Odorant receptor orthologues in conifer-feeding beetles display conserved responses to ecologically relevant odors

Short title: Conserved responses in odorant receptors

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Abstract

Insects are able to detect a plethora of olfactory cues in the environment using a divergent family of odorant receptors (ORs). Despite the divergent nature of this family, related species frequently express several evolutionarily conserved OR orthologues. In Coleoptera, it remains unknown whether OR orthologues have conserved or divergent functions in different species. We addressed this question through functional characterization of two groups of OR orthologues in three species of the Curculionidae (weevil) family, the conifer-feeding bark beetles *Ips typographus* ('Ityp') and *Dendroctonus ponderosae* ('Dpon') (both in subfamily Scolytinae), and the pine weevil *Hylobius abietis* ('Habi'; Molytinae). The ORs of *H. abietis* were annotated from antennal transcriptomes. Results show highly conserved response specificities, with one group of orthologues (HabiOR3/DponOR8/ItypOR6) responding exclusively to 2-phenylethanol (2-PE), and the other group (HabiOR4/DponOR9/ItypOR5) responding to angiosperm green leaf volatile (GLV) alcohols. Both groups of orthologues belong to the major coleopteran OR group 2B, and share a common ancestor with OR5 in the cerambycid *Megacyllene caryae*, also tuned to 2-PE, suggesting a shared evolutionary history of 2-PE receptors across two beetle superfamilies. The detected compounds are ecologically relevant for conifer-feeding curculionids, and are probably linked to fitness, with GLVs being used to avoid angiosperm non-host plants, and 2-PE being important for intraspecific communication and/or playing a putative role in beetle-microbe symbioses. To our knowledge, this study is the first to reveal evolutionary conservation of OR functions across several beetle species and hence sheds new light on the functional evolution of beetle ORs.

Introduction

Insects are able to detect thousands of different chemical cues that convey important information about the environment, including plant volatiles, microbial odors, and pheromones (Dahanukar, et al. 2005; Hansson and Stensmyr 2011; Stensmyr, et al. 2012; Kandasamy, et al. 2019). This sophisticated discernment of odors is possible via large suites of odorant receptors (ORs), which bind and detect odor molecules in peripheral olfactory sensory neurons (OSNs), triggering neuronal signals to be processed by the central nervous system (Clyne, et al. 1999; Vosshall, et al. 1999; Sato, et al. 2008; Wicher, et al. 2008; Brand, et al. 2018). These seven-transmembrane proteins form heteromeric complexes with a highly conserved co-receptor (Orco), which is necessary for odor responses in most insects by

contributing to the formation of a ligand-gated ion channel (Larsson, et al. 2004; Butterwick, et al. 2018).

The OR gene family evolves according to a birth-and-death model, in which duplication events represent the birth of genes, and deletion or pseudogenization their death (Nei, et al. 2008). Accordingly, OR genes are often found in tandem arrays on insect chromosomes, with significant variation in the size of OR repertoires between species (Andersson, et al. 2015; Benton 2015; Brand and Ramírez 2017). Frequently, the majority of ORs in a given species are present within species- or taxon-specific phylogenetic OR-lineage radiations (Mitchell, et al. 2020). Within such radiations, novel olfactory functions may evolve due to relaxed constraints or positive selection post gene duplication, provided the duplicated gene is retained and expressed (Andersson, et al. 2015; Hou, et al. 2021). Despite the general divergent nature of this receptor family, certain ORs are conserved across species, with simple (1:1) orthologous OR-pairs typically being present among related species. However, such clear orthology is usually rare or absent when comparing more distantly related insect species from different families, which has been shown in e.g., beetles (Coleoptera) (Mitchell and Andersson 2020; Mitchell, et al. 2020). Whether OR orthologues share the same or similar olfactory functions, or if functions have diverged in different species, has been studied primarily in moths (Lepidoptera) and mosquitos (Diptera). Such studies are important for advancing our understanding of the functional evolution of the insect OR family, as they may inform shared ecological relevance of certain compounds in different species, hence shared selection pressures acting on the olfactory sense of insects. For example, the host- and oviposition cues 1-octen-3-ol, indole and skatole, respectively, are detected by orthologous groups of ORs across several mosquito species (Dekel, et al. 2016; Ruel, et al. 2019). In moths, some olfactory functions are widely conserved among OR orthologues, including both the detection of specific plant odors and sex pheromone compounds, whereas other orthologues are functionally different (Gonzalez, et al. 2015; Guo, Huang, et al. 2021; Guo, Du, et al. 2021).

In contrast, nothing is known about the functions of OR orthologues in beetles, which is not surprising given the very few ORs that have been functionally characterized in this large order. The response profiles of seven ORs have been characterized from the Eurasian spruce bark beetle *Ips typographus* ('Ityp'; Curculionidae) (Hou, et al. 2021; Roberts, et al. 2021; Yuvaraj, et al. 2021), two ORs from the red palm weevil *Rhynchophorus ferrugineus*

(Curculionidae) (Antony, et al. 2021; Ji, et al. 2021), one OR from the dark black chafer *Holotrichia parallela* (Scarabaeidae) (Wang, et al. 2020), one OR from the Adonis ladybird *Hippodamia variegata* (Coccinellidae) (Xie, et al. 2022) and three ORs from the hickory borer *Megacyllene caryae* (‘Mcar’; Cerambycidae) (Mitchell, et al. 2012). Among the characterized ORs of this cerambycid, McarOR5 responded strongly to the male-produced pheromone component 2-phenylethanol (2-PE) (Mitchell, et al. 2012). McarOR5 belongs to the beetle OR subfamily named Group 2B, which contains conserved OR lineages with receptors from several beetle families, including the large family of true weevils, Curculionidae (Mitchell, et al. 2020). This beetle family harbors the damaging conifer-feeding bark beetles of the Scolytinae subfamily and many other weevils that are pests of agriculture and forestry, such as the pine weevil *Hylobius abietis* (‘Habi’; Molytinae) (Shin, et al. 2018). The 2-PE is ecologically relevant for several conifer-feeding beetles. For instance, it is part of the attractive odor bouquets released by the fungal symbionts of *I. typographus* (Kandasamy, et al. 2019) and the compound has also been identified from the hindgut of male beetles, where it may be produced by yeasts (Leufvén, et al. 1984), in highest amounts before the acceptance of females (Birgersson, et al. 1984). The compound is the primary odorant for one of the characterized OSN classes of *I. typographus*, suggesting that this species is likely to have an OR tuned to this compound. Also *Dendroctonus* bark beetles produce this compound (Sullivan 2005), including the mountain pine beetle *D. ponderosae* (‘Dpon’; Curculionidae), in which 2-PE reduces the attraction to the aggregation pheromone (Pureswaran, et al. 2000). In the pine weevil *H. abietis*, 2-PE operates as a strong anti-feedant present in deterrent non-host plants (Eriksson, et al. 2008). Interestingly, 2-PE is also produced by gut bacteria of *H. abietis*, and the behavior of this species, to cover their laid eggs with feces and frass containing deterrent compounds, may protect the eggs from being eaten by conspecifics (Borg-Karlson, et al. 2006; Axelsson, et al. 2017).

Due to the widespread use of 2-PE in the ecologies of conifer-feeding curculionids, we hypothesized that the compound may be detected by evolutionarily and functionally conserved ORs and that these receptors may be related to McarOR5. To test this hypothesis, we used HEK293 cells to functionally characterize two clades with simple OR orthologues from three curculionids (*I. typographus*, *D. ponderosae*, and *H. abietis*), i.e., the orthologues in Group 2B that are positioned closest to McarOR5 in the OR phylogeny. The OR repertoires of the two bark beetles have been previously reported (Andersson, et al. 2013; Andersson, et al. 2019; Yuvaraj, et al. 2021); however, to obtain the OR sequences from *H. abietis* we

sequenced, analyzed, and annotated male and female antennal transcriptomes. Our results show that 2-PE is indeed detected by functionally conserved and highly specific OR orthologues in all three curculionids. These ORs share a common ancestor with MearOR5 suggesting functional conservation also across two beetle superfamilies (Curculionoidea and Chrysomeloidea). Additionally, green leaf volatile (GLV) alcohols, abundant in non-host angiosperm plants and generally avoided by conifer-feeding beetles (Zhang and Schlyter 2004), were detected by the second assayed clade of curculionid ORs. These receptors share evolutionary history with the ORs detecting 2-PE. Altogether, our findings suggest strong functional conservation in ORs detecting ecologically important odors, and thereby expand our knowledge of the functional evolution of the OR family in the largest order of insects.

Results

HabiOR annotation, expression, and phylogenetic analysis of ORs

The HabiOrco gene and 78 HabiOR genes were annotated from the antennal transcriptome assembly, of which 51 transcripts encoded full length proteins. The 28 partial HabiORs encompassed 174 to 397 amino acids. Three of the ORs were extended by joining overlapping sequences from two different transcripts. Annotation details and sequences of the HabiORs are presented in Supplementary Table 1. Sequence reads from the male and female samples were mapped to the open reading frames (ORFs) of annotated OR genes for estimation of relative OR gene expression levels. This analysis showed that the HabiOrco gene is clearly more highly expressed than any of the 78 HabiORs (Supplementary Table 1). The ORs showed a range of expression levels (from 0.03% to 4.2% of the Orco expression in males; from 0% to 5.7% in females), with no specific OR standing out as being particularly highly expressed compared to the others. Expression was similar in the two sexes; the only putative exceptions being the partial HabiOR27NC and HabiOR46NC that showed twice the expression in females compared to males, and HabiOR48 with twice the expression in males compared to females. Expression levels of the functionally characterized HabiOR3 and HabiOR4 were intermediate, with a somewhat higher estimate for HabiOR4 in both sexes.

Recently, a phylogenetic analysis of the ORs across several coleopteran superfamilies defined and revised nine main monophyletic groups of ORs (Mitchell, et al. 2020). Our phylogenetic analysis including ORs from the Curculionidae and Cerambycidae shows that the distribution of HabiORs among the nine OR groups is similar to that of the other two curculionids (*I.*

typographus and *D. ponderosae*) in the analysis (Figure 1), with most (55) ORs located within Group 7, followed by Group 1 (9 ORs), Group 5A and 2A (5 ORs in each), and 2B (4 ORs). The main differences between *H. abietis* and the two bark beetle species are the stronger bias of HabiORs towards Group 7, including a large HabiOR-radiation of 26 receptors (HabiOR23-HabiOR48), and comparatively few HabiORs in Group 5A. The latter may be explained by the generally poor antennal expression of Group 5A ORs (Yuvaraj, et al. 2021); indeed, the vast majority of Group 5A ORs from *D. ponderosae* was not found in the initial transcriptome analyses (Andersson, et al. 2013), but later recovered from the genome (Andersson, et al. 2019). As with other curculionids, our analysis indicates that *H. abietis* entirely lacks ORs from Groups 3, 4, 5B, and 6. Similar to our previous study, the OR phylogeny did not recapitulate the monophyly of Group 2B, which is likely explained by the few species and narrow taxonomic range sampled in this study (Yuvaraj, et al. 2021).

Our OR phylogeny suggests 12 highly supported clades of simple (1:1:1) OR orthologues conserved across the three curculionids (Figure 1). We also found that two of the MearORs appear to have representative orthologues in at least some curculionids, i.e., MearOR2 grouped together with HabiOR1 and ItypOR11, and MearOR4 with DponOR6NTE and ItypOR51 (Figure 1). Within OR Group 2B, two orthologous groups of curculionid ORs (ItypOR5/DponOR9/HabiOR4 and ItypOR6/DponOR8/HabiOR3) were positioned close to MearOR5, responding to 2-PE –a pheromone component in this species (Figure 1). Hence, these six ORs were targeted for functional characterization to investigate whether evolutionarily related beetle ORs within and between coleopteran superfamilies may have the same response specificities. The amino acid identities between ItypOR5, DponOR9, HabiOR4 range from 50.5% to 57.8%, and for ItypOR6, DponOR8, and HabiOR3 between 60.0% and 67.5%.

Conserved responses to green leaf volatiles and 2-phenylethanol in OR orthologues

The HEK cells transfected with each of the six above-mentioned beetle OR genes were analyzed for OR protein detection using Western blots. Except for HabiOR4, the OR proteins were clearly detected, and only from cells induced to express the exogenous receptor genes, demonstrating proper regulation by the repressor system (Supplementary Figure 1). Gene sequences codon-optimized for expression in human cells were used for the DponORs because we had no access to biological material from this species (Roberts, et al. 2021). Additionally, because the HabiOR4 protein was not detected from cell lines transfected with

the wildtype OR gene, this gene was also codon-optimized and used in functional assays. The superscript HsCO (*Homo sapiens* Codon Optimized) was added to the names of codon optimized OR genes.

In the OR phylogeny, the orthologous curculionid ORs HabiOR4, ItypOR5, and DponOR9 group most closely to the 2-PE receptor in the cerambycid *M. caryae* (McarOR5; Figure 1). Screening experiments testing 62 ecologically relevant compounds at 30 μ M concentration showed that neither of these curculionid ORs responded to 2-PE. Instead, HabiOR4^{HsCO} responded to five six-carbon green leaf volatile (GLV) alcohols, abundant in angiosperm non-host plants, with significantly stronger responses in induced vs. non-induced cells (Figure 2A). Z3-hexenol elicited the strongest response in the screening experiments (7.3% increased fluorescence; $F_{1,14} = 183.8$; $p < 0.001$), followed by slightly weaker and similar responses to each of E2-hexenol, Z2-hexenol, and 1-hexanol (5.2-5.7%; $F_{1,14} = 48.9-118.8$; all $p < 0.001$), and a yet weaker response to E3-hexenol (3.6%; $F_{1,14} = 40.5$; $p < 0.001$). Subsequent dose-response experiments largely recapitulated the screening data in terms of response magnitudes elicited by the five compounds at the higher concentrations (Figure 2B). Nevertheless, these experiments indicated similar sensitivities to the four most active ligands (EC_{50} values: Z3-hexenol – 7.99 μ M, E2-hexenol – 3.88 μ M, 1-hexanol – 2.85 μ M, and Z2-hexenol – 7.34 μ M), whereas the sensitivity to E3-hexenol was lower as shown by its weaker response at most tested concentrations and the non-sigmoid shape of the dose-response curve (EC_{50} could not be estimated).

A similar response profile was apparent for DponOR9^{HsCO} (Figure 2C). Again, Z3-hexenol elicited the highest response in the screening experiments (4.5%; $F_{1,14} = 51.5$; $p < 0.001$), followed by E2-hexenol (3.2%; $F_{1,14} = 44.2$; $p < 0.001$), Z2-hexenol (2.3%; $F_{1,14} = 48.3$; $p < 0.001$), and 1-hexanol (2.0%; $F_{1,14} = 24.6$; $p < 0.001$). The slightly increased fluorescence seen upon stimulation with E3-hexenol was not statistically significant after correction for multiple statistical comparisons. In contrast to HabiOR4, (+)-*trans*-4-thujanol elicited a significant response in DponOR9^{HsCO} (3.5%; $F_{1,14} = 17.0$; $p = 0.001$); however, half of this response was also evident in the non-induced control cells suggesting that factors unrelated to the DponOR contributed to the cells' response. The two GLV compounds eliciting responses above 3% increased fluorescence in the screening were further examined in dose-response trials, showing somewhat stronger responses to Z3-hexenol as compared to E2-hexenol at the higher

concentrations but similar responses at intermediate and lower concentrations (Figure 2D). EC₅₀ values could not be estimated due to the shape of the dose-response curves.

ItypOR5 responded to the same four GLV alcohols as did DponOR9^{HsCO}, albeit with overall lower response magnitudes (Figure 2E), which is in accordance with this OR being detected as a fainter band on Western blot (Supplementary Figure 1) compared to the other orthologues (see also Roberts, et al. 2021). The rank order between compounds was slightly different, with *E2*-hexenol eliciting the highest response (3.3%; $F_{1,24} = 39.8$; $p < 0.001$), followed by *Z2*-hexenol (2.2%; $F_{1,23} = 19.3$; $p < 0.001$), and similar responses to *Z3*-hexenol (1.6%; $F_{1,24} = 27.9$; $p < 0.001$) and 1-hexanol (1.6%; $F_{1,24} = 30.5$; $p < 0.001$). As with DponOR9^{HsCO}, the slightly increased fluorescence elicited by *E3*-hexenol in induced cells was not statistically significant after correction for multiple comparisons. *E2*-hexenol activated ItypOR5 in a dose-dependent manner (Figure 2F; EC₅₀ could not be estimated due to the shape of the dose-response curve); the other GLVs were not assayed in dose-response experiments due to their screening responses being below 3%.

Compared to the GLV-responding ORs, the orthologous receptors HabiOR3, ItypOR6, and DponOR8 are positioned a bit further away from McarOR5 in the OR phylogeny; yet they are part of the same OR clade (Figure 1). The three ORs all responded exclusively to 2-PE, with significantly stronger responses in induced compared to non-induced cells. The highest response was recorded for HabiOR3 (10.5% increased fluorescence; $F_{1,19} = 324.7$; $p < 0.001$; Figure 3A), followed by ItypOR6 (5.7%; $F_{1,14} = 85.9$; $p < 0.001$; Figure 3E), and DponOR8^{HsCO} (4.0%; $F_{1,14} = 80.4$; $p < 0.001$; Figure 3C). Each of these receptors responded to 2-PE in a dose-dependent manner with estimated EC₅₀ values at 8.54 μ M, 3.45 μ M, and 5.12 μ M for HabiOR3, ItypOR6, and DponOR8^{HsCO}, respectively (Fig. 3B, D, F).

Discussion

To allow for functional characterization of *H. abietis* ORs, antennal transcriptomes were sequenced and the ORs annotated, suggesting a similarly sized OR repertoire (79 ORs) as in *I. typographus* (73 ORs) and *D. ponderosae* (86 ORs) (Andersson, et al. 2019; Yuvaraj, et al. 2021). The largest number of HabiORs was found in OR Group 7, and *H. abietis* stands out by its large OR-radiation within this group. This differs from both the bark beetles and the red palm weevil *R. ferrugineus*, but displays similarity with the large radiation of Group 7 ORs in

the sweetpotato weevil *Cylas formicarius* (Antony, et al. 2016; Bin, et al. 2017). Our previous study indicated that the two scolytine bark beetles *I. typographus* and *D. ponderosae* share 17 highly-supported simple OR orthologues (Yuvaraj, et al. 2021). Here, we included also the pine weevil from the Molytinae subfamily in the analysis, and found that 12 simple orthologues are conserved across these three species. The observation of fewer orthologues in this broader analysis is consistent with the general positive correlation between species relatedness and occurrence of OR orthology (Mitchell, et al. 2020). Our OR phylogeny also support the notion that OR orthology is rare across beetle superfamilies (Mitchell and Andersson 2020; Mitchell, et al. 2020).

To investigate whether OR orthologues are functionally conserved in the three curculionids, and whether evolution of OR functionality may be traced beyond the beetle superfamily level, we characterized two curculionid OR clades within beetle OR subfamily 2B, both of which are evolutionarily related to the cerambycid 2-PE receptor McarOR5 (Mitchell, et al. 2012). Our results show conserved functions across the tested OR orthologues, with one group (HabiOR3/DponOR8/ItypOR6) responding exclusively to 2-PE, and the other group (HabiOR4/DponOR9/ItypOR5) responding to several six-carbon GLV alcohols. Our results further show that the 2-PE receptors in the curculionids share a common ancestor with McarOR5, which is also the case for their GLV-responding ORs. Interestingly, the GLV receptors in the curculionids are the most closely related to McarOR5, while their 2-PE receptors are part of a sister clade. This suggests that the response to 2-PE may be ancestral and that new functions such as GLV-responsiveness have subsequently evolved (neofunctionalization) in the more recent OR lineages of the curculionids. Another possibility would be that the ancestral receptor was broadly tuned to both 2-PE and GLVs and that the more recent OR lineages, derived from gene duplication, may have evolved higher specificity for either GLVs or 2-PE in the process of subfunctionalization (Andersson, et al. 2015). In fact, this scenario may be supported by recent data from single-sensillum recordings (SSR) from *I. typographus* showing secondary responses to 1-hexanol in the ‘2-PE’ OSN class and secondary responses to 2-PE in the ‘GLV-OH’ OSN class (Kandasamy, et al. 2019). Revealing the responses of the remaining six related McarORs (McarORs 8, 10, 13, 22, 39, and 56; see Figure 1) is needed to conclusively inform the evolutionary history of these OR functions; in particular in this context, it could be enlightening to search for GLV-responding ORs among the orphan McarORs. The clade containing the curculionid 2-PE receptors is also intriguing due to the presence of orthologues in numerous additional species from several

beetle families, including *Anoplophora glabripennis* (Cerambycidae), *R. ferrugineus* (Curculionidae), *Leptinotarsa decemlineata* (Chrysomelidae), *Tribolium castaneum* (Tenebrionidae), *Nicrophorus vespilloides* (Silphidae), and *Onthophagus taurus* (Scarabaeidae) (Mitchell, et al. 2020; Antony, et al. 2021). Unraveling the functions of the orthologues in these species should inform how widely conserved the ORs for 2-PE are across the Coleoptera. In moths, it was shown that OR orthologs detecting the flower compound phenylacetaldehyde were functionally conserved across eleven species from several families of the Glossata suborder although the majority of the other investigated orthologues were functionally divergent, even among related species (Guo, Du, et al. 2021).

The strong functional conservation among the examined OR orthologues suggests that 2-PE and GLV alcohols convey important fitness-related information to the three curculionids. Indeed, GLVs are abundant in the leaves of angiosperms and less so in conifers, and are regarded as non-host cues that typically inhibit the attraction of conifer-feeding bark beetles to their aggregation pheromones (reviewed in Zhang and Schlyter 2004). Hence, GLVs are likely used by the beetles to avoid colonizing angiosperm trees in which they cannot reproduce (Dickens, et al. 1992; Schiebe, et al. 2011). A role of GLVs in host choice has also been proposed for *H. abietis* (Pettersson, et al. 2008; Kännaste, et al. 2013). Extensive SSRs from the OSNs of *I. typographus* have identified a single OSN class responding with similar sensitivity to several GLV alcohols (*E2*-hexenol, *Z3*-hexenol, and 1-hexanol) (Andersson, et al. 2009; Kandasamy, et al. 2019), which is in accordance with the data from the GLV-responsive ORs characterized here. In *I. typographus*, the three GLVs have similar inhibitory effects on pheromone attraction and the use of a single compound (e.g., 1-hexanol) can replace a three-component GLV mixture at an equivalent release rate without compromising the inhibitory effect (Zhang and Schlyter 2003; Unelius, et al. 2014). These findings support the idea that this conifer specialist employs a single neuronal channel, hence likely a single OR (ItypOR5), for the detection of several, structurally similar compounds that all convey the message of a non-host plant that should be avoided (Andersson, et al. 2009; Raffa, et al. 2016). In *D. ponderosae*, coupled gas chromatographic-electroantennographic detection (GC-EAD) demonstrated antennal detection of the GLV alcohols that activate DponOR9 (Wilson, et al. 1996; Huber, et al. 2000). The strongest inhibitory effect on pheromone attraction was observed for *Z3*-hexenol and *E2*-hexenol (Wilson, et al. 1996), that is, the two compounds that elicited the strongest responses in DponOR9. However, since (to our knowledge) no SSRs have been performed, it remains unknown whether *D. ponderosae* also has only a single

OSN class responding to six-carbon GLV compounds. Likewise, SSR studies testing GLV compounds in *H. abietis* appear to be missing in the published literature (Wibe, et al. 1997). In contrast to *I. typographus*, beetles feeding on angiosperms typically possess several OSN classes detecting GLVs, each with their unique response specificity (Hansson, et al. 1999; Larsson, et al. 2001; Andersson, et al. 2012; Carrasco, et al. 2019).

Also 2-PE is tightly connected to the chemical ecologies - and potentially reproductive fitness - of the three investigated species. In *H. abietis* it is a potent anti-feedant, present in the gut of the beetle and in non-host plants (Eriksson, et al. 2008; Axelsson, et al. 2017). Through deposition of feces containing 2-PE over the laid eggs, the compound may contribute to reducing egg predation by conspecifics (Borg-Karlson, et al. 2006; Axelsson, et al. 2017). It is also present in the guts of both *D. ponderosae* and *I. typographus*. 2-PE inhibits attraction to the aggregation pheromone in the former species, suggesting it may contribute to termination of aggregation and induction of dispersal (Pureswaran, et al. 2000). In contrast, 2-PE appears to have no effect on pheromone attraction in *I. typographus* (Schlyter, et al. 1987); however, it is part of the odor blends released by several species of ophiostomatoid symbiotic fungi, which are attractive to beetles in laboratory bioassays (Kandasamy, et al. 2019). These fungi, inoculated by beetles inside their galleries under the tree bark, are likely to benefit beetles by providing nutrients and through metabolism of the tree's chemical defenses (Kandasamy, et al. 2019; Kandasamy, et al. 2021). *I. typographus* has an OSN class that primarily responds to 2-PE and secondarily to phenethyl acetate, 3-methyl-1-butanol and a few more compounds (including 1-hexanol) with weaker activity (Kandasamy, et al. 2019). These secondary responses were not evident in any of the three 2-PE receptors (HabiOR3, DponOR8, and ItypOR6) characterized in the present study. Higher response specificities in ORs when tested in HEK293 cells as compared to those seen in putatively corresponding OSN classes have been observed also previously, such as for ItypOR46 and ItypOR49, responding to the beetle-produced compounds ipsenol and ipsdienol, respectively (Yuvaraj, et al. 2021). Similarly, several of the weakly active secondary compounds for the GLV-responsive OSN class in *I. typographus*, including hexanal, *E2*-hexenal, 1-octen-3-ol, and 2-PE failed to activate ItypOR5 (as well as DponOR9 and HabiOR4) (Andersson, et al. 2009; Kandasamy, et al. 2019). The reasons for this discrepancy remain unknown but could potentially be due to lower sensitivity of the HEK cell assay as compared to SSR or a consequence of the “unnatural” cellular environment in HEK cells, which may affect protein folding and hence access of ligands to their binding sites (see also Hou, et al. 2020; Yuvaraj, et al. 2022).

In conclusion, we report the functional characterization of six ORs from three species of the Curculionidae family, including the first characterized ORs from the devastating forest pests *H. abietis* and *D. ponderosae*. We reveal highly conserved responses to ecologically relevant odors within the two groups of assayed OR orthologues, suggesting that the detection of 2-PE and GLVs is important for the fitness of conifer-feeding curculionids. The characterized ORs were shown to be evolutionarily related with the 2-PE receptor in *M. caryae*, sharing a common ancestral OR protein. Our findings demonstrating conserved responses among beetle ORs from two taxonomic superfamilies provide new insight into the functional evolution of the OR family in this large insect order.

Materials and Methods

Sequencing, assembly, annotation, and analyses of the Hylobius abietis antennal transcriptome

Wild beetles were collected by hand at Balungstrand's sawmill in Enviken, close to Falun in mid-Sweden, and kindly provided by Prof. Göran Nordlander (Swedish University of Agricultural Sciences, Uppsala, Sweden). The antennae from 20 males and 20 females were excised and collected separately in tubes kept on dry ice and then stored at -80°C . The antennae were homogenized using Tissue-tearor model 98370-365 (Bartlesville, OK, USA), and total RNA was isolated using the RNeasy Minikit (Qiagen, Hilden, Germany). Extractions yielded 4.8 μg and 3.1 μg of high-quality total RNA from male and female antennal samples, respectively. These RNA samples were used for both transcriptome sequencing and molecular cloning.

The RNA samples were DNase-treated and then underwent poly-A enrichment and library construction using a TruSeq v2 Library Preparation Kit (Illumina, San Diego, CA, USA), followed by 150 bp paired-end sequencing on an Illumina HiSeq 3000 platform at the Max Planck-Genome center (Cologne, Germany). The sequencing produced 36,455,021 and 35,770,733 paired-end reads from the male and female samples, respectively. Adaptor sequences and low-quality reads were removed using Trimmomatic (v 0.36) (Bolger, et al. 2014) with a custom screening database, before performing *de novo* assemblies with Trinity version 2.4.0 (Grabherr, et al. 2011). Reads from males and females were assembled separately, and also combined. Contigs from the Trinity output were clustered to reduce the

number of redundant transcripts using CD-HIT-EST (v 4.6.8) (Li and Godzik 2006) with a sequence identity threshold of 0.98. Primarily, the sex-combined non-redundant assembly was used for downstream annotation of OR-encoding transcripts, and was comprised of 46,669 predicted protein-coding ‘genes’ with their respective isoforms and together with other non-coding genes totaled 199,035 transcripts. The average transcript length was 824 bp with an N50 of 1,570 bp. The completeness of the sex-combined assembly was firstly assessed using the Benchmarking Universal Single-Copy Orthologs (BUSCO v5.2.2; <https://busco.ezlab.org/>) tool performed against the Insecta odb10 dataset, including 1,367 reference genes (Manni, et al. 2021). This analysis revealed 96.6% complete (C) BUSCOs, of which 59.8% were present as single copy genes (S) and 40.2% as duplicated genes (D). Only 30 (2.3%) BUSCOs were missing (M) from the assembly and 17 (1.3%) BUSCOs were fragmented (F), indicating the majority of transcripts were represented and were full length. Mapping of the clean reads to the non-redundant assembly resulted in an overall alignment rate of 94.06%, further demonstrating a high level of completeness for this assembly. The RNAseq reads have been deposited in the SRA database at NCBI under the BioProject accession number PRJNA783427.

H. abietis OR genes were annotated through exhaustive tBLASTn searches against the assemblies at an *e*-value cut-off at 1.0. The OR query sequences were obtained from *I. typographus* (Andersson, et al. 2013; Yuvaraj, et al. 2021), *D. ponderosae* (Andersson, et al. 2013; Andersson, et al. 2019), *Anoplophora glabripennis* (McKenna, et al. 2016), *M. caryae* (Mitchell, et al. 2012), and *Leptinotarsa decemlineata* (Schoville, et al. 2018). All annotated HabiOR sequences were included in additional tBLASTn searches against the *H. abietis* assemblies until all novel OR hits were exhausted. Except for the HabiOrco gene which was only assembled to full length in the male-specific assembly, all OR genes were annotated from the sex-combined assembly, and no OR genes were uniquely found or assembled to higher completeness in the two sex-specific assemblies. A few partial OR genes could be extended by joining overlapping transcripts with identical sequences. The names of these genes were given a ‘JOI’ suffix according to established nomenclature (Andersson, et al. 2019, and references therein). Likewise, OR genes missing the N-terminus or C-terminus were given the suffixes ‘NTE’ and ‘CTE’, respectively, to their names. Single letter abbreviations were used in combinations (i.e., J, N, C) for genes with multiple suffixes. Transcripts encoding partial OR sequences below 170 amino acids and those that did not overlap with other OR sequences in multiple sequence alignments were discarded as they may

not represent unique genes. Likewise, for ORs sharing >96% amino acid identity, only one transcript was kept in the dataset to exclude potential assembly isoforms or allelic variants. The identified HabiOR genes were given names from HabiOR1 to HabiOR78 following their groupings in the OR phylogeny (Fig. 1), with consecutive numbering within the major coleopteran OR clades (Mitchell, et al. 2020).

To analyze the expression levels of OR genes in male and female antennae, clean reads were mapped to the ORFs of annotated HabiOR genes using the `align_and_estimate_abundance.pl` script from the Trinity v2.4.0 software package (Haas, et al. 2013) with default parameters except for `--est_method RSEM--aln_method bowtie2 --trinity_mode`. The rationale for mapping to the ORFs of OR genes, and not to all transcripts in the assembly, was because some OR transcripts contained misassembled fragments in non-coding regions, which could bias the estimated expression level of the OR gene.

The HabiOR amino acid sequences were aligned with the OR sequences from *D. ponderosae* (Andersson, et al. 2019), *I. typographus* (Yuvaraj, et al. 2021), and *M. caryae* (Mitchell, et al. 2012) using MAFFT v7.450 (Katoh, et al. 2002; Katoh and Standley 2013), implemented in Geneious Prime v.2020.0.5 (Biomatters Ltd. Auckland, New Zealand). The alignment of a few partial OR sequences were corrected manually. Three miss-aligned partial McarOR sequences (McarORs 41PAR, 50PAR, and 53PAR) were excluded from analysis since their alignments could not be corrected with confidence. Uninformative regions were excised using trimAl v.1.2 (Capella-Gutiérrez, et al. 2009) with the following settings: similarity threshold 0, gap threshold 0.7, and minimum 25% conserved positions. The trimmed alignment was used to construct a phylogenetic tree of ORs using FastTree v.2.1.11 at default settings (Price, et al. 2010). Local node support values were calculated using the Shimodaira-Hasegawa (SH) test implemented within FastTree. The tree was rooted with the Orco lineage, and color coded in FigTree v.1.4.3 (Rambaut 2014). Final graphical editing was performed using Adobe Illustrator.

Molecular cloning and generation of HEK293 cell lines

The protocols for cloning, cell line generation, and culturing have been previously described (Corcoran, et al. 2014; Andersson, et al. 2016; Yuvaraj, et al. 2021). Briefly, OR (and Orco) genes with added 5' Apa1 and 3' Not1 restriction sites, *cacc* Kozak sequence, and N-terminal epitope tags (Myc for Orco, V5 for ORs) were ligated into the pcDNATM4/TO (Orco) or

pcDNATM5/TO (ORs) mammalian expression vectors (Thermo Fisher Scientific). For OR genes that required codon optimization for functional expression in HEK293 cells (Roberts, et al. 2021), the nucleotide sequences were submitted to the Thermo Fisher Scientific GeneArt Portal and codon optimized for *Homo sapiens*, excluding the start methionine and the epitope tag, then synthesized and ligated into the pcDNATM5/TO expression vector. Because we had no access to biological material from *D. ponderosae*, the two functionally assayed DponOR genes were synthesized as codon optimized versions directly, and also because several wildtype beetle OR genes are not functionally expressed in HEK cells (Roberts, et al. 2021). A codon optimized gene of HabiOR4 was also tested since Western blots (below) failed to detect this protein from cells transfected with the wildtype gene. Sequences of codon optimized genes from the two species are provided in Supplementary Table 2. Sequences of OR genes cloned from antennal cDNA (ItypOR5, ItypOR6, HabiOR3, and HabiOR4) have been deposited in GenBank under the accession numbers OL865310-OL865313.

OR genes in expression vectors were transformed into HB101 ampicillin-resistant competent cells (Promega), plated on ampicillin-containing agar, and incubated overnight at 37 °C. Resulting colonies were PCR-screened with vector-specific primers, spread onto a new plate, and incubated at 37 °C for 4-6 hours. Positive colonies were sub-cultured overnight at 37°C in LB broth containing ampicillin. Plasmid DNA from the overnight cultures was harvested via extraction with the PureLink™ HiPure Plasmid Filter Midiprep kit (Thermo Fisher Scientific), and insert sequence was confirmed by Sanger sequencing at the on-site DNA Sequencing Facility (Dept. Biology, Lund University) using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific). Positive plasmids were linearized with FspI, PciI, or BstZ17I (New England Biolabs, Ipswich, MA, United States) restriction enzymes and incubated overnight at 37 °C.

Linearized plasmids containing the ItypOrco gene were transfected into HEK293 cells containing a tetracycline-inducible repressor (TREx) using Lipofectamine 2000 (Thermo Fisher Scientific) (Corcoran, et al. 2014). Twenty-four hours post-transfection, antibiotics (blastidicin for TREx, zeocin for Orco; both NEB) were added to select successfully transfected cells. Once a stable cell line was generated, protein expression of the ItypOrco gene was confirmed via Western blot analysis and functionality of Orco was confirmed using the Orco agonist VUAA1 (described below) (Jones, et al. 2011). We recently found that the DponOrco gene is not functionally expressed in HEK cells (Roberts, et al. 2021); we

therefore co-expressed the DponOR genes with the ItypOrco gene to allow functional characterization of these ORs. For consistency, the HabiOR genes were also tested together with the ItypOrco. Due to the conserved function of Orco and the relatedness among the three beetle species, we assumed that this strategy would not affect the OR response specificities. This assumption is supported by previous studies showing that combinations of OR and Orco proteins from different taxa assemble into functional receptor complexes responding properly to known ligands (Corcoran, et al. 2018). The linearized OR gene-containing plasmids were transfected into the stably expressing TREx/ItypOrco cell line as described above, and cultured with the antibiotic hygromycin (Gold BioTech) to select successfully transfected cells. Resulting cell lines were frozen at -80°C before functional assays.

Protein extraction and Western blot analysis

Cells were cultured without antibiotics for 24 hours before the expression of Orco and OR genes was induced with doxycycline (Sigma). At 16 hours post-induction, cells were pelleted via centrifugation and total protein extraction was performed as described by Corcoran et al. (2014). Protein extractions from non-induced cells served as negative controls. Western blot was performed using 25 μg of total protein from each sample and standard protocols for mixed molecular weight proteins. Primary antibodies (rabbit anti-Myc for Orco, rabbit anti-V5 for ORs) were added at a ratio of 1:2000, and the secondary antibody (anti-rabbit +IgG, HRP-linked for both Orco and ORs) was added at a ratio of 1:5000 (all antibodies from Cell Signaling Technology), as described by Andersson et al. (2016).

Functional characterization of odorant receptors

Ligand-binding activity of cell lines co-expressing Orco and ORs was tested via a calcium fluorescence assay using a CLARIOstar Omega plate reader (BMG Labtech, Ortenberg, Germany) according to previously described protocols (Corcoran, et al. 2014; Andersson, et al. 2016; Yuvaraj, et al. 2021). Briefly, cells were plated in black-walled poly-D-lysine coated 96-well plates (Corning Costar) and incubated overnight. Cells in half the wells were treated with doxycycline to induce expression of exogenous Orco and OR genes 16 hours prior to testing, leaving the non-induced cells as negative controls. The calcium-sensitive fluorophore Fluo-4AM (Life Technologies) was loaded into all wells, and plates were incubated in the dark at room temperature for 30 min before being washed with assay buffer, then incubated for another 30 min in the dark at room temperature prior to the assay.

The test odor panel included 62 compounds (Supplementary Table 3) that are ecologically relevant for conifer-feeding curculionids, including pheromone compounds, volatiles from conifers and angiosperms as well as odorants produced by bark beetle fungal symbionts (Kandasamy, et al. 2019; Kandasamy, et al. 2021; Yuvaraj, et al. 2021). The Orco agonist VUAA1 was tested (at 50 μ M) on each cell line as a positive control for functional Orco expression. Test odors were diluted in DMSO and assay buffer with a final concentration of 30 μ M in the plate wells for screening experiments. Compounds were tested over a minimum of three biological replicates (plates), and were pipetted into three induced and three non-induced wells per plate, creating three technical replicates per plate. A negative control of 0.5% DMSO in assay buffer (vehicle) was tested on each cell line. Background fluorescence was measured for both induced and non-induced cells immediately before compounds were added to the wells, with ligand-binding response of cells measured as the percentage increase in fluorescence from background readings 10 s post stimulation. Mean responses of cells to the added ligands were calculated using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, United States).

A response of $\geq 1\%$ increase in fluorescence in induced cells was required for a compound to be considered active, provided a significantly higher response in induced compared to non-induced cells. Hence, a general linear model (GLM) with “induction” as a fixed factor and “plate” as a random factor (to account for inter-plate variation) was performed using IBM SPSS statistics v.25. Bonferroni correction to maintain the α -level at 0.05 (for up to 12 multiple comparisons within a cell line) was undertaken to avoid reporting false positives (Type I statistical error). Compounds eliciting an increase in fluorescence of 3% or more at the 30 μ M screening concentration were tested in subsequent dose-response experiments. Half-maximal effective concentrations (EC_{50}) were calculated using the non-linear curve fit regression function in GraphPad Prism (version 6). Calculations of EC_{50} values were only performed for compounds with (reasonably) sigmoid dose-response curves.

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Author contributions

MNA conceived the project. RER and MNA conceptualized and designed the study. RER, TB, JKY, and MNA performed molecular work. RER, TB, and JKY performed cell culturing and experimental assays. EG-W, BSH, and MNA coordinated the sequencing of *H. abietis*. EG-W and DP performed transcriptome assemblies, with DP assessing OR gene expression and completeness of final assemblies. MNA annotated the HabiORs, constructed the OR phylogeny, and performed statistical analysis of the HEK cell data. MNA, JKY, and CL contributed to project supervision. MNA and RER drafted the manuscript. All authors contributed to the final version of the manuscript, and have read and approved it for submission.

Data availability

The *H. abietis* RNAseq reads have been deposited in the SRA database at NCBI under the BioProject accession number PRJNA783427. Sequences of cloned OR genes ItypOR5, ItypOR6, HabiOR3, and HabiOR4 have been deposited in GenBank under the accession numbers OL865310-OL865313. Raw data from all HEK cell assays are reported in the supplementary materials of this article.

References

- Andersson MN, Corcoran JA, Zhang D-D, Hillbur Y, Newcomb RD, Löfstedt C. 2016. A sex pheromone receptor in the Hessian fly *Mayetiola destructor* (Diptera, Cecidomyiidae). *Front Cell Neurosci.* 10:212.
- Andersson MN, Grosse-Wilde E, Keeling CI, Bengtsson JM, Yuen MM, Li M, Hillbur Y, Bohlmann J, Hansson BS, Schlyter F. 2013. Antennal transcriptome analysis of the chemosensory gene families in the tree killing bark beetles, *Ips typographus* and *Dendroctonus ponderosae* (Coleoptera: Curculionidae: Scolytinae). *BMC Genomics.* 14:198.

- Andersson MN, Keeling CI, Mitchell RF. 2019. Genomic content of chemosensory genes correlates with host range in wood-boring beetles (*Dendroctonus ponderosae*, *Agrilus planipennis*, and *Anoplophora glabripennis*). *BMC Genomics*. 20:690.
- Andersson MN, Larsson MC, Schlyter F. 2009. Specificity and redundancy in the olfactory system of the bark beetle *Ips typographus*: Single-cell responses to ecologically relevant odors. *J Insect Physiol*. 55:556-567.
- Andersson MN, Larsson MC, Svensson GP, Birgersson G, Rundlöf M, Lundin O, Lankinen Å, Anderbrant O. 2012. Characterization of olfactory sensory neurons in the white clover seed weevil, *Apion fulvipes* (Coleoptera: Apionidae). *J Insect Physiol*. 58:1325-1333.
- Andersson MN, Löfstedt C, Newcomb RD. 2015. Insect olfaction and the evolution of receptor tuning. *Front Ecol Evol*. 3:53.
- Antony B, Johnny J, Montagné N, Jacquín-Joly E, Capoduro R, Cali K, Persaud K, Al-Saleh MA, Pain A. 2021. Pheromone receptor of the globally invasive quarantine pest of the palm tree, the red palm weevil (*Rhynchophorus ferrugineus*). *Mol Ecol*. 30:2025-2039.
- Antony B, Soffan A, Jakše J, Abdelazim MM, Aldosari SA, Aldawood AS, Pain A. 2016. Identification of the genes involved in odorant reception and detection in the palm weevil *Rhynchophorus ferrugineus*, an important quarantine pest, by antennal transcriptome analysis. *BMC Genomics*. 17:69.
- Axelsson K, Konstanzer V, Rajarao GK, Terenius O, Seriot L, Nordenhem H, Nordlander G, Borg-Karlson A-K. 2017. Antifeedants produced by bacteria associated with the gut of the pine weevil *Hylobius abietis*. *Microb Ecol*. 74:177-184.
- Benton R. 2015. Multigene family evolution: perspectives from insect chemoreceptors. *Trends Ecol Evol*. 30:590-600.
- Bin S-Y, Qu M-Q, Pu X-H, Wu Z-Z, Lin J-T. 2017. Antennal transcriptome and expression analyses of olfactory genes in the sweetpotato weevil *Cylas formicarius*. *Sci Rep*. 7:11073.
- Birgersson G, Schlyter F, Löfqvist J, Bergström G. 1984. Quantitative variation of pheromone components in the spruce bark beetle *Ips typographus* from different attack phases. *J Chem Ecol*. 10:1029-1055.
- Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 30:2114-2120.

- Borg-Karlson A-K, Nordlander G, Mudalige A, Nordenhem H, Unelius CR. 2006. Antifeedants in the feces of the pine weevil *Hylobius abietis*: identification and biological activity. *J Chem Ecol.* 32:943-957.
- Brand P, Ramírez SR. 2017. The evolutionary dynamics of the odorant receptor gene family in corbiculate bees. *Genome Biol Evol.* 9:2023-2036.
- Brand P, Robertson HM, Lin W, Pothula R, Klingeman WE, Jurat-Fuentes JL, Johnson BR. 2018. The origin of the odorant receptor gene family in insects. *eLife.* 7:e38340.
- Butterwick JA, del Marmol J, Kim KH, Kahlson MA, Rogow JA, Walz T, Ruta V. 2018. Cryo-EM structure of the insect olfactory receptor Orco. *Nature.* 560:447-452.
- Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics.* 25:1972-1973.
- Carrasco D, Nyabuga FN, Anderbrant O, Svensson GP, Birgersson G, Lankinen Å, Larsson MC, Andersson MN. 2019. Characterization of olfactory sensory neurons in the red clover seed weevil, *Protopion trifolii* (Coleoptera: Brentidae) and comparison to the closely related species *P. fulvipes*. *J Insect Physiol.* 119:103948.
- Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR. 1999. A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron.* 22:327-338.
- Corcoran JA, Jordan MD, Carraher C, Newcomb RD. 2014. A novel method to study insect olfactory receptor function using HEK293 cells. *Insect Biochem Mol Biol.* 54:22-32.
- Corcoran JA, Sonntag Y, Andersson MN, Johanson U, Löfstedt C. 2018. Endogenous insensitivity to the Orco agonist VUAA1 reveals novel olfactory receptor complex properties in the specialist fly *Mayetiola destructor*. *Sci Rep.* 8:3489.
- Dahanukar A, Hallem EA, Carlson JR. 2005. Insect chemoreception. *Curr Opin Neurobiol.* 15:423-430.
- Dekel A, Pitts RJ, Yakir E, Bohbot JD. 2016. Evolutionarily conserved odorant receptor function questions ecological context of octenol role in mosquitoes. *Sci Rep.* 6:37330.
- Dickens JC, Billings RF, Payne TL. 1992. Green leaf volatiles interrupt aggregation pheromone response in bark beetles infesting southern pines. *Experientia.* 48:523-524.
- Eriksson C, Månsson PE, Sjödin K, Schlyter F. 2008. Antifeedants and feeding stimulants in bark extracts of ten woody non-host species of the pine weevil, *Hylobius abietis*. *J Chem Ecol.* 34:1290-1297.

- Gonzalez F, Bengtsson JM, Walker WB, Sousa MF, Cattaneo AM, Montagné N, de Fouchier A, Anfora G, Jacquin-Joly E, Witzgall P. 2015. A conserved odorant receptor detects the same 1-indanone analogs in a tortricid and a noctuid moth. *Front Ecol Evol.* 3:131.
- Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat Biotechnol.* 29:644.
- Guo H, Huang L-Q, Gong X-L, Wang C-Z. 2021. Comparison of functions of pheromone receptor repertoires in *Helicoverpa armigera* and *Helicoverpa assulta* using a *Drosophila* expression system. *Insect Biochem Mol Biol.* 141:103702.
- Guo M, Du L, Chen Q, Feng Y, Zhang J, Zhang X, Tian K, Cao S, Huang T, Jacquin-Joly E, et al. 2021. Odorant receptors for detecting flowering plant cues are functionally conserved across moths and butterflies. *Mol Biol Evol.* 38:1413–1427.
- Haas BJ, Papanicolaou A, Yassour M, Grabherr M, Blood PD, Bowden J, Couger MB, Eccles D, Li B, Lieber M, et al. 2013. *De novo* transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. *Nat Protoc.* 8:1494-1512.
- Hansson BS, Larsson MC, Leal WS. 1999. Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiol Entomol.* 24:121-126.
- Hansson BS, Stensmyr MC. 2011. Evolution of insect olfaction. *Neuron.* 72:698-711.
- Hou X, Zhang D-D, Yuvaraj JK, Corcoran JA, Andersson MN, Löfstedt C. 2020. Functional characterization of odorant receptors from the moth *Eriocrania semipurpurella*: a comparison of results in the *Xenopus* oocyte and HEK cell systems. *Insect Biochem Mol Biol.* 117:103289.
- Hou X-Q, Yuvaraj JK, Roberts RE, Zhang D-D, Unelius CR, Löfstedt C, Andersson MN. 2021. Functional evolution of a bark beetle odorant receptor clade detecting monoterpenoids of different ecological origins. *Mol Biol Evol.* 38:4934-4947.
- Huber DPW, Gries R, Borden JH, Pierce Jr HD. 2000. A survey of antennal responses by five species of coniferophagous bark beetles (Coleoptera: Scolytidae) to bark volatiles of six species of angiosperm trees. *Chemoecology.* 10:103-113.
- Ji T, Xu Z, Jia Q, Wang G, Hou Y. 2021. Non-palm plant volatile α -pinene is detected by antenna-biased expressed odorant receptor 6 in the *Rhynchophorus ferrugineus* (Olivier)(Coleoptera: Curculionidae). *Front Physiol.* 1187.

694 Jones PL, Pask GM, Rinker DC, Zwiebel LJ. 2011. Functional agonism of insect odorant
695 receptor ion channels. *Proc Natl Acad Sci USA*. 108:8821-8825.

696 Kandasamy D, Gershenzon J, Andersson MN, Hammerbacher A. 2019. Volatile organic
697 compounds influence the interaction of the Eurasian spruce bark beetle (*Ips*
698 *typographus*) with its fungal symbionts. *ISME J*. 13:1788–1800.

699 Kandasamy D, Zaman R, Nakamura Y, Zhao T, Hartmann H, Andersson MN, Hammerbacher
700 A, Gershenzon J. 2021. Bark beetles locate fungal symbionts by detecting volatile
701 fungal metabolites of host tree resin monoterpenes. *bioRxiv*.
702 <https://doi.org/10.1101/2021.1107.1103.450988>, last accessed 22 February 2022.

703 Katoh K, Misawa K, Kuma Ki, Miyata T. 2002. MAFFT: a novel method for rapid multiple
704 sequence alignment based on fast Fourier transform. *Nucleic Acids Res*. 30:3059-3066.

705 Katoh K, Standley DM. 2013. MAFFT multiple sequence alignment software version 7:
706 improvements in performance and usability. *Mol Biol Evol*. 30:772-780.

707 Kännaste A, Zhao T, Lindström A, Stattin E, Långström B, Borg-Karlson A-K. 2013. Odors
708 of Norway spruce (*Picea abies* L.) seedlings: differences due to age and chemotype.
709 *Trees*. 27:149-159.

710 Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB. 2004. Or83b
711 encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction.
712 *Neuron*. 43:703-714.

713 Larsson MC, Leal WS, Hansson BS. 2001. Olfactory receptor neurons detecting plant odours
714 and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J Insect*
715 *Physiol*. 47:1065-1076.

716 Leufvén A, Bergström G, Falsen E. 1984. Interconversion of verbenols and verbenone by
717 identified yeasts isolated from the spruce bark beetle *Ips typographus*. *J Chem Ecol*.
718 10:1349-1361.

719 Li W, Godzik A. 2006. Cd-hit: a fast program for clustering and comparing large sets of
720 protein or nucleotide sequences. *Bioinformatics*. 22:1658-1659.

721 Manni M, Berkeley MR, Seppey M, Zdobnov EM. 2021. BUSCO: Assessing genomic data
722 quality and beyond. *Curr Protoc*. 1:e323.

723 McKenna DD, Scully ED, Pauchet Y, Hoover K, Kirsch R, Geib SM, Mitchell RF,
724 Waterhouse RM, Ahn S-J, Arsala D. 2016. Genome of the Asian longhorned beetle
725 (*Anoplophora glabripennis*), a globally significant invasive species, reveals key
726 functional and evolutionary innovations at the beetle–plant interface. *Genome Biol*.
727 17:227.

- Mitchell RF, Andersson MN. 2020. Olfactory genomics of the Coleoptera. In: Blomquist GJ, Vogt RG, editors. *Insect Pheromone Biochemistry and Molecular Biology* 2nd Edition. Oxford: Academic Press. p. 547-590.
- Mitchell RF, Hughes DT, Luetje CW, Millar JG, Soriano-Agatón F, Hanks LM, Robertson HM. 2012. Sequencing and characterizing odorant receptors of the cerambycid beetle *Megacyllene caryae*. *Insect Biochem Mol Biol.* 42:499-505.
- Mitchell RF, Schneider TM, Schwartz AM, Andersson MN, McKenna DD. 2020. The diversity and evolution of odorant receptors in beetles (Coleoptera). *Insect Mol Biol.* 29:77-91.
- Nei M, Niimura Y, Nozawa M. 2008. The evolution of animal chemosensory receptor gene repertoires: roles of chance and necessity. *Nat Rev Genet.* 9:951-963.
- Pettersson M, Kännaste A, Lindström A, Hellqvist C, Stattin E, Långström B, Borg-Karlson A-K. 2008. Mini-seedlings of *Picea abies* are less attacked by *Hylobius abietis* than conventional ones: is plant chemistry the explanation? *Scand J For Res.* 23:299-306.
- Price MN, Dehal PS, Arkin AP. 2010. FastTree 2—approximately maximum-likelihood trees for large alignments. *PloS one.* 5:e9490.
- Pureswaran DS, Gries R, Borden JH, Pierce Jr HD. 2000. Dynamics of pheromone production and communication in the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and the pine engraver, *Ips pini* (Say)(Coleoptera: Scolytidae). *Chemoecology.* 10:153-168.
- Raffa KF, Andersson MN, Schlyter F. 2016. Chapter one-Host selection by bark beetles: Playing the odds in a high-stakes game. In: Tittiger C, Blomquist GJ, editors. *Adv Insect Physiol.* Oxford: Academic press. Vol 50. p. 1-74.
- Rambaut A. 2014. FigTree v1.4.0, a graphical viewer of phylogenetic trees. <http://tree.bio.ed.ac.uk/software/figtree/>
- Roberts RE, Yuvaraj JK, Andersson MN. 2021. Codon optimization of insect odorant receptor genes may increase their stable expression for functional characterization in HEK293 cells. *Front Cell Neurosci.* 15:744401.
- Ruel DM, Yakir E, Bohbot JD. 2019. Supersensitive odorant receptor underscores pleiotropic roles of indoles in mosquito ecology. *Front Cell Neurosci.* 12:533.
- Sato K, Pellegrino M, Nakagawa T, Vosshall LB, Touhara K. 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature.* 452:1002-1006.
- Schiebe C, Blaženec M, Jakuš R, Unelius CR, Schlyter F. 2011. Semiochemical diversity diverts bark beetle attacks from Norway spruce edges. *J Appl Entomol.* 135:726-737.

- Schlyter F, Birgersson G, Byers JA, Löfqvist J, Bergström G. 1987. Field response of spruce bark beetle, *Ips typographus*, to aggregation pheromone candidates. *J Chem Ecol.* 13:701-716.
- Schoville SD, Chen YH, Andersson MN, Benoit JB, Bhandari A, Bowsher JH, Brevik K, Cappelle K, Chen M-JM, Childers A, et al. 2018. A model species for agricultural pest genomics: the genome of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae). *Sci Rep.* 8:1931.
- Shin S, Clarke DJ, Lemmon AR, Moriarty Lemmon E, Aitken AL, Haddad S, Farrell BD, Marvaldi AE, Oberprieler RG, McKenna DD. 2018. Phylogenomic data yield new and robust insights into the phylogeny and evolution of weevils. *Mol Biol Evol.* 35:823-836.
- Stensmyr MC, Dweck HKM, Farhan A, Ibba I, Strutz A, Mukunda L, Linz J, Grabe V, Steck K, Lavista-Llanos S, et al. 2012. A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. *Cell.* 151:1345-1357.
- Sullivan BT. 2005. Electrophysiological and behavioral responses of *Dendroctonus frontalis* (Coleoptera: Curculionidae) to volatiles isolated from conspecifics. *J Econ Entomol.* 98:2067-2078.
- Unelius RC, Schiebe C, Bohman B, Andersson MN, Schlyter F. 2014. Non-host volatile blend optimization for forest protection against the European spruce bark beetle, *Ips typographus*. *PLoS ONE.* 9:e85381.
- Vosshall L, Amrein H, Morozov P, Rzhetsky A, Axel R. 1999. A spatial map of olfactory receptor expression in the *Drosophila* antenna. *Cell.* 96:725-736.
- Wang X, Wang S, Yi J, Li Y, Liu J, Wang J, Xi J. 2020. Three host plant volatiles, hexanal, lauric acid, and tetradecane, are detected by an antenna-biased expressed odorant receptor 27 in the dark black chafer *Holotrichia parallela*. *J Agric Food Chem.* 68:7316-7323.
- Wibe A, Borg-Karlson A-K, Norin T, Mustaparta H. 1997. Identification of plant volatiles activating single receptor neurons in the pine weevil (*Hylobius abietis*). *J Comp Physiol A.* 180:585-595.
- Wicher D, Schäfer R, Bauernfeind R, Stensmyr MC, Heller R, Heinemann SH, Hansson BS. 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature.* 452:1007-1011.
- Wilson IM, Borden JH, Gries R, Gries G. 1996. Green leaf volatiles as antiaggregants for the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Scolytidae). *J Chem Ecol.* 22:1861-1875.

- Xie J, Liu T, Yi C, Liu X, Tang H, Sun Y, Shi W, Khashaveh A, Zhang Y. 2022. Antenna-biased odorant receptor HvarOR25 in *Hippodamia variegata* tuned to allelochemicals from hosts and habitat involved in perceiving preys. *J Agric Food Chem.* 70:1090-1100.
- Yuvaraj JK, Jordan MD, Zhang D-D, Andersson MN, Löfstedt C, Newcomb RD, Corcoran JA. 2022. Sex pheromone receptors of the light brown apple moth, *Epiphyas postvittana*, support a second major pheromone receptor clade within the Lepidoptera. *Insect Biochem Mol Biol.* 141:103708.
- Yuvaraj JK, Roberts RE, Sonntag Y, Hou X, Grosse-Wilde E, Machara A, Zhang D-D, Hansson BS, Johanson U, Löfstedt C, et al. 2021. Putative ligand binding sites of two functionally characterized bark beetle odorant receptors. *BMC Biol.* 19:16.
- Zhang Q-H, Schlyter F. 2004. Olfactory recognition and behavioural avoidance of angiosperm nonhost volatiles by conifer-inhabiting bark beetles. *Agric For Entomol.* 6:1-19.
- Zhang Q-H, Schlyter F. 2003. Redundancy, synergism, and active inhibitory range of non-host volatiles in reducing pheromone attraction in European spruce bark beetle *Ips typographus*. *Oikos.* 101:299-310.

Figure Legends

Figure 1. Maximum likelihood phylogeny of odorant receptors (ORs) from beetles of Curculionidae and Cerambycidae. Included are OR amino acid sequences from the curculionids *Hylobius abietis* ('Habi'; red), *Ips typographus* ('Ityp'; blue), *Dendroctonus ponderosae* ('Dpon'; orange), and the cerambycid *Megacyllene caryae* ('Mcar'; black). The tree is based on a MAFFT alignment, constructed using FastTree, and rooted with the conserved Orco lineage. The major coleopteran OR groups are indicated by the black arcs (Mitchell, et al. 2020). The twelve groups of simple (1:1:1) OR orthologues across the three curculionids are highlighted in yellow; two clades housing putative simple orthologues shared by the cerambycid *M. caryae* and two of the curculionids are highlighted in purple. Receptors in OR group 2B that were functionally characterized in the present study and the previously characterized McarOR5 (Mitchell, et al. 2012) are labeled by the compounds that activate them (GLV-OHs = green leaf volatile alcohols; 2-PE = 2-phenylethanol). Local node support values were calculated using the Shimodaira-Hasegawa (SH) test implemented in FastTree, and are indicated on branch nodes by the shaded circles; support increases with the brightness of the circles. The sources of sequence data and explanation of receptor suffixes are detailed in the Materials and Methods section.

Figure 2. Conserved responses to green leaf volatile (GLV) alcohols in curculionid odorant receptor (OR) orthologues. (A) Response of *Hylobius abietis* OR4 (*Homo sapiens* codon optimized; HabiOR4^{HsCO}) to select compounds in the screening experiments (30 μM stimulus concentration; $n = 3$ biological replicates, $n_{total} = 9$). (B) Dose-dependent response of HabiOR4^{HsCO} to the five active GLVs, indicating similar sensitivities to four of the compounds (see main text for EC₅₀ values; $n = 3-5$ biological replicates, $n_{total} = 9-15$). (C) Screening responses of *Dendroctonus ponderosae* OR9 (*H. sapiens* codon optimized; DponOR9^{HsCO}; $n = 3$ biological replicates, $n_{total} = 9$), and (D) dose-dependent responses of DponOR9^{HsCO} to the two most active ligands ($n = 5$ biological replicates, $n_{total} = 15$). (E) Screening response of *Ips typographus* OR5 (ItypOR5; $n = 3-5$ biological replicates, $n_{total} = 9-15$), and (F) dose-dependent response of ItypOR5 to the most active ligand ($n = 6$ biological replicates, $n_{total} = 18$). Ligand-induced activation was recorded from cells induced (+) to express the exogenous Orco and OR genes and from non-induced (−) control cells. VUAA1 was tested at 50 μM as a control for functional Orco expression. Asterisks indicate significantly stronger responses in induced compared to non-induced cells (at $p < 0.001$; see

main text for details on statistics). Ligands eliciting < 3% increased fluorescence in screening assays were excluded from dose-response trials. Error bars show SEM. All data from induced and non-induced cells to all 62 test compounds are reported in Supplementary Data 1.

Figure 3. Conserved responses to 2-phenylethanol in curculionid odorant receptor (OR) orthologues. (A) Response of *Hylobius abietis* OR3 (HabiOR3) to select compounds in the screening experiments (30 μ M stimulus concentration; $n = 3$ -4 biological replicates, $n_{total} = 9$ -12). (B) Dose-dependent responses of HabiOR3 ($n = 3$ biological replicates, $n_{total} = 9$; see main text for EC₅₀ value). (C) Screening responses of *Dendroctonus ponderosae* OR8 (*H. sapiens* codon optimized; DponOR8^{HsCO}; $n = 3$ biological replicates, $n_{total} = 9$), and (D) dose-dependent responses of DponOR8^{HsCO} ($n = 9$ biological replicates, $n_{total} = 27$; see main text for EC₅₀ value). (E) Screening responses of *Ips typographus* OR6 (ItypOR6; $n = 3$ biological replicates, $n_{total} = 9$), and (F) dose-dependent responses of ItypOR6 ($n = 3$ biological replicates, $n_{total} = 9$; see main text for EC₅₀ value). Ligand-induced activation was recorded from cells induced (+) to express the exogenous beetle Orco and OR genes and from non-induced (–) control cells. VUAA1 was tested at 50 μ M as a control for functional Orco expression. Asterisks indicate significantly stronger responses in induced compared to non-induced cells (at $p < 0.001$; see main text for details on statistics). Error bars show SEM. All data from induced and non-induced cells to all 62 test compounds are reported in Supplementary Data 1.

871 **Figure 2.**

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