Evolution of the olfactory code in the *Drosophila melanogaster* subgroup

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The *Drosophila melanogaster* subgroup has been the focus of numerous studies about evolution. We address the question of how the olfactory code has evolved among the nine sister species. By using *in vivo* electrophysiological measurements, so called single-cell recordings, we have established the ligand affinity of a defined subset of olfactory receptor neurons (ORNs) across all nine species. We show that the olfactory code as relayed by the investigated subset of ORNs is conserved to a striking degree. Distinct shifts in the code have occurred only within the *simulans* clade. However, these shifts are restricted to an altered tuning profile of the same single ORN type in all three of the *simulans* siblings and a more drastic change unique to *D. sechellia*, involving a complete loss of one sensillum type in favour of another. The alterations observed in *D. sechellia* may represent a novel host-specific adaptation to its sole host, morinda fruit (*Morinda citrifolia*). The overall high degree of similarity of the code within the subgroup is intriguing when considering the great variety in distributions as well as in habitat and host choice of the siblings, factors that could greatly affect the olfactory system.

Keywords: olfactory receptor neuron; odour coding; host adaptation

1. INTRODUCTION

The olfactory system of an animal is adapted to its niche. The colonization of a novel niche often leads to the encounter of novel sensory information, which the animal then has to accurately and efficiently assess to be competitive. Adaptations to new resources may result in speciation (Hawthorne & Via 2001; Nosil *et al.* 2002). To what extent will these processes affect the olfactory sense? Will the new species keep its ancestral olfactory system or will it evolve a unique set-up, adapted to the conditions of the novel niche?

These questions are intriguing but, as yet, poorly studied. The fruitflies within the *Drosophila melanogaster* subgroup offer excellent opportunities for studies about the evolution of olfactory coding. The nine species of the subgroup are closely related and their phylogenetic relationship is well understood (Jeffs *et al.* 1994; Lemeunier *et al.* 1986; Kliman *et al.* 2000; Lachaise *et al.* 2000). In addition, the subgroup contains endemic species and species with an almost global distribution, as well as species exhibiting differences in food choice, including specialists and generalists (Lachaise *et al.* 1988, 2000). Habitat complexity, as well as food choice, are factors that could induce shifts in the olfactory system. Another important advantage with the fruitflies is that the olfactory code can be directly studied in high resolution with *in vivo* electrophysiological measurements from olfactory receptor neurons (ORNs) (Siddiqi 1991; Clyne *et al.* 1997; De Bruyne *et al.* 1999, 2001; Stensmyr *et al.* 2003). In addition, the olfactory system of *D. melanogaster* is well characterized morphologically, physiologically as well as at the molecular level (Stocker 2001; Vosshall 2001).

The olfactory system of the fruitfly offers the possibility to study the physiological effect of single genes, as the receptive field of single ORNs reflects the ligand affinity of single odorant receptors (ORs) (Clyne *et al.* 1999; Gao & Chess 1999; Vosshall *et al.* 1999; Störtkuhl & Kettler 2001; Wang *et al.* 2003; Dobritsa *et al.* 2003). Because the species within the *melanogaster* subgroup are closely related, there is a fair chance that the tuning profiles of the ORNs are, to a large degree, conserved as a consequence of the presence of orthologous ORs. By examining the ORN physiology, it may thus be possible to follow the evolution of single ORs across the nine species.

In this study, we have addressed the question of how the olfactory code has evolved among the nine member species of the *melanogaster* subgroup. We show that the olfactory code, as relayed by a defined subset of receptor neurons is, to a remarkable degree, conserved among the nine species. Notable changes have, however, occurred in the *simulans* cluster. These changes, especially pronounced in the two island endemics *D. mauritiana* and *D. sechellia*, may represent host-specific adaptations.

2. MATERIAL AND METHODS

(a) Fly stocks

Flies were obtained from the *Drosophila* Species Stock Center (Tucson, AZ, USA), except for *D. santomea*, which was kindly donated by D. Lachaise (CNRS, Gif-sur-Yvette, France). The stocks used were: *D. melanogaster* (Oregon R), *D. simulans* (14021-0251.169), *D. sechellia* (14021-0248.0), *D. mauritiana* (14021-0241.1), *D. yakuba* (14021-0261.0), *D. teissieri* (14021-0257.0), *D. orena* (14021-0245.0) and *D. erecta* (14021-0224.0). Flies were reared on standard cornmeal-yeast-agar medium and kept in a climate- and light-controlled environment. Throughout the study, we used only male flies, 2–10 days old.

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(b) Morphology

Whole-mounted fly antennae were examined through an Olympus BX51W1 microscope equipped with a digital camera (Olympus DP11). The antennal morphology of *D. sechellia* and *D. simulans* was also examined through a scanning electron microscope (LEO 435VP, Oberkochen, Germany). Heads were dehydrated, air-dried and subsequently sputtered with a 6 nm layer of gold/platinum.

(c) Electrophysiology

For the electrophysiological recordings, a fly was mounted in a truncated pipette tip with the antennae protruding from the narrow end. The pipette tip was fixed with wax on a microscope slide, and the antennae gently placed on a cover-slip and stabilized with a glass micropipette (Clyne *et al.* 1997; Stensmyr *et al.* 2003). ORNs were contacted with tungsten microelectrodes, electrolytically sharpened in KNO_3 -solution (Hubel 1957). We positioned the recording electrode and the indifferent electrode (inserted into the eye) under a microscope (Olympus BX51W1), magnification of $\leq \times 1500$. For the recording electrode we used a motor-controlled micromanipulator (Märzhauser DC-3K, Wetzlar, Germany) equipped with a piezo unit (Märzhauser PM-10) and for the reference a manually controlled micromanipulator (Narishige MM33, Tokyo, Japan). The signal was amplified 1000 times (Syntech UN-06, Hilversum, The Netherlands), digitally converted (Syntech IDAC-4 USB) and then fed into a PC (IBM, Pentium IV) and visualized (Syntech AutoSpike 3.2). The signal was also fed to a loudspeaker for audio monitoring. Recordings of action potentials were stored on the PC and all analysis was done with the AUTOSPIKE software. Separation of activity of collocated ORNs in single sensilla was based on differences in spike amplitude. The ORN with the largest spike amplitude was termed A, the second largest B and so forth. As spike amplitudes frequently change during extensive firing, we often had to complement the computerized template for spike sorting with manual sorting, where we took into account changing amplitudes and relied on spike shape for classification. Contacts where problems arose in assigning ORN identity were excluded from further analysis. To avoid over-sampling certain areas of the antennae, the position of the recording electrode was noted on template drawings of the *Drosophila* antennae (Shanbhag *et al.* 1999).

(d) Odour stimuli

The odour stimuli used were previously identified efficient ligands for the ORNs under scrutiny (De Bruyne *et al.* 2001; Stensmyr *et al.* 2003). The odorants used were obtained from Aldrich and Fluka and had purity greater than 98%. Neat compounds were diluted in redistilled hexane or CH_2Cl_2 down to a concentration of $1 \mu\text{g} \mu\text{l}^{-1}$. From these solutions, we pipetted $10 \mu\text{l}$ onto small pieces of filter paper placed inside Pasteur pipettes. Blank cartridges, with only filter paper plus solvent served as control. We frequently renewed the test cartridges. Once contact with ORNs was established, the test cartridges were screened in random order. A glass tube, with its outlet *ca.* 5 mm from the antenna, delivered a constant flow of charcoal-filtered and humidified air at a velocity of 0.5 m s^{-1} over the preparation. Stimulation was performed by inserting the tip of the test cartridge into a hole in the glass tube, *ca.* 15 cm before the outlet. The test cartridge was connected to a stimulus controller (Syntech CS-01) that generated air puffs (2.5 ml for 0.5 s)

through the cartridge into the constant air stream in the glass tube.

(e) Statistical analysis

We analysed the response spectra of the neurons with a hierarchical cluster algorithm. Cluster analysis is a multivariate procedure that is used to detect natural groupings in data. To form clusters we used Ward's minimum variance method, which assesses cluster membership by calculating the total sum of squared deviations from the mean of a cluster (Ward 1963). The cluster analysis was performed with CHAMELEON STATISTICS v. 1.22 (7th Sense Software, Dhaka, Bangladesh). All other statistics were performed with JMP 4.02 (SAS Institute Inc., Cary, NC).

3. RESULTS

To study how the olfactory code has evolved, the receptive field of a set of ORNs that could be identified in all the species was investigated, thus ensuring comparison of homologous structures. ORNs situated in large basiconic (LB) sensilla were chosen, as the receptive range of these neurons is well documented in *D. melanogaster* (De Bruyne *et al.* 2001; Dobritsa *et al.* 2003; Stensmyr *et al.* 2003) and for several of the LB ORNs there are good candidate key ligands, i.e. biologically relevant and behaviourally significant stimuli (Stensmyr *et al.* 2003). In addition, the LB sensilla occur in only a specific area of the *D. melanogaster* antenna, the proximo-medial side, where other types of sensilla are absent (Shanbhag *et al.* 1999).

Do all *melanogaster* sister species have LB sensilla? Antennae of all nine species were examined at high magnification and LB sensilla were located across the whole subgroup. In all the species the LB sensilla occupied the proximo-medial side of the antenna (figure 1). As the overall morphological architecture of the system seemed to be conserved, we deemed it possible to record from similar structures across all the nine species.

How do the ORNs in these sensilla code odours? We approached this question by recordings of neural activity from single LB ORNs. In previous studies the receptive range of LB ORNs was established in *D. melanogaster*. Three functionally different classes of LB sensilla were found, denoted ab1, ab2 and ab3 (De Bruyne *et al.* 2001) or S1, S2 and S3 (Stensmyr *et al.* 2003), here we follow the first terminology. In the present study, we confirmed the presence of these three types in *D. melanogaster*. The ab1 type houses four neurons, whereas the ab2 and ab3 house two neurons each. The odorant set used yielded distinct and characteristic responses from each of the eight ORN types. Having confirmed the response spectra of the *D. melanogaster* LB ORNs, we next aimed to locate homologous ORNs in the other eight sister species.

(a) ab1 sensillum

We found a LB sensillum type housing four ORNs (type ab1), distinguishable by firing action potentials of different amplitudes (figure 2), across the whole subgroup (figure 3). In all the species, the ligand affinity of these ORNs displayed a high degree of similarity. All had the A ORN (the

Figure 1. The fruitfly antenna. (a) Detail image of the proximo-medial side of the third antennal segment showing large basiciconic (LB) sensilla (arrows) intermingled by non-innervated spinules (arrowheads). The image was obtained from a whole-mounted living fly (*D. simulans*) through the microscope of the recording set-up, and illustrates the high magnification that allowed us to accurately locate LB sensilla for the electrophysiological recordings. Scale bar represents 10 μm . (b–d) The LB sensilla occupied the same area across all the species of the subgroup. The distribution of LB sensilla is marked by the dashed line on the schematic 3D model of the antenna.

largest spiking neuron) tuned to the same range of esters, the B ORN primarily to acetoin, the C ORN to carbon dioxide, and the D ORN primarily triggered by methylsalicylate and acetophenone. Although highly similar, we observed some variation, for example, 2,3-butanediol produced weak responses from the D ORN in *D. simulans* and *D. sechellia*, whereas not in the other species.

We also examined the spike amplitudes produced by the neurons. We chose to measure the amplitudes as relative ratios, as this should be independent of, for example, the recording quality. Setting the amplitude of the A neuron to 100%, the relative amplitude of the B ORN was calculated and so forth. The average ratios of the B, C and D ORNs were $59.1 \pm 1.4\%$, $37.6 \pm 3.2\%$ and $11.5 \pm 1.4\%$ (mean $\pm$ s.d.), respectively. The amplitude ratios for corresponding ORNs were not significantly different between any of the species ($p > 0.05$, one-way ANOVA followed by *post hoc* Tukey–Kramer on arcsin-transformed percentages).

(b) *ab2* sensillum

LB sensilla displaying *ab2* characteristics were found in eight of the species (figure 3). Across these eight species, ethyl 3-hydroxybutyrate produced very strong responses from the B ORNs, and although producing weak to moderate responses, acetoin was still the most potent ligand

for the A ORN. Another feature common to all the *ab2A* ORNs was the inhibitory response elicited by methylsalicylate. As with the *ab1* type neurons, we noted some minor variation in the response pattern of both neurons. Interestingly, the *ab2* type sensillum and its neurons were not observed in *D. sechellia*.

Although, the *ab2* were missing, the overall number of LB sensilla in *D. sechellia* did not deviate from that in *D. simulans* or *D. melanogaster*. We found between 87 and 90 LB sensilla in *D. sechellia* ($n = 4$), similar to what we found in *D. simulans* (88–91) and from what has previously been reported from *D. melanogaster* (90 in males; Shanbhag *et al.* 1999). Considering how strictly conserved the *ab2* type is among the other species in the subgroup, this observation is striking.

As with the odour tuning, the amplitude ratio of the A and B ORNs was conserved in all eight species; average amplitude ratio of the B ORNs was $24.6 \pm 1.0\%$ ($p > 0.05$, one-way ANOVA, *post hoc* Tukey–Kramer).

(c) *ab3* Sensillum

Neurons sharing *ab3* type characteristics were found in all the species, although with some distinct differences (figure 3). In *D. melanogaster* as well as in the other species, exempt for the *simulans* siblings, the *ab3A* type neurons were primarily tuned to ethyl hexanoate and, to a

Figure 2. Recordings of neural activity from olfactory receptor neurons (ORNs). (a) The presence of two distinct action potential (spike) amplitudes (denoted a and b) indicate the presence of two neurons. The recording example comes from *D. erecta*, sensillum subsequently classified as ab2 type. (b) Spikes extracted from a 1 min recording of spontaneous activity from the two neurons in (a). The two neurons give two distinct populations of spikes when sorted for amplitude, which allow for separation of activity. (c) Simultaneous recording of activity from four collocated ORNs in a single sensillum (ab1 type from *D. teissieri*, ORNs denoted a–d). The d-cell responds to methylsalicylate, whereas the other three neurons are unaffected. (d) In another type of neuron (ab2A type), methylsalicylate inhibited spike firing (example from *D. teissieri*). (e) During extensive firing the spike amplitudes often changes, here exemplified by a recording from *D. mauritiana* where the large spiking neuron responds strongly to ethyl butyrate (ab3A type ORN). (f) Extended view of the spike train in (e). Horizontal bars represent 0.5 s.

lesser degree, methyl hexanoate. However, the ab3A ORNs in the *simulans* clade displayed interesting specificity shifts. In both *D. simulans* and *D. sechellia* ab3 hom-

ologue sensilla were present, though in both species the key ligand for the ab3A type ORN was shifted from ethyl to methyl hexanoate, which in both species produced a considerably stronger response. In *D. mauritiana*, contrary to its two closest sister species and to all the other species in the *melanogaster* subgroup, the most efficient ligand for the A neuron was neither methyl hexanoate nor ethyl hexanoate, but ethyl butyrate. Both ethyl and methyl hexanoate produced weak responses and, in contrast to the other species, ethyl 3-hydroxybutyrate also elicited a weak response. The ab3B type neurons responded to 3-octanol and ethyl 3-hydroxybutyrate in all species.

In *D. sechellia* we noted a high proportion of ab3 type sensilla, comprising 84% of the contacted LB sensilla, and as the ab2 was not detected, only 16% were of the ab1 type. In the other siblings, the average proportion of the ab1 and the ab3 type sensilla were 38.3 ± 8.8 and 32.8 ± 8.8 , respectively. The high proportion of ab3 compared to ab1 sensilla in *D. sechellia* and the observation that the overall number of sensilla have not decreased compared with its siblings strongly suggests that the missing ab2 type has been replaced by an increased number of ab3 type sensilla.

The spike amplitude ratio between the A and the B ORNs was conserved for eight of the species; average ratio of $52.3 \pm 0.7\%$ for the B ORNs ($p > 0.05$, one-way ANOVA, *post hoc* Tukey–Kramer). However, in *D. mauritiana* where we found a shift in the receptive range of the A neuron we also observed a significant shift towards a larger difference in amplitude, with a ratio of $25.2 \pm 4.9\%$ for the B ORN ($p < 0.01$).

(d) Cluster analysis

The response spectra of the identified neurons were subjected to a hierarchical cluster analysis. Although not a statistical method *per se*, cluster analysis is a powerful technique for resolving groupings in datasets. We based the cluster classification on the average responses from each neuron type to the complete odour set. The resulting dendrogram shows the most similar cases linked most closely together (figure 4). The cluster analysis separates the eight different classes of ORNs. The ab3A type neurons of *D. simulans* and *D. sechellia* cluster together, separate from the ab3A type neurons of the other species. The ab3A type neuron of *D. mauritiana* is even more distantly grouped from the other ab3A neurons and is joined closer to the ab1A neurons. Within a cluster of a given ORN class, the proposed relationship between the species fails to crystallize, i.e. species more closely related to each other do not cluster together in a clear fashion.

4. DISCUSSION

We show that the olfactory code as relayed by the investigated subset of ORNs is, to a remarkable degree, conserved among the nine species of the *D. melanogaster* subgroup. The ligand affinity, pairing rule and action potential amplitude of the eight distinct ORN types found in *D. melanogaster* were mirrored in its sibling species. In the three species where we observed changes, the differences were restricted to a shift in tuning profile of a single neuron type (the ab3A in *D. simulans*, *D. sechellia* and *D.*

mauritiana) and the loss of a sensillum type (the ab2 in *D. sechellia*); apart from these changes, these three species shared the same characteristics as the other siblings.

The olfactory code in the *melanogaster* subgroup has, to a large extent, been conserved over millions of years, speciation events, and across changing environments. The level of similarity is quite surprising. Today, two of the species are cosmopolitan (*D. melanogaster* and *D. simulans*), three occur only in the Afrotropics (*D. yakuba*, *D. teissieri* and *D. erecta*), whereas another four are endemics, of which three are confined to islands (*D. sechellia*, *D. mauritiana* and *D. santomea*) and one restricted to a single mountain top (*D. orena*) (Lachaise *et al.* 1988, 2000). The differing environments, characterized by very different sensory information, should have exerted equally varying selective pressures, shaping the olfactory sense of the flies to the different unique settings. However, despite this, we noted pronounced shifts in only three out of nine species in the subset of ORNs under study. Evidently, the odorants detected by the ORNs we studied can be used to navigate in a multitude of settings and are clearly important to fruitflies. Although very similar, the ligand affinity of the neurons was not identical, as can be seen in figure 3. The well-defined shift we observe in *D. simulans* and *D. sechellia* from ethyl hexanoate to methyl hexanoate as key ligand for the ab3A neuron is in accordance with what could be expected from a minor substitution in the binding region of the OR. Support for this notion comes from the finding that the ab3A ORNs in *D. melanogaster* express the receptor *Or22a*, which has an orthologous counterpart in *D. simulans* (*DsOr22a*) that shows 94% sequence homology (Dobritsa *et al.* 2003). Assuming the receptive range of the ab3A ORNs in *D. simulans* is due to the activity of the *DsOr22a* would imply that the minor shift in ligand affinity is accomplished by a 6% difference in coding sequence. The differences observed among the other neurons are probably caused by minor shifts in orthologous ORs. The distinct shift in response profile of the ab3A ORNs of *D. mauritiana* from ethyl and methyl hexanoate to ethyl butyrate may indicate the presence of an OR with a different origin than the ORs expressed in the other ab3A neurons. In addition, the *D. mauritiana* ab3 sensillum was the only case where we noted a significant change in ORN spike amplitude ratio, which may further suggest a separate origin. However, the ligand affinity of the neuron has actually only shifted from a C-6 ethyl ester to a C-4 ethyl ester specificity, a switch that perhaps does not require a substantially different OR. A more drastic shift would be the loss of an entire sensillum type, and its replacement by another, as we observed in *D. sechellia*: a change that probably requires a substantial re-wiring of peripheral as well as central nervous circuits.

Both species where we observed alterations in the olfactory code are insular endemics, perhaps not unexpected, as islands have long been associated with evolutionary processes (Wallace 1880; MacArthur & Wilson 1967). Are the alterations we observe the outcome of genetic drift or driven by natural selection? In *D. sechellia* we found in place of the missing ab2 sensilla an increased proportion of ab3 sensilla. This could possibly represent a host-specific adaptation towards the *Morinda citrifolia*

fruit, the single host of *D. sechellia* (Tsacas & Bächli 1981; Louis & David 1986). The specialization of *D. sechellia* towards morinda fruit involves specific adaptations, as fresh and ripe morinda fruit is presumably toxic to all *Drosophila* species, except for *D. sechellia* (R'Kha *et al.* 1991; Legal *et al.* 1992; Farine *et al.* 1996; Jones 1998). ripe morinda fruit has a high content of esters, including ethyl and methyl hexanoate, which give the fruit a characteristic aroma, apart from the cheese-like odour, which is primarily due to a high content of octanoic acid. The odorants detected by the missing ab2 neurons are, however, not found in morinda fruit (Farine *et al.* 1996). Having a larger proportion of neurons devoted to the detection of ethyl and methyl hexanoate should enhance the ability of the flies to locate their host over long distances. As the toxic fatty acids have been shown to be important for inducing oviposition (R'Kha *et al.* 1991; Amlou *et al.* 1998), we propose that the esters are important cues used by the flies to detect morinda fruit over distance, necessary as patches of morinda trees are dispersed (R'Kha *et al.* 1991). The lost capacity in *D. mauritiana* to efficiently detect ethyl and methyl hexanoate is quite surprising. Ethyl hexanoate has been shown to be highly attractive to *D. melanogaster* (Stensmyr *et al.* 2003). As *D. mauritiana* shares food preference with *D. melanogaster* (David *et al.* 1989), i.e. being a generalist rotting-fruit feeder, it is notable that in *D. mauritiana* the ability to efficiently detect these compounds has been lost, especially because the compounds are common fruit volatiles (Stensmyr *et al.* 2003) and should thus be of major importance. The present day population of *D. mauritiana* feeds primarily on non-native plants, introduced by man during the past centuries (David *et al.* 1989). The host/s of the founding population, before the arrival of man, can of course only be speculated upon; however, the shift to ethyl butyrate may represent an adaptation for an as yet unknown primitive resource. If so, a possible candidate host may be fruits of the *Spondias borbonica* tree, as relatives of this Mauritius endemic have been shown to have ethyl butyrate as a major or prominent volatile constituent (Jirovetz *et al.* 1999; Augusto *et al.* 2000). However, with the original host unknown, whether or not the shift in tuning is the result of a host-specific adaptation remains to be explored.

Endemic distribution and resource specialization have not generally resulted in shifted olfactory physiology of the LB ORNs in the *melanogaster* subgroup. The two other endemics, *D. santomea* and *D. orena*, share the same olfactory characteristics as their presumed closest ancestors as well as with the other siblings. *Drosophila santomea*, apart from being an insular endemic, may also be a host specialist as specimens have been found only on fallen figs from *Ficus clamydocarpa* trees (Cariou *et al.* 2001). In addition, host specialization may also occur in *D. erecta*, which can be found only in *Panadamus* spp. swamps. *Drosophila yakuba*, although a generalist, actually uses few genera of plants and prefers fruit of the *Parinari excelsa* tree (Lachaise *et al.* 1988). Why changes in the LB ORNs are found only in the *simulans* clade and not in the species with similar life histories is intriguing and should be the focus of future studies.

In the present study, we examined a part of the olfactory code, as defined by ORNs in LB sensilla. By judging OR

Figure 3. Response spectra of ORNs housed in LB sensilla across the *melanogaster* subgroup. Neuron activity is shown as spike frequency (spikes per second $\pm$ s.e.m.). ab1, ab2 and ab3 refer to the three functional types of LB sensilla, characterized by the receptive fields of their ORNs. A–D refer to ORNs, with A being the largest spiking neuron, B second largest and so forth. The response spectra of the ORNs were highly conserved, and corresponding ORN types only displayed a minor variance in ligand affinity. Notable shifts were found in only four places: in *D. simulans*, *D. sechellia* and *D. mauritiana* the ab3A type neurons displayed an altered ligand affinity and also in *D. sechellia*, the ab2 type sensilla was not detected. *n*-values represent the number of recordings used to establish the spectra and within brackets the total number found. *Dmel*, *D. melanogaster*; *Dsim*, *D. simulans*; *Dsec*, *D. sechellia*; *Dmau*, *D. mauritiana*; *Dyak*, *D. yakuba*; *Dsan*, *D. santomea*; *Dtei*, *D. teissieri*; *Dere*, *D. erecta*; *Dore*, *D. orena*.

expression patterns (Clyne *et al.* 1999; Gao & Chess 1999; Vosshall *et al.* 1999, 2000; Scott *et al.* 2001), we have examined approximately one-fifth of the antennal ORN types. It is possible that shifts have also occurred among the ORNs not studied. However, a drastic change of the code among those ORNs would probably also have affected the code as relayed by the studied subset of ORNs. That relatively minor shifts, like those observed among the

LB neurons, have occurred among the other neurons is quite likely, but does not detract from the overall picture that the code at large has been conserved to a remarkable degree across the subgroup.

The authors thank members of the Hansson laboratory for valuable comments on the manuscript. This work was supported by the Swedish Research Council.

Figure 4. Hierarchical dendrogram. The cluster classification was based on the average responses from each neuron type, of each species to the complete odour set. The cluster analysis separates the eight different classes of ORNs, in addition it also clustered the ab3A type neurons of the *simulans* clade (clusters denoted ab3A' and ab3A'') separate from those of the other ab3A type neurons. Abbreviations as in figure 3.

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