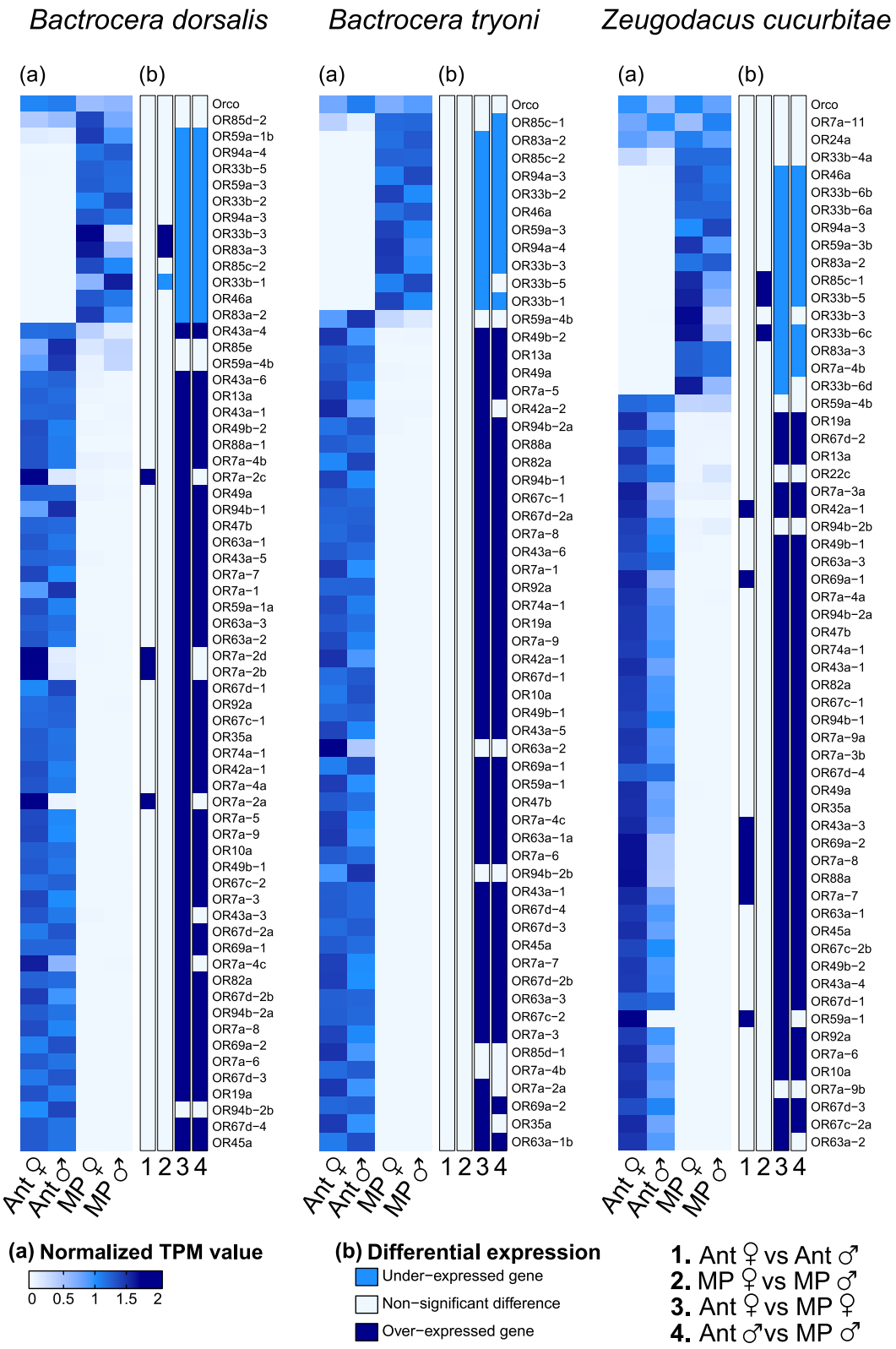

Fig. 3. Differential expression profiles of *B. dorsalis*, *B. tryoni* and *Z. cucurbitae* ORs according to tissues and sexes. (a) Heatmaps showing the normalized TPM values of ORs in female antennae (Ant ♀), male antennae (Ant ♂), female maxillary palps (MP ♀), and male maxillary palps (MP ♂). The colour coding is based on TPM values normalized by gene, ranging from light blue (low expression) to dark blue (high expression). **(b)** Differential expression status of ORs (FDR < 0.1, $-1 <$ logFC > 1) between the different conditions: (1) female antennae *vs* male antennae; (2) female maxillary palps *vs* male maxillary palps; (3) female antennae *vs* female maxillary palps; (4) male antennae *vs* male maxillary palps. ORs that showed no significant differential expression between two conditions are shown in light blue, ORs that were significantly under-expressed in the first condition compared to the second are shown in blue, and ORs that were significantly over-expressed in the first condition compared to the second are shown in dark blue. TPM values and differential expression raw data are available in **Suppl. Table S4**.

between host range and phylogenetic distance (Mantel test, r = 0.15, p = 0.17).

First, we found that the number of OR orthogroups shared between two species, while significantly correlated with species phylogenetic distance (Mantel test, r = 0.572, $p < 10^{-3}$), was also correlated with host range category (partial Mantel test, ρ = 0.57, p = 0.005). More specifically, by looking at the residuals after subtracting the linear effect of phylogenetic distance, we found on average 6.27 more orthogroups shared between two polyphagous species than shared between other pairs of species (bootstrap 95 % confidence interval: 4.5–8.0) (Fig. 4**a**). For instance, OR7a-12 was expressed in all eight-polyphagous species and no oligophagous species (Fig. 2**b**). OR85d-1 was expressed in six polyphagous species (four *Bactrocera* and two *Ceratitis*) and no oligophagous one. Three ORs were found in all polyphagous *Bactrocera* species and specifically in them, but not in the oligophagous *B. umbrosa*: OR7a-2e, OR7a-4c and OR43a-6 (Fig. 2**b** and **Suppl. Fig. 5**).

Secondly, we found that the relative expression level of ORs in antenna and maxillary palp, normalised by the expression level of Orco, was correlated most of the time between two species (F(1,82 to 124) = 1.24 to 574; $p < 0.05$ for 73 out of 78 pairs of species). The Pearson's correlation coefficient depended significantly on host range category (partial Mantel test, ρ = 0.35, $p = 0.02$) and species phylogenetic distance (Mantel test, r = 0.23, $p = 0.055$) (Fig. 4**b**). Thus, correlation of OR expression levels was stronger between polyphagous species than between other combinations of species. Looking at each olfactory organ independently, the effect of host range category was significant for the maxillary palp (partial Mantel test, ρ = 0.35, $p = 0.03$) but not for the antenna (partial Mantel test, ρ = −0.54, $p = 0.38$).

Finally, we selected 38 sets of orthologous ORs for which we got complete amino acid sequences for at least two polyphagous species and two oligophagous species in different genera. For 14 among them, the percent of amino acid substitutions between two species correlated significantly with host range category of the pair (Fig. 4**c**). Eleven of them were more conserved among polyphagous species (Fig. 4**d–o**). These were OR13a (partial Mantel test, ρ = 0.63, $p = 0.002$), OR45a (ρ = 0.63, $p = 0.002$), OR47b (ρ = 0.50, $p = 0.005$), OR19a (ρ = 0.43, p = 0.009), OR7a-6 (ρ = 0.45, $p = 0.009$), OR85c-1 (ρ = 0.45, $p = 0.01$), OR67d-3 (ρ = 0.38, $p = 0.014$), OR49b-1 (ρ = 0.42, $p = 0.031$), OR67d-1 (ρ = 0.30, $p = 0.034$), OR63a-1 (ρ = 0.46, $p = 0.039$), and OR69a-2 (ρ = 0.32, $p = 0.044$). Inversely, the sequences of three ORs were less conserved among polyphagous than among oligophagous species: OR43a-1 (ρ = −0.54, $p = 0.007$), OR92a (ρ = −0.51, $p = 0.003$), and OR67c-1 (ρ = −0.55, p = 0.003) (Fig. 4**p–r**).

4. Discussion

At evolutionary time scales, genomic modifications that alter an OR repertoire accumulate. These modifications can affect the OR sequence itself via punctual mutations, change the total number of ORs in the repertoire of a species via gene duplication and loss, or alter the relative abundance and expression pattern of OR transcripts when the modifications occur in regulatory regions (Miller and Carlson, 2010; Song et al., 2012; Andersson et al., 2015; Barish and Volkan, 2015). Thus, OR sequence identity and number, as well as their relative abundance and expression pattern, are bound to differ increasingly with phylogenetic distance between species. This process of diversification is tempered by the selection of olfactory properties adapted to the species' ecological needs.

Here, we cumulated new transcriptomic data and conducted a comprehensive comparative analysis of the OR repertoires from 13 Tephritidae species adequately chosen as distributed over the phylogenetic and ecological range within this family. Taken together, our results bring convincing evidences that species' host range may have contributed in shaping OR characteristics in Tephritidae.

First, we *de novo* assembled olfactory transcriptomes from adult antennal and palp tissues of 13 Tephritidae species. Remarkably, while the number of published Tephritidae OR sequences multiplied exponentially in the recent years (Papanicolaou et al., 2016; Miyazaki et al., 2018; Cheng et al., 2020; Ono et al., 2020; Wu et al., 2020; Jiang et al., 2022; Guo et al., 2023; Xu et al., 2023) our high-quality sequencing and assemblies enabled us to identify 698 entirely novel ORs in Tephritidae. For instance, we could identified novel ORs that were not reported previously in the emblematic and deeply studied species *B. dorsalis*, *Z. cucurbitae*, and *C. capitata* (Papanicolaou et al., 2016; Ono et al., 2020; Xu et al., 2023). We failed to retrieve some ORs previously identified through genome analyses (Papanicolaou et al., 2016; Xu et al., 2023), suggesting that these ORs are not expressed in our specific conditions (sexually mature adult stage, antennae and palp tissues) and possibly expressed at a different developmental stage or in different tissues (legs, ovipositor, etc). More, as we sequenced antenna and maxillary palp transcriptome independently, we revealed new ORs specific to the maxillary palp, e.g. OR33b or OR59a-3, that have been missed in transcriptomic studies focused only on antennae in *B. dorsalis* and *Z. cucurbitae* (Wu et al., 2020). In addition, we identified the OR repertoires of Tephritidae species never investigated before, such as *B. curvipennis*, *B. psidii*, *B. umbrosa*, *B. zonata*, *C. catoirii*, *C. quilicii*, *D. ciliatus*, *D. demmerezi*, and *N. cyanescens*.

Then, we used this newly established OR dataset to reconstruct a maximum likelihood phylogeny of ORs within the Tephritidae family. *Drosophila melanogaster* ORs were included in the phylogeny because they have been functionally studied extensively and can serve as a benchmark for comparison. Consistent with the findings by Jacob et al. (2017), we reported that approximately half of the *D. melanogaster* OR repertoire was conserved across Tephritidae species. Some OR clades exhibited limited evolutionary expansion, with only one OR member per Tephritidae and *Drosophila* species. These highly conserved ORs may play fundamental roles in basic olfactory functions shared among Tephritidae members and *D. melanogaster*. For instance, a few Tephritidae ORs belonging to these clades have been functionally characterized and indeed shared the same ligand as their *D. melanogaster* ortholog: BdorOR13a responds to 1-octen-3-ol (Xu et al., 2023); BdorOR82a responds to geranyl acetate (Miyazaki et al., 2018); and BdorOR74a responds to 1-nonanol (Ono et al., 2020). Some other OR clades exhibited considerable divergence between *D. melanogaster* and Tephritidae but also within the Tephritidae family, such as the OR7a subfamily (Lin et al., 2015; Miyazaki et al., 2018; Guo et al., 2023) that contained only one *D. melanogaster* representative and many Tephritidae OR expansions. *Drosophila* OR7a encodes, in part, for plant or fruit compounds (Hallem and Carlson, 2006; Lin et al., 2015). The most apparent ecological difference between Tephritidae and Drosophilidae is the way they exploit fruit resources, with the species included in our study ovipositing essentially in fresh fruits (White and Elson-Harris, 1992; Charlery de la Masselière et al., 2017a). Thus, the significant expansion of the OR7a clade may have resulted from an ancient divergence in how the two families exploit plant volatiles. Cases of adaptation to specific ecological niches or behaviours in Tephritidae might be revealed by further functional studies of those ORs.

Next, we compared OR number, conservation, and expression pattern and level between the 13 Tephritidae species, according to their phylogenetic distance and degree of specialisation. OR number, expression pattern in antennae and palps, and sex enrichment were globally homogenous within the considered species, with slight variations according to species. Consistent with previous findings by Xu et al. (2023), the two organs expressed distinct panels of ORs, with few overlaps. The majority of ORs identified in the 13 Tephritidae species were present in the antennae, while only a quarter were observed in the maxillary palps, in low to high abundance. Few ORs were found to be expressed in both antennae and maxillary palps in all the species studied, which extends a previous observation in *B. dorsalis* (Xu et al., 2023). Similar overlap in OR expression in both structures was also observed in other species including the Diptera *Anopheles gambiae* (Iatrou and Biessmann, 2008), the Lepidoptera *Manduca sexta* (Tom et al., 2022),

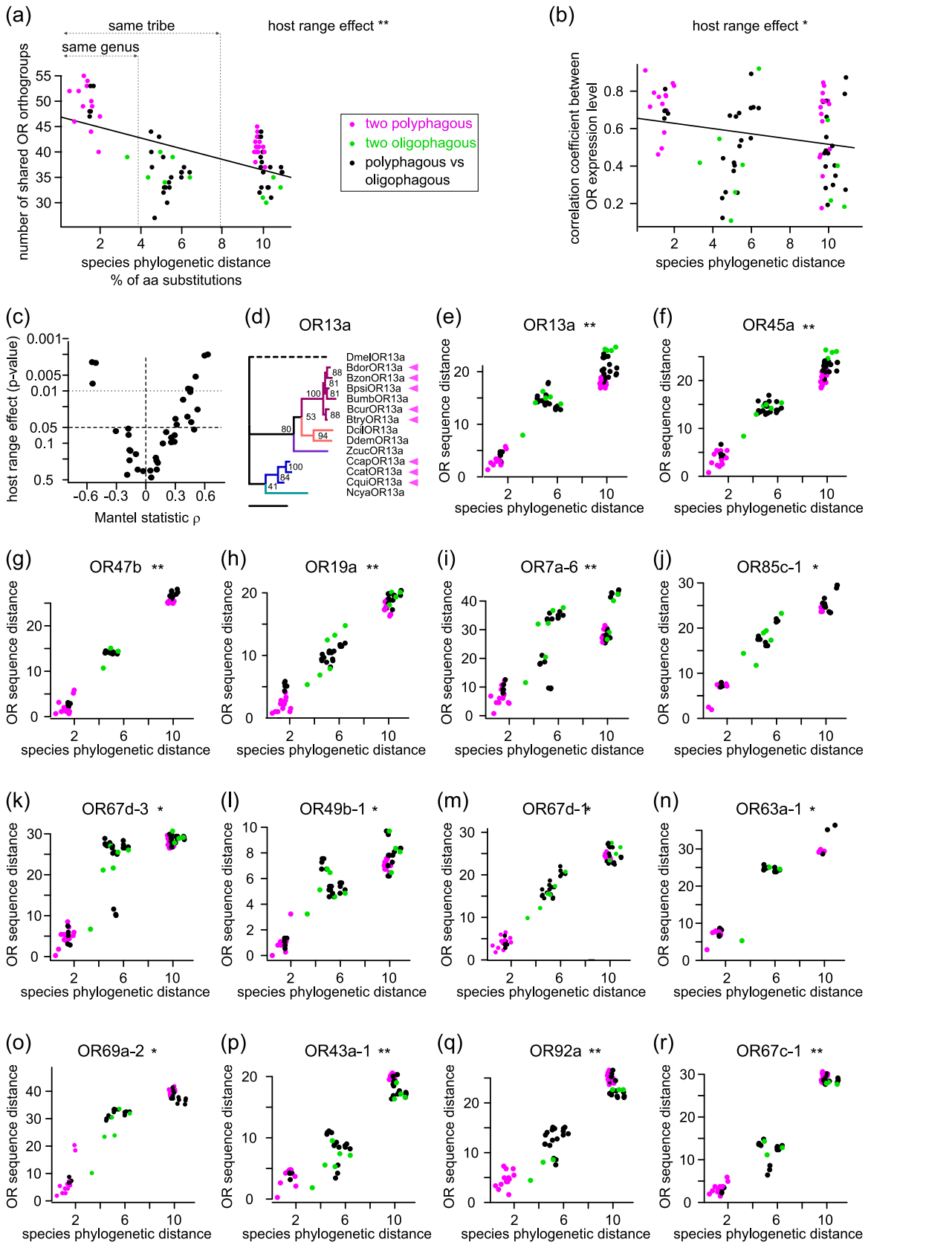

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Fig. 4. Molecular distance of ORs between pairs of species depends on host range. (a) The number of OR orthogroups between two species correlates significantly with host range. Each dot represents a comparison between two Tephritidae species. Host range category for combinations of two species are color-coded. Index of species phylogenetic distance stands for a percentage of amino acid substitutions between sets of reference genes from the BUSCO repertoire (X-axis). Values corresponding to a pair of species from the same genus or from the same tribe are indicated. The oblique line shows the linear regression between this number and species phylogenetic distance. Partial Mantel test, effect of host range conditioned on phylogenetic distance: ** p-value < 0.01. **(b)** Correlation between the relative expression level of ORs is significantly higher between two polyphagous species than for other combinations of host range (Partial Mantel test, effect of host range conditioned on phylogenetic distance: * p-value < 0.05). Same convention as in panel (a). **(c)** Volcano-type plot showing, for each OR clades with one OR per species, the p-value of a partial Mantel test comparing OR sequence distance (% aa substitution) with host range, conditioned on phylogenetic distance. The x-axis shows the Mantel statistic ρ (partial Pearson correlation coefficient). ORs more conserved between polyphagous species than others have a positive value, ORs less conserved have a negative value. **(d)** Phylogenetic tree reconstructed for OR13a, the clade with the highest p-value. Magenta arrowheads design polyphagous species, which have shorter branches. **(e–o)** Eleven OR clades whose sequences are significantly more conserved among polyphagous species than among others (partial Mantel test, *: p-value < 0.05, **: p-value < 0.01). Same conventions as in panel (a). **(p–r)** Three OR clades whose sequences are significantly less conserved among polyphagous species than among others. Same conventions as in panel (e).

and the Orthoptera *Locusta migratoria* (Li et al., 2018). In *D. melanogaster*, however, a mutually exclusive expression of ORs in antenna and palp tissues was observed, which might be specific to this lineage (Boronat-Garcia et al., 2022). Functional investigation of palp ORs in Tephritidae might be particularly relevant in the future, since this organ plays a crucial role in detecting male attractants and sexual pheromones (Chieng et al., 2018; Park et al., 2018; Verschut et al., 2018; Oh et al., 2019; Biswas et al., 2020; Noushini et al., 2020; Jacob et al., 2023). As some olfactory behaviours are sex-specific and involve specific volatile compounds, such as pheromones, male attractants or host-plant volatiles, we analysed the differential expression of ORs between sexes in three species, *B. dorsalis*, *B. tryoni*, and *Z. cucurbitae*. Few ORs were significantly over-expressed in females compared to males in *B. dorsalis* and *Z. cucurbitae*, but not in *B. tryoni*, as was the case for the *B. dorsalis* OR7a-2 variants. While this observation is in accordance with the previous study by Wu et al. (2020), other studies in *B. dorsalis* did not evidence expression level difference between male and female ORs (Miyazaki et al., 2018; Ono et al., 2020; Xu et al., 2023). The female-enriched ORs may detect volatile cues particularly determinant for females, such as oviposition cues.

Looking at the diversity of the OR repertoire across Tephritidae, we revealed that a few ORs specifically equipped polyphagous species, irrespectively of their phylogenetic position. Additionally, the sequences of 11 ORs were more conserved in polyphagous species that what would be expected based on phylogenetic distance and shared selection pressures acting on all Tephritidae species. Indeed, apart from their host range, the overall ecology of polyphagous and oligophagous species does not differ fundamentally, with adults feeding on the same sources of proteins (Piñero et al., 2011). The polyphagous species included in our study are attracted to and develop on the same range of fruits, while the oligophagous species are focused on different and specific range of fruits (Charlery de la Masselière et al., 2017a, 2017b; Moquet et al., 2021). Accordingly, similar antennal response to fruit volatile compounds were observed in two polyphagous but not in an oligophagous Tephritidae species (Biasazin et al., 2019). These functional observations may be underpinned by the polyphagous-related ORs we have reported. These ORs would be interesting targets for functional investigation related to fruit volatile compound detection. Moreover, the relative abundance of palp ORs was remarkably similar between most polyphagous species, which echoes a recent functional study that showed correlated palp responses to fruit compounds between two polyphagous species, *B. dorsalis* and *C. capitata*, whereas the palp responses of the oligophagous *Z. cucurbitae* was tuned to other volatile compounds (Dekker et al., 2024).

5. Conclusions

Altogether, our work provides abundant new resources in Tephritidae, including species whose chemosensory proteins were never investigated so far. We pinpoint ecologically relevant ORs for further functional studies, and, last but not least, provide molecular insights into the ecological driving force behind Tephritidae OR evolution, as OR divergence and expression level clearly correlated with the species degree of specialisation.

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CRediT authorship contribution statement

Emma Persyn: Writing – original draft, Methodology, Formal analysis, Data curation. **Pierre-François Duyck:** Writing – review & editing, Resources. **Marie-Christine François:** Writing – review & editing, Resources, Methodology. **Christian Mille:** Writing – review & editing, Resources. **Vincent Jacob:** Writing – review & editing, Resources, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Emmanuelle Jacquin-Joly:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2025.108322.

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