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ORIGINAL PAPER

Functional and molecular evolution of olfactory neurons and receptors for aliphatic esters across the *Drosophila* genus

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Abstract Insect olfactory receptor (*Or*) genes are large, rapidly evolving gene families of considerable interest for evolutionary studies. They determine the responses of sensory neurons which mediate critical behaviours and ecological adaptations. We investigated the evolution across the genus *Drosophila* of a subfamily of *Or* genes largely responsible for the perception of ecologically relevant aliphatic esters; products of yeast fermentation and fruits. Odour responses were recorded from eight classes of olfactory receptor neurons known to express this *Or* subfamily in *D. melanogaster* and from homologous sensilla in seven other species. Despite the fact that these species have diverged over an estimated 40 million years, we find that odour specificity is largely maintained in seven of the eight species. In contrast, we observe extensive changes in most neurons of the outgroup species *D. virilis*, and in two neurons across the entire genus. Some neurons show small shifts in specificity, whilst some dramatic changes correlate with gene duplication or loss. An olfactory receptor neuron response similarity tree did not match an Or sequence similarity tree, but by aligning Or proteins of likely functional equivalence we identify residues that may be relevant for odour specificity. This will inform future structure–function studies of *Drosophila* Ors.

Keywords Olfaction · Evolution · Odour response profile · Receptor neurons · Olfactory receptors

Abbreviations

ORN	Olfactory receptor neuron
Or	Olfactory receptor
PCR	Polymerase chain reaction

Introduction

Insect olfactory systems are excellent models to study interactions between animal genomes and the environment, as they have evolved specifically to monitor volatile chemicals that provide information about vital resources and potential dangers. Insects inhabit many diverse ecological environments and exhibit vast differences in food sources. For example many *Drosophila* species are generalists that feed on a broad diet of fermenting fruits, whilst some specialise on particular host plants (Dekker et al. 2006), and others utilise other food sources such as fungi or tree sap (Markow and O'Grady 2005). In *D. melanogaster* odours are detected by different functional classes of olfactory receptor neurons (ORNs), with individual ORNs responding to multiple odorants, and most odorants being detected by multiple classes of ORN, though typically with different sensitivities (de Bruyne et al 1999; de Bruyne et al. 2001). Insect behavioural responses to ecologically relevant odours typically involve mixtures of odorants and multiple ORN classes (Hildebrand and Shepherd 1997). It is likely that insect olfactory systems have adapted to detect particular ecologically relevant odorants or odorant combinations. However, the evolution of odour detection has predominantly been studied in pheromone communication systems. A comparison of a set of non-pheromonal ORNs suggests

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that response properties are mostly conserved across closely related *Drosophila* species within the melanogaster subgroup (Stensmyr et al. 2003).

The responses of most insect ORNs are reliant on members of an insect-specific family of odorant receptor (*Or*) genes (Clyne et al. 1999; Vosshall et al. 1999), which constitutes one of the largest and most diverse gene families in insects. Insect *Or* families have been identified from the pea aphid (Smadja et al. 2009), the honeybee (Robertson and Wanner 2006), the flour beetle (Engsontia et al. 2008), the silkmoth (Wanner et al. 2007), two species of mosquitoes (Hill et al. 2002; Bohbot et al. 2007) and 12 species of *Drosophila* (Guo and Kim 2007; Nozawa and Nei 2007; McBride and Arguello 2007; Gardiner et al. 2008). Comparison of *Or* genes within a species or between species clearly shows that the *Or* gene family has undergone rapid evolution. This raises two interesting questions, how conserved are ORN identities, and to what extent do *Or* sequence changes reflect changes in functional properties?

The insect Or proteins are quite different to vertebrate Ors, which are G protein-coupled receptors. Insect Ors also have seven-transmembrane domains, but they have an inverted membrane topology compared to G protein-coupled receptors (Benton et al. 2006; Smart et al. 2008), and appear to form a novel class of heteromeric cation channels that are gated directly by odorants (Sato et al. 2008; Wicher et al. 2008). It is currently unknown how these receptors bind odorants and regulate ion permeability. Molecular evolutionary studies of Ors in the 12 *Drosophila* species whose genomes have been sequenced have indicated positive selection at particular sites (Guo and Kim 2007; McBride and Arguello 2007; Tunstall et al. 2007; Gardiner et al. 2009), however, the role of these sites in Or function has not been explored. Thus, the consequences of receptor sequence changes for ORN physiology and behavioural ecology are not known.

Conservation of neuronal identity and odour response has been shown in some ORNs of closely related species of moths (Stranden et al. 2003), tephritid flies (Olsson et al. 2006) and *Drosophila* (Stensmyr et al. 2003), but how does ORN response vary when species are more divergent and Or sequences change considerably? We aimed to determine response variability across more distantly related species to explore how ORNs evolve and to assess the functional consequences of *Or* gene evolution. *Drosophila* species are ideal for such a study because *Or* genes have been identified from the sequenced genomes of 12 species of *Drosophila* (Guo and Kim 2007; McBride and Arguello 2007) and their expression in *D. melanogaster* can be linked to clearly defined and unique ORN phenotypes and thus to their ligands (de Bruyne et al 1999; de Bruyne et al. 2001; Couto et al. 2005; Hallem and Carlson 2006). The combination of *Or* sequence information from many species with the ability to measure their function provides a unique opportunity to study sequence-response relationships.

Here, we determine the responses of a group of ORNs to a specific set of ligands over more than 40 million years of evolution in the *Drosophila* genus. We show conservation of neuronal phenotypes in many species, as well as divergence at high phylogenetic distance, and some examples of specific functional changes. By comparing receptors that are highly likely to be functionally equivalent with those that are not, we determine conserved functional residues and putative specificity determining residues for this Or subfamily. This provides important information for future structure–function studies.

Materials and methods

Fly stocks and rearing conditions

All flies were reared on yeasted semolina/syrup medium in 40 ml vials at 22°C and normal daylight. *D. melanogaster* was the standard CS-5 strain used in many other olfactory studies (Helfand and Carlson 1989). The following stocks were from the Tucson *Drosophila* stock centre: *D. simulans* (4021-0251.169), *D. mauritiana* (14021-0241.01), *D. pseudoobscura* (14011-0121.94), *D. willistoni* (14030-0814.10), and *D. virilis* (15010-1051.87). The SE Asian species *D. ananassae* and *D. serrata* were collected from Northern Queensland and reared in the lab for at least 30 generations.

Electrophysiological recordings from single olfactory sensilla

The basic recording technique was described elsewhere (de Bruyne et al 1999; de Bruyne et al. 2001). A 4–10-day-old male fly was immobilized in a plastic pipette tip. Recordings were made from AgCl-coated silver wire inserted in saline filled glass capillaries (0.015 M KCl). One microelectrode was inserted through the wall of a single olfactory sensillum to contact the lymph surrounding the dendrites of the ORNs. The reference electrode was inserted in the eye. Signals were amplified 1,000× via a 10× active probe fed into an AD converter with digital amplification (USB-IDAC, Syntech, Hilversum, the Netherlands). Responses were analysed offline using Autospike software (Syntech, Hilversum, the Netherlands). Odour responses (ΔF) were calculated from action potentials counts during, and prior to stimulation (500 ms). We analysed only recordings in which action potentials could be reliably attributed to the activity of a single neuron, based on amplitude differences (de Bruyne et al. 2001). Odour response spectra were

established from 7 to 15 sensilla from at least three different flies.

Odour stimulation

Stimulation with odorants was as in de Bruyne et al. (2001). Specifically, a glass tube held 5 mm from the preparation supplied continuous humidified air at 66 cm/s (zero grade, BOC, Sydney, Australia). Volatiles were injected into the air for a 500 ms period from 5 ml disposable syringes holding 10 µl of odorant solution on filter paper, giving a headspace dilution factor of 10%. All odorants were at highest available purity (>98%, Aldrich, Milwaukee, WI or Fluka, Buchs, Switzerland) and dissolved in paraffin oil (Fluka, Buchs, Switzerland) at 10^{-2} or 10^{-4} v/v (see Fig. 2).

Odour response data analysis

To verify our classification of ORNs and determine the similarity of response properties we performed a hierarchical cluster analysis of all mean odour response spectra using Ward's method in the SPSS software. To detect differences in responses to single odorants between response spectra considered similar by the cluster analysis we used a *T* test with Bonferroni corrections for multiple odorant comparisons.

Or gene sequences

Sequences for *D. melanogaster*, *D. simulans*, *D. ananassae*, *D. pseudoobscura*, *D. willistoni* and *D. virilis Or* genes were obtained from Flybase (http://flybase.bio.indiana.edu). For *D. mauritiana* the *Or42a*, *Or22a* and *Or22b* sequences are from Tunstall et al. (2007). We amplified the remaining *D. mauritiana* genes (*Or7a*, *Or42b*, *Or43b*, *Or59b*, *Or59c* and *Or85a*) from genomic DNA using polymerase chain reaction (PCR) primers designed to 5′ and 3′ regions outside the coding region of the respective *D. melanogaster* and *D. sechellia* orthologues. PCR amplification was performed in a Thermo Hybaid PCR machine in 50 µl reaction volumes containing 1–2 µg DNA, 1 µl Taq polymerase, 0.4 mM dNTPmix, 1× reaction buffer, 2.5 mM magnesium chloride, and 1 µM of each primer. The programme included an initial denaturation step for 5 min at 95°C and 33 amplification cycles (95°C for 30 s, 55–60°C for 1 min, 72°C for 2 min), then held at 72°C for 10 min. Annealing temperatures varied according to primer pairs used. Genes were amplified using proofreading Expand High Fidelity Taq polymerase (Roche, Mannheim, Germany). PCR products were cloned into the pGEM-T Easy vector. At least two independent clones were sequenced for each gene. Cycle sequencing was performed using Big Dye version 3.1 (Applied Biosystems, CA) under standard conditions with gene specific forward, reverse, or internal primers. Sequencing reactions were purified using the Applied Biosystems ethanol/NaAc/EDTA precipitation protocol and resolved on an ABI 3100 automated sequencer. All newly amplified sequences are available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank) (accession numbers GU339213-18).

Results

A subfamily of *Or* genes mediates responses to aliphatic esters

In order to study whether ORN identity is conserved in the *Drosophila* genus, and whether differences in odour response properties correlate with differences in *Or* gene sequence, we chose eight *Drosophila* species spanning 40 million years of evolution (Fig. 1a). *D. melanogaster*, *D. simulans* and *D. mauritiana* belong to the African Melanogaster species subgroup. *D. annanassae* and *D. serrata* are representative species of the Oriental/Australasian Annanassae and Montium species groups, respectively. *D. pseudoobscura* and *D. willistoni* represent successive sister lineages within the subgenus Sophophora, whilst *D. virilis* is the outgroup representing the subgenus Drosophila.

The response profiles of 37 *D. melanogaster* Or proteins to a fairly large set of odorants have previously been analysed (Hallem and Carlson 2006; Kreher et al. 2008). We performed a comparative analysis of these response spectra that clearly shows that the majority of the responses to aliphatic esters reside in subfamily H (Fig. 1b), the largest and most highly conserved subfamily. Seven of the nine members of this subfamily preferentially respond to esters, which are important components of fruit aromas and also products of yeast fermentation. We chose to focus on this *Or* subfamily to investigate the evolution of odour coding in general, and of responses to aliphatic esters in particular, and will refer to it as the ester subfamily.

For all nine receptors in this subfamily expression has been mapped to a known ORN class in *D. melanogaster*, and thus detailed ligand information is known for this species (Hallem and Carlson 2006; Couto et al. 2005). The ORN class in which each receptor is expressed, and its best known ligand, is shown in Fig. 1c.

Homology of ester-responsive ORNs across *Drosophila* species

We targeted eight ORN classes that express ester subfamily members in *D. melanogaster* and recorded their odour response spectra across eight *Drosophila* species, a total of

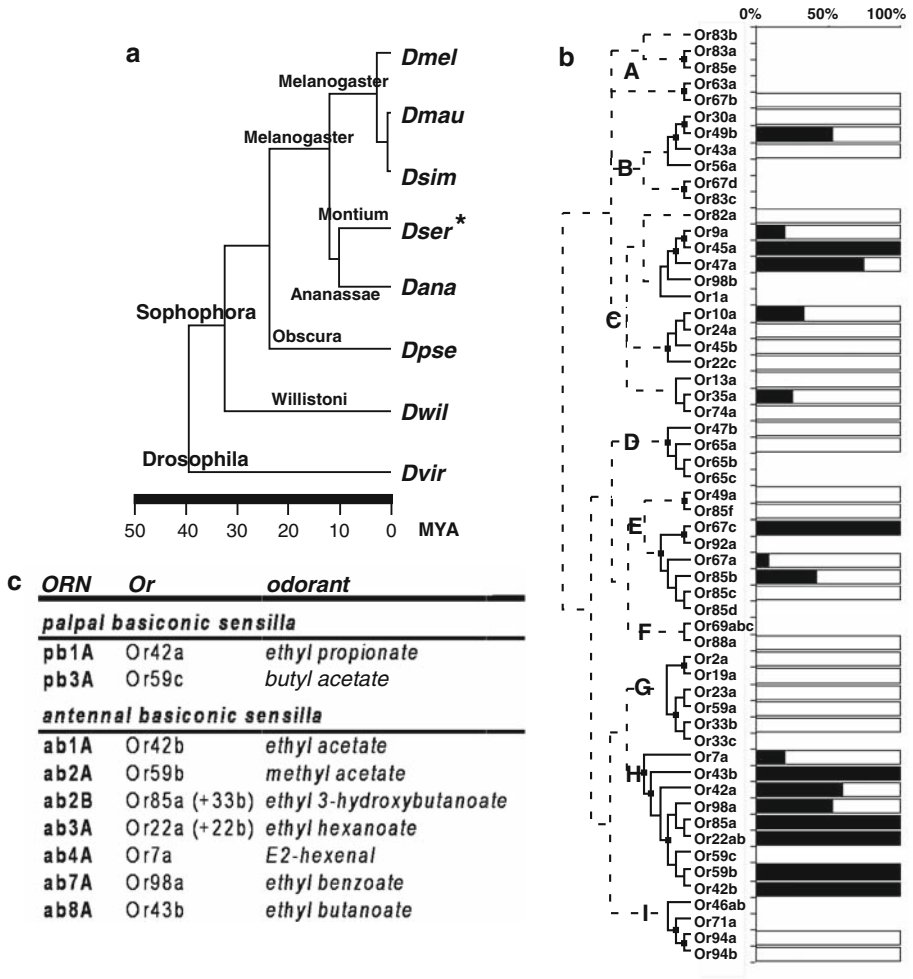


Fig. 1 A subfamily of Or proteins in Drosophila preferentially responds to esters. **a** Phylogenetic tree of the species used in this study, from (Russo et al. 1995) modified with data from Schawaroch (2002). *Dmel*, *D. melanogaster*; *Dmau*, *D. mauritiana*; *Dsim*, *D. simulans*; *Dser*, *D. serrata*; *Dana*, *D. ananassae*; *Dpse*, *D. pseudoobscura*; *Dwil*, *D. willistoni*; *Dvir*, *D. virilis*. The *asterisk* indicates that we have no *Or* sequences for *D. serrata*. **b** Summary of the phylogenetic relationship of Or proteins and their odour responses in *D. melanogaster*. *Dotted lines* in the tree are clades that vary in different studies. Subfamilies (*capitals*) are as defined by McBride and Arguello (2007). The *black bars* show the percentage of high responses (>180 spikes/s) of each receptor which is to aliphatic ester compounds (data from Hallem and Carlson 2006; Kreher et al. 2008). No bars are shown for receptors with no available ligand data. **c** Summary of the subfamily H receptors, the neuronal class in which they are expressed, and their best known ligands (data from de Bruyne et al 1999: Hallem and Carlson 2006; Kreher et al. 2008). Note that the best known ligands for Or7a and Or98a are not aliphatic esters

64 neuron types. We did not include *Or98a* in our analysis because the ab7A neuron which expresses this gene is difficult to differentiate from ab6A. We define homology of ORNs based on three criteria; occurrence in large or small basiconic sensilla, conservation of pairing with a particular ORN class seen in *D. melanogaster*, and similarity of odour response to the ORNs of *D. melanogaster*. The set of odorants we used to identify the eight targeted neuron classes includes nine aliphatic esters, as well as odorants from several other chemical classes (Fig. 2a). The odorant set was designed such that each neuron class has a robust response (>45 spikes/s) to at least two of the odorants. Four odorants were presented at a 100-fold lower dose (shaded grey in Fig. 2a) as the standard 10^{-2} dilution saturates the receptors in *D. melanogaster*.

We found that the organisation of ORN classes on the antenna and palp is generally conserved across these eight species. In most cases we were able to record from homologous sensilla that are similar to those in *D. melanogaster*. On the maxillary palps, the targeted pb1 and pb3 type sensilla were found in all species, except in *D. virilis* where we could not find pb3 sensilla. On the antenna, in all species the targeted ab1, ab2 and ab3 type sensilla were always large basiconic sensilla in the medio-distal region, and, as in *D. melanogaster*, ab1 sensilla invariably contained 4 ORNs, one of which responds to CO_2 (de Bruyne et al. 2001) (not

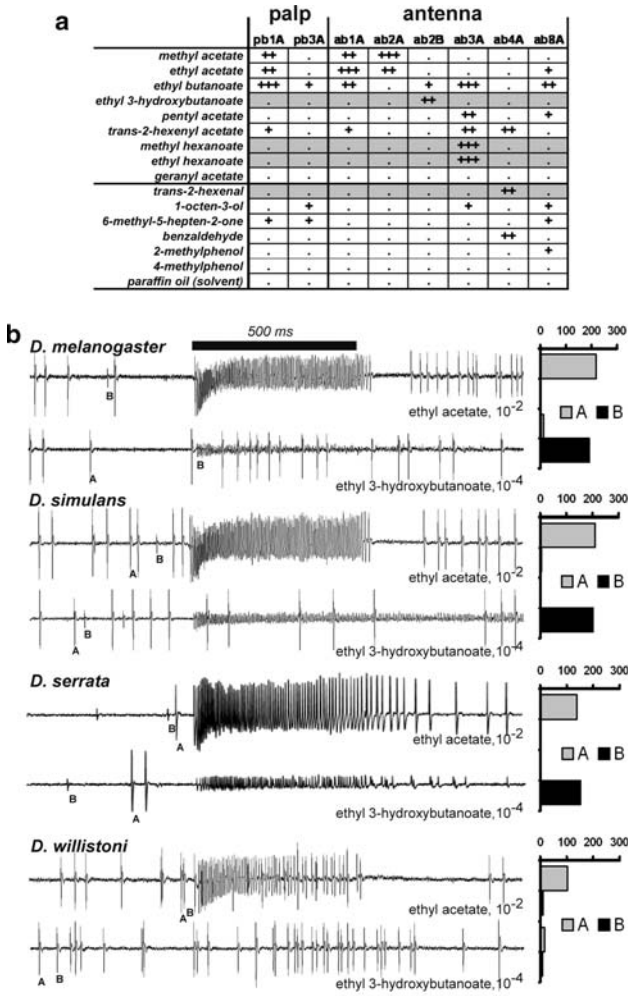


Fig. 2 Homologous sensilla and neuron classes can be identified across *Drosophila* species. **a** Diagnostic odour set containing nine aliphatic esters (shown in upper section of table) and the responses of the eight neuron classes in *D. melanogaster*. Responses are given as: high, +++, >245 spikes/s; medium, ++, 145–245 spikes/s; or low, +, 45–145 spikes/s. Neuronal nomenclature—*pb* palpal basiconic, *ab* antennal basiconic. Most odorants are tested as 10^{-2} dilutions in paraffin oil. Odorants for which responses are shaded in *grey*, which are strong stimulants, were tested at 10^{-4}. **b** Differences observed in ab2B sensillum phenotypes from four species. Traces show 1.5 s of activity around a 500 ms stimulation period (*horizontal bar*). Two different neurons (A and B) can be differentiated based on action potential (spike) amplitudes. *Bar graphs* to the right show responses in spikes/s. Note the similarity of responses in *D. melanogaster*, *D. simulans* and *D. serrata* and the consistently different amplitudes of spikes from A and B neurons. In contrast *D. willistoni* has a higher amplitude of B spikes and a lack of response to ethyl 3-hydroxybutanoate

shown). Other aspects of neuronal phenotypes were also mostly conserved, such as the typically large ratio between the spike amplitudes of ab2A and B neurons (Fig. 2b) and the very small amplitude of ab1D spikes (not shown). However, we did observe a few differences. For example, the ab2B neurons of *D. willistoni* and *D. virilis* fire spikes with much larger amplitudes than in *D. melanogaster* (Fig. 2b).

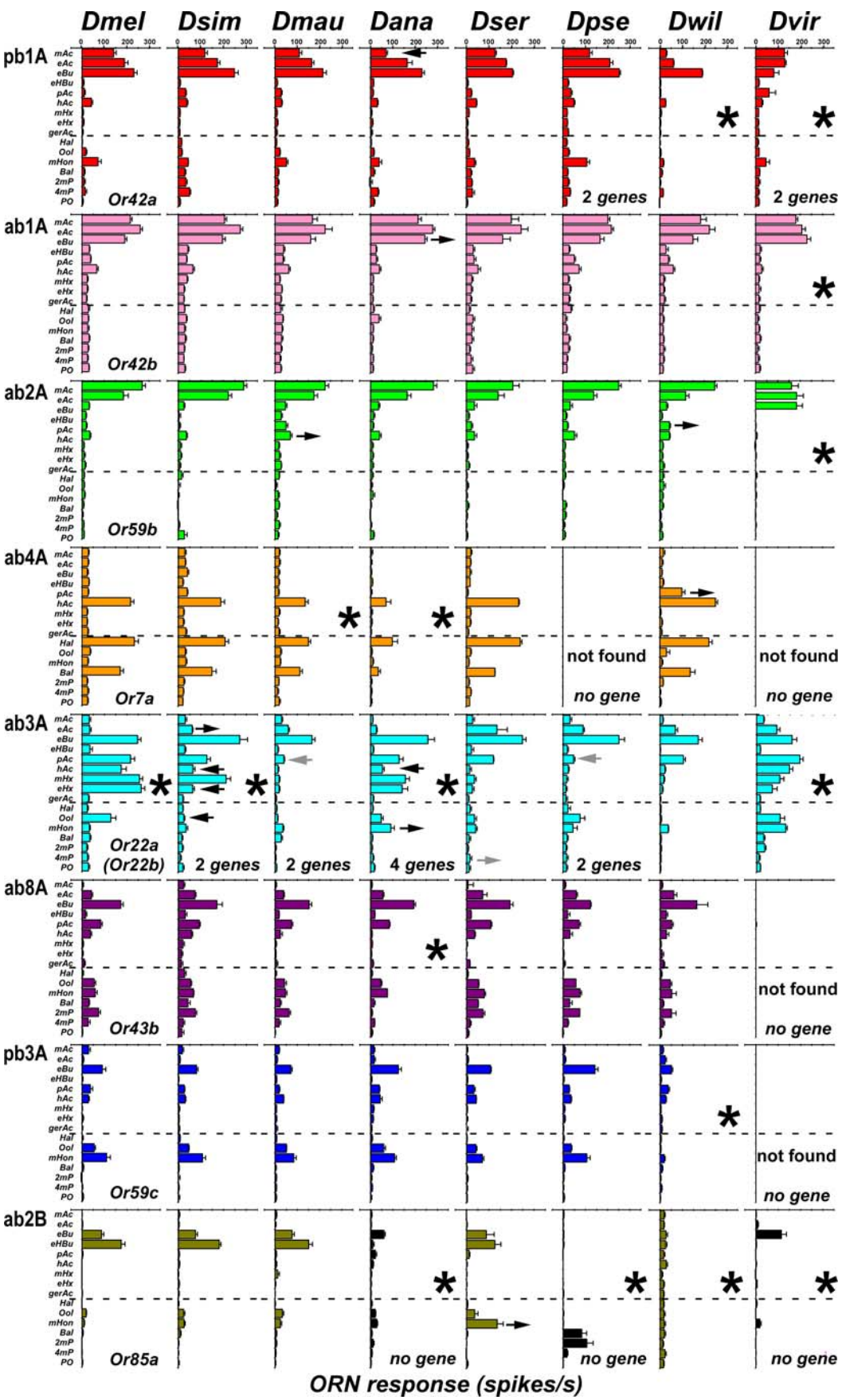
Many odour response spectra are conserved across Sophophora species

We determined responses to the set of 16 odorants for the eight ORN classes across the different species (Fig. 3). In order to objectively determine whether ORN responses have diverged from each other across the *Drosophila* genus we classified these response spectra using a hierarchical cluster analysis (Fig. 4). In general members of individual ORN classes tend to cluster together, indicating that the different ORN classes are broadly conserved in the *Drosophila* genus and thus may have originated prior to it.

For six of the eight classes (pb1A, ab1A, ab2A, ab4A, ab8A and pb3A) we found a high level of conservation of characteristic features of response spectra within the Sophophora subgenus (Fig. 3). However, in each case we see changes in the outgroup species *D. virilis*, which is in the Drosophila subgenus. The changes are of two types. Firstly, for three of the six classes, ab1A, ab2A, and pb1A, the *D. virilis* neuron is still present, but its response has changed. These three neuron classes are the most highly conserved in the Sophophora and show only minor changes within the subgenus (arrows). They form a subgroup in Fig. 4 which share distinctive responses to the short esters methyl acetate, ethyl acetate and ethyl butanoate (Fig. 3). By contrast, the *D. virilis* pb1A, ab1A and ab2A neurons are all classified outside the main cluster for the neuron type (Fig. 4). Secondly, and more dramatically, in the other three of the six classes, ab4A, ab8A and pb3A, the neuron was not found in *D. virilis*.

As well as these major differences in the outgroup species *D. virilis*, there are some other response changes (asterisks) in particular species of the Sophophora subgenus for some of these six neuron classes, for example, the ab4A neuron. This neuron has a very different response profile to the others, with the best ligand being E2-hexenal, not an ester. Its very characteristic spectrum, showing three distinct peaks, is basically conserved in all Sophophora species. However, in *D. mauritiana* and *D. ananassae* responses are significantly lower, whilst in *D. willistoni* a moderate response to an ester, pentyl acetate, is added to the spectrum. These changes are, however, not substantial enough to affect the neuron clustering in Fig. 4.

There are also three examples where we see change in a particular neuron in a single species. For two of these the change is in *D. willistoni* (pb1A and pb3A), the member of the Sophophora most distant from *D. melanogaster*. However, an interesting example is a change in the response of ab8A in *D. ananassae*, where the response seen in *D. melanogaster* to 2-methylphenol, an aromatic compound, is missing in *D. ananassae* but not in its sister species *D. serrata*, nor in other species more evolutionarily distant from *D. melanogaster*.


Dmel
Dsim
Dmau
Dana
Dser
Dpse
Dwil
Dvir
pb1A
Or42a
2 genes
2 genes
ab1A
Or42b
ab2A
Or59b
ab4A
Or7a
not found
no gene
not found
no gene
ab3A
Or22a
(Or22b)
2 genes
2 genes
4 genes
2 genes
ab8A
Or43b
not found
no gene
pb3A
Or59c
not found
no gene
ab2B
Or85a
no gene
no gene
no gene
mAc
eAc
eBu
eHBu
pAc
hAc
mHx
eHx
gerAc
Hal
Ool
mHon
Bal
2mP
4mP
PO
0
100
200
300
ORN response (spikes/s)

◀ **Fig. 3** Odour response spectra of homologous ORNs in eight species of *Drosophila*. Mean response (±SEM, $n = 8–14$) to 500 ms stimulations with the odorants listed in Fig. 2a. *Dotted lines* separate esters (*top*) from other compounds. The *Or* gene expressed in each ORN class in *D. melanogaster* is indicated. For other species we indicate when more or less than one orthologous gene was found in the genome. *Asterisks* highlight substantial changes in spectra as indicated by the cluster analysis of Fig. 5, i.e. cases in which the ORN does not cluster with its homologous group. *Arrows* indicate smaller changes to individual odorants compared to *D. melanogaster* (*black*) or *D. willistoni* (*light grey*) as determined by a Bonferroni-corrected *t* test ($P < 0.003$) on individual odorants. Some ORNs were absent from our recordings (not found). Spectra of ab2B neurons for which the *Or85a* gene was absent in the genome are in *black*

Two ORNs show many response changes across *Drosophila* species

Two of the eight neuron classes, ab3A and ab2B, show a quite different pattern of evolution as they show high variability in response spectra across many species. A high response to ethyl butanoate is fundamental to ab3A spectra, but depending on the species we see many different secondary responses. Whilst the spectra of *D. mauritiana*, *D. serrata*, *D. pseudoobscura* and *D. willistoni* are all similar, *D. melanogaster*, *D. simulans*, *D. ananassae* and *D. virilis* each exhibit the addition of different secondary responses, with particularly strong responses to methyl- and/or ethyl hexanoate (Fig. 3).

The ab2B neuron also shows high variation in response properties. In the melanogaster subgroup (*D. melanogaster*, *D. simulans*, *D. mauritiana*) the spectrum of ab2B is conserved, showing a typical high sensitivity to ethyl 2-hydroxybutanoate, an odour that no other neuron responds to at low doses (Fig. 3). However, in several other species, *D. ananassae*, *D. pseudoobscura*, *D. willistoni* and *D. virilis*, the response spectra of the neurons do not show the characteristic response to ethyl 2-hydroxybutanoate, and in the case of *D. pseudoobscura* the spectrum is completely changed and the neuron now responds to two very different odorants; benzaldehyde and 2-methylphenol (Fig. 3).

Correlating ORN spectra with Or protein sequence

We next asked the question of how well Or sequence variation correlates with variation in ORN response spectra. We aligned amino acid sequences derived from *Or* genes in the ester group, as found in public databases for the genomes of *D. melanogaster*, *D. simulans*, *D. ananassae*, *D. pseudoobscura*, *D. willistoni* and *D. virilis*. In addition, we cloned and sequenced the following *Or* genes from *D. mauritiana*: *Or7a*, *Or42b*, *Or43b*, *Or59b*, *Or59c* and *Or85a*. Sequences for *Dmau Or42a*, *Or22a* and *Or22b* were obtained previously in our lab (Tunstall et al. 2007). All these amino acid sequences (orthologues and paralogues) align fairly well

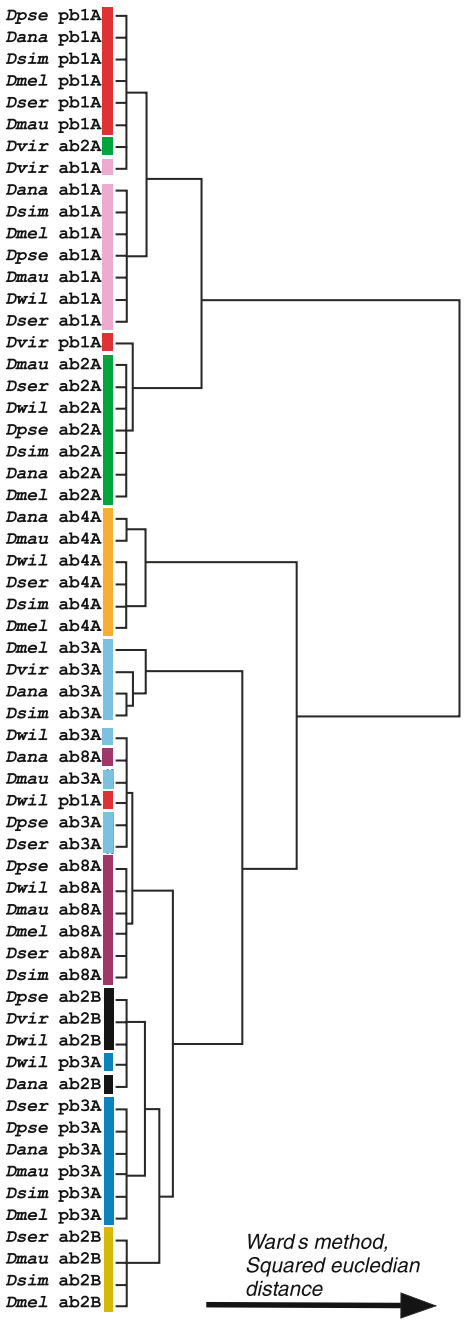


Fig. 4 Classification of ORNs across species. A hierarchical cluster analysis was performed on the mean odour responses of all ORNs using Ward's method. Branch length is inversely proportional to the similarity between odour response spectra of eight neuron classes across eight *Drosophila* species

(Supplemental Fig. 1) although amino acid identities between some orthologues can be as low as 43%. Figure 5 shows how these proteins are related in a phylogenetic tree. We have included all putatively functional copies of genes with duplications (*Or22a*, *Or42a*), as in most cases we do not know which copy is functional.

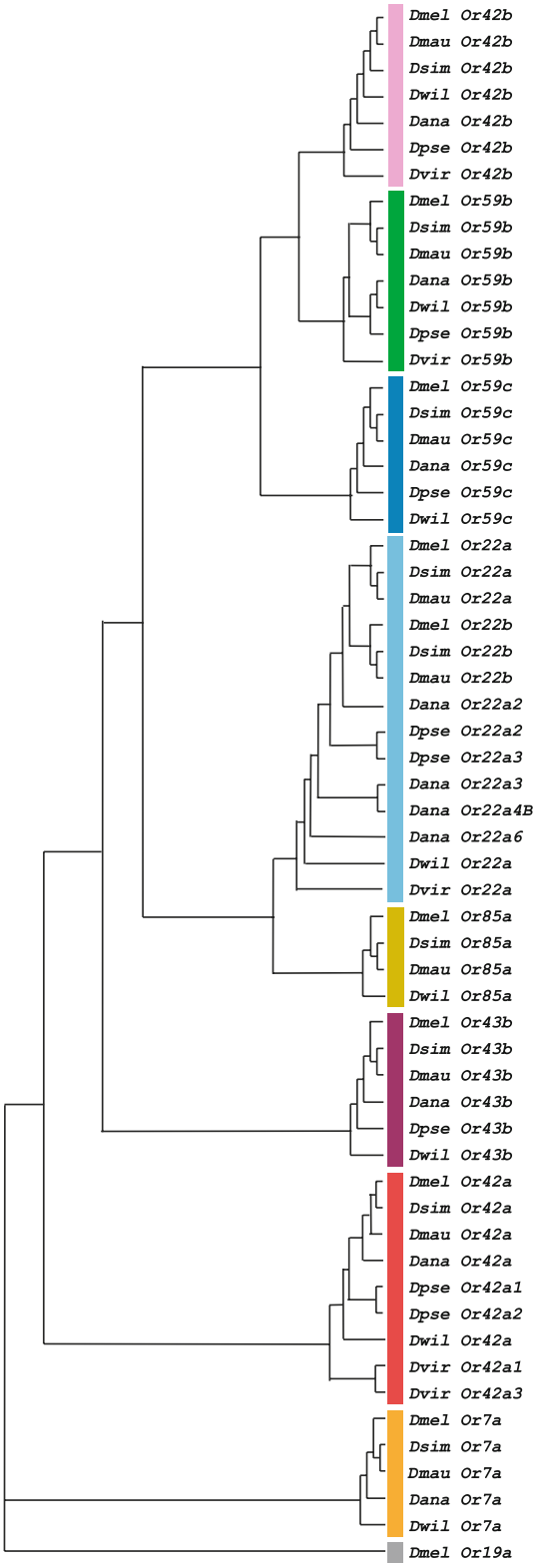


Fig. 5 Protein similarity tree of Or proteins in the ester subfamily using JTT distance. Colour coding scheme is as in Fig. 4, assuming that each gene is expressed in the ORN homologous to *D. melanogaster*. *DmelOr19a* is used as the outgroup but the tree is unrooted

Comparing the tree diagrams of neurons (Fig. 4) with receptor proteins (Fig. 5) indicates that there is no obvious relation between the two, i.e. similarity of *Or* sequence does not predict similarity of odour response spectrum in any straight forward manner. For example, all Or42a sequences are more similar to each other than to other Or proteins (Fig. 5) but the pb1A spectrum of *D. willistoni* actually looks more similar to the ab3A spectrum of *D. mauritiana* (Fig. 4). We also note that the most similar paralogous genes do not consistently show the most similar spectra. For example, the most similar odour response spectra in our set are those of the pb1A, ab1A and ab2A neurons (Fig. 4), with pb1A and ab1A being most similar. However, looking at the three relevant receptors, whilst Or42b (ab1A) and Or59b (ab2A) are very similar, Or42a (pb1A) does not cluster with these two proteins. Finally, tree topology for each *Or* orthologous group mostly reflects the species phylogeny (compare Fig. 5 with Fig. 1a) with only a few exceptions. However, several ORNs are classified outside their main clusters, and these are not always in the most distantly related species (Fig. 4). For example, the tree for Or43b reflects the established phylogeny for the species (Fig. 5) and does not reflect the altered nature of the ab8A spectrum of *D. ananassae* (Fig. 4).

Putative functional residues in Or proteins

As the ORN similarity tree (Fig. 4) does not always correlate with the Or similarity tree (Fig. 5) we can not automatically assume homologous ORNs always express homologous genes. However, many of the response spectra of homologous neurons are nearly identical between species. As in all cases to date ORNs with differences in response spectra have been shown to express different *Or* genes, it is highly likely that these identical response spectra across species are mediated by orthologous *Or* genes, although we acknowledge that expression studies are needed to formally prove this. We therefore assumed for the following analysis that where we see identical or near identical response spectra between homologous neurons that these responses are mediated by orthologous *Or* genes. In this case, as the relevant receptors show no apparent difference in odour response it seems likely that residues that vary between these orthologues do not play a critical role in odour binding. In these cases, representing seven of the eight *Or* genes, for each *Or* we generated sequence alignments only of those orthologues that do not show large changes in ligand-binding properties, as defined by all that do not have asterisks in Fig. 4 (Fig. 6a, Supplemental Fig. 2). We designated the residues that are conserved across functionally equivalent orthologous Ors as putative functional residues, with those that vary being relatively

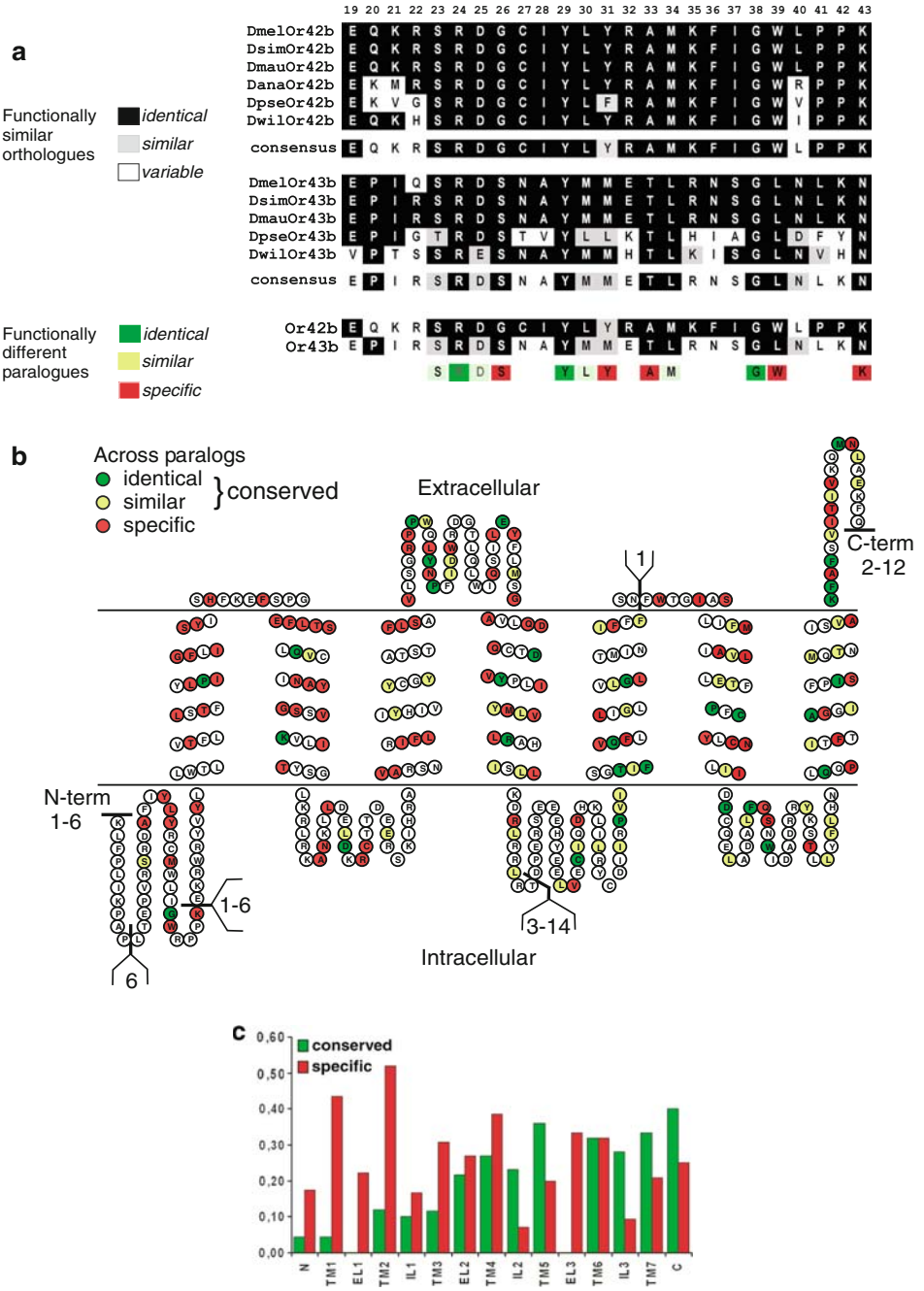


Fig. 6 Putative functional residues in Or proteins. **a** Shown is a small portion of the amino acid sequence alignments (amino acid numbers indicated above the alignment) of two Or proteins for all orthologues that have similar response spectra, to illustrate the procedure for determining putative functional residues (see Supplemental Fig. 2). For each set of orthologues a consensus sequence is generated and conserved (*black*/*grey*) and variable (*white*) residues indicated. The conserved residues within a set of orthologues are putative functional residues. The consensus sequences of these Or proteins are aligned and conservation across paralogues is determined. Any of the functional residues that are also conserved across the paralogues we have called conserved functional residues, shown in green. Residues that are conserved between orthologues but vary across paralogues we have called specific functional residues, shown in *red*. **b** Model of the Or proteins in the ester group uses the consensus amino acid sequence from the alignment of all Or proteins conserved functional residues are *green*, specific functional residues are *red*. Variable numbers of residues in insertions, N-terminus and C terminus are indicated. Positions of predicted TM domains are taken from Smart et al. (2008). **c** The proportion of conserved and specific functional residues per protein domain. *TM* transmembrane, *EL* extracellular loop, *IL* intracellular loop

unimportant for function. The latter total 56% of the residues, allowing us to exclude these as unimportant for Or function. For each of the seven Ors we generated a consensus sequence and we then aligned these seven consensus sequences to generate an alignment of paralogues that have different response spectra. Any of the functional residues that are also conserved across the paralogues, which we have called "conserved functional residues", are likely to be important in general Or function, but are not likely to contribute to differences in odour response spectra within the ester subfamily. We have indicated their positions in an Or protein model (Fig. 6b). The conserved functional residues make up 20% of Or residues, giving a total of 76% of residues that do not contribute to the different ligand response properties among this group of Ors. Conversely, functional residues that vary between the paralogues may contribute to determining the specific odour response properties of each receptor. We have called these "specific functional residues" similar to the "specificity determining residues" of Guo and Kim (Guo and Kim 2009). They make up the remaining 24% of residues (Fig. 6b). The proportions of conserved and specific functional residues for each Or protein domain are represented in Fig. 6c. The domain comparison reveals a number of interesting points. Firstly,

the conserved residues are more highly represented in the C terminal half of the protein, from EL2 to the C terminus. In contrast there are a relatively high number of specific residues in some of the domains in the N terminal half of the protein, for example TMs 1 and 2 have a very high proportion. We also see high proportions of specific residues in the extracellular loops.

Discussion

Conservation of neuronal identity in the evolution of ORN classes

We determined the response profiles of a set of eight ester-responsive neurons across *Drosophila* species spanning 40 million years of evolution. Esters are products of yeast fermentation and generally occur in fruits, and our analysis strongly suggests that a dedicated subfamily of *Or* genes has evolved in *Drosophila* species. Interestingly, the genomes of three mosquito species do not show evidence of this subfamily of Ors (Hill et al. 2002; Bohbot et al. 2007) suggesting it may have originated in the higher Dipteran lineage. As many *Drosophila* species feed on yeast in various fermenting plant tissues these genes are likely to play an important role in locating feeding and oviposition sites (Dekker et al. 2006; Semmelhack and Wang 2009). In the *Drosophila* ester-responsive neurons we studied here we generally found a high level of conservation. For six of the eight neurons within the Sophophora subgenus major changes occur only in our outgroup *D. virilis*, which belongs to the Drosophila subgenus. *D. virilis* thus potentially perceives odorants quite differently, however, the precise behavioural responses of the species we studied to the odorants reported here remain largely unexplored.

We did, however, find that even for the six conserved neuron classes there are some cases of response spectra changes in some of the Sophophora species. For example, the response profile of ab4A exhibits changes in *D. mauritiana*, *D. ananassae* and *D. willistoni*, and, more dramatically, we could not find this neuron in *D. pseudoobscura.* It is thus possible that this neuron is evolving to suit different species needs.

There are also three examples where we see changes in a particular neuron only in a single Sophophora species. For two of these the change is in *D. willistoni* (pb1A and pb3A), the most distant member of the Sophophora from *D. melanogaster*, and thus may simply reflect phylogeny. The third case is a change in the response of ab8A in *D. ananassae*, where the response seen in *D. melanogaster* to 2-methylphenol, an aromatic compound, is missing in *D. ananassae* but not in its sister species *D. serrata*, nor in other species more evolutionarily distant from *D. melanogaster*. Such a specific change in one species is perhaps more likely to be driven by selection for ecological adaptation. However, a more accurate assessment awaits behavioural analysis.

Dramatic changes of odour response correlate with gene loss and duplication

For two of the neuron classes we found extensive variability across the species. One of these, ab3A, was previously shown to exhibit differences in odour response between *D. melanogaster*, *D. mauritiana*, *D. simulans* and *D. sechellia* in sensitivity to methyl- and/or ethyl hexanoate (Stensmyr et al. 2003), and changes in ab3A response have been associated with the highly specialised feeding habits of *D. sechellia* (Dekker et al. 2006). However, our results show this neuron to be variable across several generalist species, and it is thus unclear how this variability relates to differences in their feeding ecology. What might be causing the high variability for this particular neuron type? Interestingly, *Or22a*, the gene associated with this neuron in *D. melanogaster*, shows increased sequence divergence and duplications in many of the species, including in *D. melanogaster* (Guo and Kim 2007; Nozawa and Nei 2007). In *D. melanogaster Or22a* and *Or22b* are thought to both be expressed in ab3A neurons, however, rescue experiments of a mutant strain in which both genes are deleted have clearly shown that *Or22a* alone is responsible for the response spectrum (Dobritsa et al. 2003). This might not be the case for the other species, where we do not know which of the copies constitute the functional receptor(s). It is possible that recent gene duplications, coupled with relaxed selective pressure on the non-functional copy(s), could provide the opportunity for accelerated evolution of response properties of single neuron classes. Testing this hypothesis would require determination of which genes mediate ab3A responses in the other species.

We also do not know what causes the high variation in response properties of the second neuron, ab2B, but it may be related to gene loss and/or changes in gene expression. In three of the four species that show dramatic changes (*D. ananassae*, *D. pseudoobscura* and *D. virilis*, Fig. 3) this change correlates with a loss of the relevant *Or* gene, *Or85a*. These species still have responsive ab2B neurons but the neurons have very different response spectra to that of *D. melanogaster*, in particular for *D. pseudoobscura* where the ab2B neuron responds to two very different odorants. The loss of *Or85a*, combined with the changes in response spectra, indicate a different *Or* is expressed in these ab2B neurons, and it would be very interesting to investigate which *Or* gene is responsible. As the spectrum of *D. pseudoobscura* ab2B is very different from the response spectra seen in *D. ananassae* and *D. virilis* different

Or genes may underly these response spectra. In *D. melanogaster* the ab2B neurons have been shown to co-express the *Or33b* gene together with *Or85a* (Fishilevich and Vosshall 2005), and it is thus possible that some of these altered spectra may be due to *Or33b*. However, *DmelOr33b* ligands have not been identified as it did not give responses in an in vivo expression system (Hallem and Carlson 2006).

For three neuron types in *D. virilis*, ab4A, ab8A and pb3A, and for ab4A in *D. pseudoobscura,* the apparent absence of a homologous neuron correlates with the fact that the relevant *Or* gene is either absent from the genome (*Dvir Or7a*, *Or43b* and *Or59c*) or is a pseudogene (*DpseOr7a*) (Guo and Kim 2007; Nozawa and Nei 2007). If we could not find a homologous A neuron in a species we would normally use the B neuron to identify homologous sensilla. However, we were unable to use the paired B neuron to detect ab4 or ab8 sensilla as we did not have odorants that stimulate the *D. melanogaster* ab4B and ab8B neurons. In the cases of missing ab4A and ab8A neurons it is thus possible that these neurons are still present but express different *Or* genes and are no longer recognisable to us using our criteria for homology. In this regard we note that the *Or56a* and *Or9a* genes, expressed in the *D. melanogaster* ab4B and ab8B neurons, respectively, have putative functional orthologues in the *D. virilis* genome (Guo and Kim, 2007). It is thus possible that these sensillum types are retained, perhaps expressing different *Or* genes in the A neurons. However, any novel neuron types were not identified in our analysis. In the case of pb3A, in *D. virilis* we did not detect the response of the pb3B neuron to pentyl acetate, however, our sampling was not extensive and thus the absence of this neuron is not conclusive.

Relating ORN changes to Or sequence evolution

Perhaps the most surprising finding from this study is how ORN responses can be highly conserved over many million years of evolution. A previous study of closely related species in the melanogaster subgroup found high conservation (Stensmyr et al. 2003), but here we have examined much more distant relatives. If we assume that identical response spectra in different species are mediated by orthologous *Or* genes, this means that response spectra can be highly conserved despite considerable sequence change in the receptor protein. For example the ab8A neuron responses of *D. melanogaster* and *D. willistoni* are equivalent but the orthologous Or43b amino acid sequences are only 60% identical. Interestingly other *Ors* that underly high levels of response conservation are evolving much more slowly, for example *D. melanogaster* and *Dwillistoni* Or42b, underlying the conserved ab1A neuron, are 90% identical. The high level of functional conservation that we see supports previous findings that most *Or* genes are under purifying selection (Guo and Kim 2007; Nozawa and Nei 2007; McBride and Arguello 2007; Tunstall et al. 2007), but why some *Ors* are evolving much faster within this selective constraint than others is not clear.

By comparing ORN spectrum variation and receptor sequence variation we found that similarity of Or sequence does not predict similarity of response spectrum in any logical manner. The tree topology for each Or reflects the species phylogeny and thus suggests overall neutral evolution. However, tree topology for ORN spectra does not. The fact that evolution of ORN response properties is not always strictly correlated with lineage suggests that in some cases it is adaptive. For instance *D. mauritiana* often differs from the other two closely related species of the *melanogaster* subgroup (*D. melanogaster*, *D. simulans*). However, *D. mauritiana* is an island isolate and it is unclear to what extent neutral factors such as founder effects and genetic drift contribute to genetic differences in such species. This comparative analysis thus suggests that most of the Or sequence variation does not affect ligand-binding properties.

Identifying putative functionally important amino acid residues

We compared orthologous Ors of assumed functional equivalence with functionally different paralogues in order to distinguish residues of functional importance. Within this Or subfamily we found that 44% of the amino acid residues are putative functional residues, of these 20% are conserved across paralogues and thus likely to be important in general function, whilst 24% are not conserved across paralogues and these specific functional residues are thus candidates for specificity determining residues (SDR, Guo and Kim 2009). Comparing the location of both types of putative functional residues within domains of the receptors showed that conserved residues are more highly represented in the C terminal half of the protein, from EL2 to the C terminus. This fits well with an experiment where a chimeric receptor was used to show that the C terminal half (TM4—C terminus) of Or43a mediates its association with the highly conserved co-receptor Or83b (Benton et al. 2006). Conversely, specific residues are more highly represented in some of the domains in the N terminal half of the protein, for example TMs 1 and 2, and the extracellular loops. These specific residues should include residues involved in odour binding in this subfamily of odorant receptors. For vertebrate receptors modelling and functional studies have proposed residues in the central part of TMs 3, 5 and 6 as forming part of a binding pocket (Kato and Touhara 2009). However, insect Ors have a very different protein conformation and our analysis suggests important roles for TMs 1 and 2. Narrowing down the

ligand-binding region in insect Ors will require detailed structure–function studies, and would be greatly assisted by the availability of an X-ray crystal structure.

In a recent study Guo and Kim (2009) used comparative sequence analysis of *Drosophila* Ors to predict specificity determining residues (SDRs), but in the absence of functional data they assumed equivalent function across orthologues, which, as our study shows, does not always hold. They also studied a larger set of less related receptors and used more closely related species, therefore our results are not directly comparable. Nevertheless, all their 15 conserved residues are also identified in our analysis, and our set of predicted specific functional residues includes 7 of the 12 predicted SDRs in their study.

In summary, our study reveals that ORN response spectra in *Drosophila* species can be conserved across many millions of years of evolution despite considerable variation in odorant receptor sequence. Conversely, some ORNs have undergone major response changes during evolution, and receptor gene duplications or changes in expression pattern may underly some of these. Our comparative analysis of Or proteins that correlate with conserved ORN responses suggests that less than 24% of amino acid residues in this subfamily of Ors are important for odour specificity. This data will help elucidate how insect olfactory receptors bind specific ligands.

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