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Evolution and function of chemoreceptors

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Title and subtitle: Olfactory genomics of bark- and ambrosia beetles: Evolution and function of chemoreceptors

Abstract:

Insect behaviours, such as host and mate selection, are often mediated by chemical cues. The chemical cues are detected by large and rapidly evolving families of chemoreceptors which include odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs). In this thesis, I used a model system of beetle (Coleoptera) species to study the functional evolution of insect ORs, their ligand binding mechanism, and the diversification of the three chemoreceptor gene families in relation to differences in species ecology. Specifically, I targeted beetles in the Curculionidae family that are pests on conifer trees, including Eurasian spruce bark beetle *Ips typographus* and mountain pine beetle *Dendroctonus ponderosae*, striped ambrosia beetle *Trypodendron lineatum* and the large pine weevil *Hylobius abietis*.

In **paper I**, orthologous ORs from *I. typographus*, *D. ponderosae, H. abietis* were functionally characterised. This study revealed conserved responses across all species, with one set of orthologues responding to 2-phenylethanol while the other set of orthologues responded to green leaf volatiles (GLVs) which serves as a cue to avoid non-host angiosperms. **Paper II** focuses on the functional characterisation of two paralogous *I. typographus* ORs (ItypORs) which responded to *I. typographus* pheromone compounds with different specificity. The phylogenetic position of these ORs suggested multiple origins of pheromone receptors in bark beetles. This study also revealed conserved amino acid residues in the binding pockets of the two ORs, and site-directed mutagenesis confirmed direct involvement of two amino acids in the ligand binding. In **paper III,** I recorded neuronal responses of the ambrosia beetle *T. lineatum*, which has a different ecology compared to bark beetles, specifically in terms of fungal symbiosis. Using single sensillum recordings, thirteen olfactory sensory neuron (OSN) classes were characterised. I found several OSNs responding specifically to volatiles produced by the nutritional fungal mutualist *Phialophoropsis ferruginea*, indicating the importance fungal odors in this association. In **paper IV**, I annotated the chemoreceptor gene families in the *T. lineatum* genome for evolutionary comparisons with such receptors in bark beetles. This study revealed a comparatively small chemoreceptor repertoire, which could relate to the specialized ecology of *T. lineatum*. Also, I found that *T. lineatum* has lost several sugar receptors, and has comparatively few bitter taste GRs.

In conclusion, the overall work included in this thesis revealed that: 1) orthologous and paralogous receptors may respond to the same or similar odors in various curculionid species; 2) pheromone receptors in bark beetles have multiple evolutionary origins; 3) several OSNs in the ambrosia beetle are tuned to odors from the obligate fungal mutualist, and; 4) the ambrosia beetle's reduced chemoreceptor gene repertoire correlates with its specialised diet.

Key words: Curculionidae; functional characterisation; odorant receptors (ORs); gustatory receptors; ionotropic receptors; OR-ligand interaction; orthologues; paralogues; olfacotry sensory neurons (OSNs).

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आत्मैव ह्यात्मनो बन्धुरात्मैव रिपुरात्मनः।।

The mind alone is one’s friend as well as one’s enemy...!

Table of Contents

Abstract

Insect behaviours, such as host and mate selection, are often mediated by chemical cues. The chemical cues are detected by large and rapidly evolving families of chemoreceptors which include odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs). In this thesis, I used a model system of beetle (Coleoptera) species to study the functional evolution of insect ORs, their ligand binding mechanism, and the diversification of the three chemoreceptor gene families in relation to differences in species ecology. Specifically, I targeted beetles in the Curculionidae family that are pests on conifer trees, including Eurasian spruce bark beetle *Ips typographus* and mountain pine beetle *Dendroctonus ponderosae*, striped ambrosia beetle *Trypodendron lineatum* and the large pine weevil *Hylobius abietis*.

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Popular science summary

Coleoptera (beetles) is a very diverse order of insects, with approximately 400,000 species. Several of them are severe pests on conifer forests; many belong to the Curculionidae family (true weevils). Like many other insects, these beetles use olfaction to find hosts, mates, food, and to avoid non-hosts and natural enemies. Generally, hosts are located using host volatiles and aggregation pheromones, while odors like green leaf volatiles (GLVs) and anti-aggregation pheromones are used to avoid non-hosts and overcrowded trees, respectively. These odors are detected by divergent families of chemoreceptors, which are expressed in sensory neurons present inside sensory hairs on their chemosensory organs. In part, these chemoreceptors are thought to rapidly evolve in order to fulfil the ecological requirements of the insect.

Understanding the function and evolution of chemoreceptors is essential for comprehending their significance in the chemical ecology of insects and the evolution of olfactory specialization, which can be further used in integrated pest management. Partly due to the high demand for components directly benefiting human interests, chemoreceptors have been extensively studied mainly in moths, flies and mosquitos. This thesis includes the study of olfactory sensory neurons (OSNs) and chemoreceptors, especially the odorant receptors (ORs), in different species of the Curculionidae family, which are known to cause severe economic loss to forestry, including the Eurasian spruce bark beetle *Ips typographus*, the mountain pine beetle *Dendroctonus ponderosae*, the pine weevil *Hylobius abietis* and the striped ambrosia beetle *Trypodendron lineatum*. All these beetles have well characterised chemical ecologies, which facilitates comparative studies of their OSNs and chemoreceptors.

In the first two chapters of this thesis, the aim was to investigate whether OR orthologues (genes that are evolutionarily conserved in different species) and paralogues (duplicated genes within a species) respond to the same or different compounds. In one of the studies (**paper I**), two sets of orthologous ORs from *I. typographus*, *D. ponderosae*, *H. abietis* were targeted. The OR orthologues showed highly similar responses across all species, with one set responding only to 2-phenylethanol and the other set to several green leaf volatiles from non-host trees that all these beetles avoid. In **paper II** I identified a specific *I. typographus* aggregation pheromone receptor and found that its paralogue responds to the same compounds but with much lower specificity. This study also showed that pheromone receptors in bark beetles have a larger number of evolutionary origins

than the pheromone receptors in moths, which are positioned inside a few defined pheromone receptor clades. Further analysis of OR structure and the interaction with pheromone compounds revealed two amino acid residues which are likely to be directly involved in pheromone binding.

Thereafter, the aim was to compare the olfactory responses of the spruce bark beetle with those of an ambrosia beetle, which differs in ecology in terms of the relationship with fungal symbionts, pheromone communication and host tissue colonization. First, electrophysiological recordings were performed on the OSNs of *T. lineatum* to investigate which odors this ambrosia beetle is able to detect (**paper III**). Several OSN classes were identified which were responding to host, non-host, fungal symbiont and pheromone compounds. The comparison of OSN classes of *T. lineatum* with *I. typographus* reflected their shared ancestry, but the results also suggested differences in OSNs in accordance with their ecology. For example, *T. lineatum* has several OSNs that detect compounds produced by its fungal partner, suggesting that odors are important for the beetle-fungus interaction. At last, to investigate the diversification of chemoreceptors in ambrosia beetles, identification of chemoreceptor genes from the *T. lineatum* genome was performed and the phylogenetic distributions of the receptors were analysed (**paper IV**). This revealed reduced repertoires of chemoreceptor genes in *T. lineatum* compared to bark beetles, particularly reduced numbers of taste receptors for sugars and bitter compounds which may correlate with the restricted feeding ecology of *T. lineatum*, feeding only on one known fungal mutualist.

The results from this thesis contribute to increase our knowledge of the fundamental functions of insect chemoreceptors and suggest that ecological specialisations are reflected in the evolution and function of the chemoreceptor gene families.

Populärvetenskaplig sammanfattning

Coleoptera (skalbaggar) är en mycket variabel insektsordning, bestående av cirka 400 000 arter. Flera av dem är svåra skadegörare på barrskog, och av dessa tillhör många familjen Curculionidae (äkta vivlar). Liksom många andra insekter använder vivlarna luktsinnet för att hitta värdväxter, partners och föda, samt för att undvika icke-värdväxter och naturliga fiender. I allmänhet lokaliseras värdar med hjälp av flyktiga dofter från värdväxterna och aggregeringsferomoner, medan dofter från gröna blad (s.k. GLVs, green leaf volatiles) och anti-aggregationsferomoner används för att undvika icke-värdar respektive upptagna träd. Dessa lukter upptäcks med hjälp av stora familjer av kemoreceptorer, som uttrycks i sensoriska neuroner som finns inuti sensoriska borst på deras sinnesorgan. Dessa kemoreceptorer anses utvecklas snabbt, sannolikt för att uppfylla insektens olika ekologiska krav.

Att förstå funktion och utveckling av kemoreceptorer är avgörande för att förstå deras betydelse i insekternas kemiska ekologi och utvecklingen av luktspecialisering. Denna information kan även användas inom integrerad skadedjursbekämpning. Delvis på grund av den höga efterfrågan på faktorer som direkt gynnar människans intressen, har kemoreceptorer främst studerats hos nattfjärilar, flugor och myggor. Denna avhandling inkluderar studiet av luktsensoriska neuroner (OSN, olfactory sensory neurons) och kemoreceptorer, särskilt luktreceptorer (OR, odorant receptors) hos olika arter i familjen Curculionidae (äkta vivlar) som är kända för att orsaka allvarliga ekonomiska förluster för skogsbruket, som granbarkborre *Ips typographus*, contortabastborre *Dendroctonus ponderosae*, vanlig snytbagge *Hylobius abietis* och randig vedborre *Trypodendron lineatum,* som är en s.k. ambrosiabagge, som lever på svamp. Alla dessa vivlar har välkarakteriserade kemiska ekologier, vilket underlättar jämförande studier av deras luktsensoriska neuroner och kemoreceptorer.

I de två första kapitlen av denna avhandling var syftet att undersöka om luktreceptorortologer (gener som är evolutionärt bevarade i olika arter) och paraloger (dubblerade gener inom en art) svarar på samma eller olika kemiska föreningar. I den första av studierna (**artikel I**) var två uppsättningar ortologa luktreceptorer från *I. typographus*, *D. ponderosae* och *H. abietis* måltavlor. Dessa ortologer uppvisade likartade svar hos alla arterna, med en uppsättning som bara svarade på 2-fenyletanol och den andra på GLVer från lövträd, som alla dessa barrträdsattackerande arter undviker. I **artikel II** identifierade jag en specifik aggregationsferomonreceptor hos granbarkborren och fann att dess paralog svarar på samma föreningar men med mycket lägre specificitet. Denna studie visade också att feromonreceptorer hos barkborrar verkar ha fler evolutionära ursprung jämfört med feromonreceptorerna hos nattfjärilar. Ytterligare analys av luktreceptorernas struktur och interaktionen med feromonföreningar avslöjade två aminosyror som sannolikt är direkt inblandade i feromonbindningen.

Därefter var syftet att jämföra granbarkborrens doftreaktioner med dem hos en ambrosiabagge, nämligen randig vedborre, *T. lineatum*, som skiljer sig åt i ekologi när det gäller det symbiotiska förhållandet med svampar, feromonkommunikation och vilken vävnad de koloniserar i träden. Först gjordes elektrofysiologiska studier på luktsensoriska neuroner hos *T. lineatum* för att undersöka vilka dofter denna ambrosiabagge kan upptäcka (**artikel III**). Flera klasser av luktsensoriska neuroner identifierades, vilka svarade på värd-, icke-värd- och feromonföreningar. Jämförelsen av olika klasser av sensoriska neuroner hos *T. lineatum* med *I. typographus* återspeglade deras delade härkomst men antydde också skillnader hos dessa arter i enlighet med deras ekologi. *T. lineatum* har till exempel flera luktsensoriska neuroner som upptäcker föreningar som produceras av dess svamppartner, vilket tyder på att dofter är viktiga för interaktionen mellan ambrosiabagge och svamp. Slutligen, för att undersöka diversifieringen av kemoreceptorer hos ambrosiabaggar, utfördes identifiering av kemoreceptorgener från *T. lineatum*-genomet och de fylogenetiska släktskapen hos receptorerna analyserades (**artikel IV**). Detta avslöjade ett lägre antal kemoreceptorgener i *T. lineatum* jämfört med barkborrar, särskilt färre smakreceptorer för sockerarter och bitterföreningar, vilket kan relateras till den randiga vedborrens begränsade födoekologi, den livnär sig enbart på en känd svampmutualist.

Resultaten från denna avhandling bidrar till att öka vår kunskap om de grundläggande funktionerna hos insekters kemoreceptorer och visar att ekologiska specialiseringar återspeglas i evolutionen och funktionen hos kemoreceptorfamiljerna.

लोकप्रिय विज्ञान सारांश

वर्मपंखी (मूंग) कीटों का एक अत्यंत विविधतापूर्ण गण है, जिसकी लगभग 4,00,000 प्रजातियां हैं। उनमे से कई मात्र सूंडी के रूप में शंकुधारी जंगलों में मिलते हैं बहुत से कीट करकुलनॉइडी (घुन) परिवार के सदस्य हैं। अन्य कीटों की भांति ये मूंग की घ्राण महक का प्रयोग साथी, समुदाय, भोजन तथा गैर प्राकृतिकव स्वाभाविक शत्रु से बचने के लिए करते हैं। सामान्यतः उपयुक्त पेड़ों को उनके वाष्पशील और एकत्रीकरण फेरोमोन का उपयोग करके स्थित किया जाता है। जबकि हरे पत्तों की गंधेदारी (GLVs) और एंटी-एग्रीगेशन फेरोमों का उपयोग गैर-मेज़बान और अधिकभिड़ित वाले पेड़ों से बचने के लिए किया जाता है। इन महक को रसायनग्राही विभिन्न परिवारों द्वारा पहचाना जाता है जो तंत्रिका न्यूरांस पर उपस्थित होते हैं। उनके रसायन संवेदी अंकों के अंदर संवेदी रोमों के अन्दर मिलते हैं। साथ ही साथ से रसायनग्राही तेजी से साबित होने लगते हैं ताकि कीटों की पारिस्थितिक आवश्यकता ओं को पूरा किया जा सके।

कीड़ों की रासायनिक पारिस्थितिकी और घ्राण विशेषज्ञता के विकास में उनके महत्व को समझने के लिए केमोरिसेप्टर्स के कार्य और विकास को समझना आवश्यक है, जिसका उपयोग आगे एकीकृत कीट प्रबंधन में किया जा सकता है। आंशिक रूप से मानव हितों को सीधे लाभ पहुंचाने वाले घटकों की उच्च मांग के कारण, केमोरिसेप्टर्स का बड़े पैमाने पर अध्ययन किया गया है, मुख्य रूप से पतंगे, मक्खियों और मच्छरों में। इस शोध पत्र में ऑलफैक्ट्री सेंसरी न्यूरॉन (OSN) तथा रसायनग्राही का अध्ययन शामिल हैं विशेषकर गंधक रिसेप्टर्स (ORs) करकुलनॉइडी परिवार की विभिन्न प्रजातियों जो कि वन्य आर्थिक क्षति के लिए मुख्यतः जानी जाती हैं। इसमें यूरेसिया का स्प्रूस छाल भृंग *Ips typographus*, पहाड़ी देवदार भृंग *Dendroctonus ponderosae*, देवदार का घुन *Hylobius abietis* तथा धारीयुक्त एम्ब्रोसिया भृंग *Trypodendron lineatum* । सभी भृंगों में अच्छी तरह से स्थापित रासायनिक पारिस्थितिकी है, जो उनके OSN और के रसायनग्राही के तुलनात्मक अध्ययन की सुविधा प्रदान करती है।

इस थीसिस के पहले दो अध्यायों में, उद्देश्य यह जांच करना था कि क्या ओआर ऑर्थोलॉगस (वह जीन्स जो विभिन्न प्रजातियों में विकास संरक्षित होती हैं) और पैरालॉगस (एक ही प्रजाति के भीतर विकासात्मक रूप से संरक्षित जीन) एक ही या अलग-अलग यौगिकों पर प्रतिक्रिया करते हैं। पहले अध्ययन (**पेपर I**) में, *I. typographus*, *D. ponderosae*, *H. abietis*

से ऑर्थोलॉगस OR के दो सेटों को लक्षित किया गया था। ओआर ऑर्थोलॉग्स ने सभी प्रजातियों में अत्यधिक समान प्रतिक्रियाएं दिखाईं, एक सैट के साथ-साथ 2-phenylethanol प्रतिक्रिया दे रहा था और दूसरा सैट कई हरित पर्ण परिवर्तन जो कि गैर समुदाय वृक्ष थे जिनसे ये सभी बीटल बचते हैं। दूसरे अध्ययन (**पेपर II**) में मैंने एक विशेष *I. typographus* एकत्रीकरण फेरॉमान ग्राही को पहचाना और पाया कि इसका पैरालॉग समान रासायन के प्रति संवेदी है किन्तु निम्न विशिष्टता के साथ। यह अध्ययन यह भी दर्शाता है कि छाल भृंग में फैरामान ग्राही ने अन्य सूंडी फैरामॉन ग्राही से हटकर कई उत्पत्तियां हुई हैं। OR संरचना का आगे का विश्लेषण तथा फैरामॉन कम्पाउंड के साथ सम्बन्ध दो अमीनों अम्ल अवक्षेप प्रकट करता है जो प्रत्यक्ष रूप से फैरामान बंधन सम्मिलित लगते हैं।

तत्पश्चात् घ्राण संवेदों का स्प्रेस छाल भृंग का एम्ब्रोसा भृंग के साथ तुलनात्मक लक्ष्य था जो पारिस्थितिकी में भिन्न था कवक के साथ सहजीविता दर्शाने में, फैरामॉस संचार में तथा समुदायिक उत्तकों के एकत्रीकरण में। सबसे पहले, *T. lineatum* के OSN पर इलेक्ट्रोफिजियोलॉजिकल रिकॉर्डिंग की गई ताकि यह जांच की जा सके कि यह एम्ब्रोसिया बीटल कौन सी गंध का पता लगाने में सक्षम है (**पेपर III**)। कई OSN वर्गों को पहचाना गया जो समुदाय गैर समुदाय तथा फैरामॉन कम्पाउंड के प्रति संवेदी थे। टी, लिविएटम तथा आई. टाइपोग्राफस के OSN वर्गों की तुलना उनके पूर्वजों से प्राप्ति को प्रदर्शित करती है। किन्तु OSN में अंतर उनका पारिस्थितिकी से अनुकूलन दर्शाता है। उदाहरण के तौर पर टी. लिनिएटम में कई OSN हैं जो उन कम्पांउड को पहचान लेते हैं जो इसके कवक साथी ने उत्पन्न किये थे। ये सुझाव देते हैं कि भृंग व कवक सम्बन्ध के लिए घ्राण महत्वपूर्ण है। अंत में एम्ब्रोसा भृंग में रसायन ग्राहियों की विविधता की जांच के लिए *T. lineatum* के रसायनग्राही जीव का जीनोम प्राप्त किया गया तथा ग्राहियों का फाइटोजेनेटिक वितरण का विश्लेषण किया गया। (**पेपर IV**) यह छाल मूंग की तुलना ही लिबिण्टम में कम होते रसायनग्राही जीव को प्रकट करता है विशेष रूप से कम होते स्वाद ग्राही जीव को प्रकट करता है विशेष रूप से कम होते स्वाद ग्राही जो शर्करा तथा कडके कम्पाउंड के लिए जो टी लिनिएटम के प्रतिबंधिति पोषण से सहसम्बन्ध दर्शाता लगता है वह मान एक पारस्परित कवक पर ही पोषित है।

इस शोघपत्र का परिणाम कीटों के रसायनग्राही के मौलिक कार्यों के ज्ञान में वृद्धि प्राप्त करना है और सुझाव देता है कि रसायनग्राही जीन परिवार के विकास एवं पारिस्थितिकीय विशिष्टीकरण में परिलक्षित

List of Papers

Paper I

Roberts R. E., **Biswas T.**, Yuvaraj J. K., Grosse-Wilde E., Powell D., Hansson B. S., Löfstedt C., & Andersson M. N. (2022). Odorant receptor orthologues in conifer-feeding beetles display conserved responses to ecologically relevant odours. *Molecular ecology*, 31(13), 3693-3707.

Paper II

Biswas T., Sims C., Yuvaraj J. K., Roberts R. E., Löfstedt C., & Andersson M. N. Functional characterisation supports multiple evolutionary origins of pheromone receptors in bark beetles. *Manuscript*.

Paper III

Biswas T., Yuvaraj J. K., Hansson B. S., Löfstedt C., Anderbrant O., & Andersson M. N. (2023). Characterization of olfactory sensory neurons in the striped ambrosia beetle *Trypodendron lineatum*. *Frontiers in Physiology*, 14, 1155129.

Paper IV

Biswas T., Vogel H., Beidermann P., Lehenberger M., & Andersson M. N. Reduced chemoreceptor gene repertoires in the ambrosia beetle *Trypodendron lineatum* may reflect its specialized ecology. *Manuscript*.

Author contributions

Paper I

M.N.A. conceived the project. R.E.R. and M.N.A. conceptualized and designed the study. R.E.R., T.B., J.K.Y. and M.N.A. performed molecular work. R.E.R., T.B. and J.K.Y. performed cell culturing and experimental assays. E.G-W., B.S.H. and M.N.A. coordinated the sequencing of H. abietis. E.G-W. and D.P. performed transcriptome assemblies, with D.P. assessing OR gene expression and completeness of final assemblies. M.N.A. annotated the HabiORs, constructed the OR phylogeny, and performed statistical analysis of the HEK cell data. M.N.A., J.K.Y. and C.L. contributed to project supervision. M.N.A. and R.E.R. drafted the manuscript. All authors contributed to the final version of the manuscript and have read and approved it for submission.

Paper II

M.N.A. conceived and conceptualized the project. T.B., C.S., J.K.Y., C.L. and M.N.A. designed the experiments. T.B., J.K.Y. and R.E.R. performed molecular work and cell culturing. T.B. performed experimental assays with contributions from J.K.Y. T.B. and M.N.A. performed statistical analysis. C.S. conducted the homology modelling and ligand docking. T.B. and C.S. prepared the figures. T.B., C.S. and M.N.A. drafted the manuscript together; all other authors contributed to manuscript revisions.

Paper III

M.N.A. conceived and conceptualized the study. T.B., M.N.A., J.K.Y., O.A., and C.L. contributed to experimental design. M.A. and B.H. collected beetles from the field. T.B. performed the single sensillum recordings and data analysis under the supervision of J.K.Y. and M.N.A. T.B. prepared all the figures. T.B. and M.N.A. drafted the manuscript, with J.K.Y., C.L., O.A., and B.H. contributing to manuscript revision. All authors read and approved the final version of the manuscript.

Paper IV

M.N.A. conceived and conceptualized the study. P. B., M.L., and M.N.A. collected beetle specimens for sequencing. H.V. sequenced and assembled the T. lineatum genome. T.B. and M.N.A performed gene annotations and phylogenetic analyses. T.B. and M.N.A prepared the figures and manuscript drafting with contributions from H.V. on the sequencing and assembly methodology. All authors contributed to manuscript revisions.

Abbreviations

AP	Action potential
GLVs	Green leaf volatiles
GR	Gustatory receptor
HEK cells	Human embryonic kidney cells
iGluR	Ionotropic glutamate receptor
IR	Ionotropic receptor
OBP	Odorant binding protein
ODE	Odorant degrading enzyme
OR	Odorant receptor
Orco	Odorant receptor co-receptor
OSN	Olfactory sensory neuron
PCR	Polymerase chain reaction
SEM	Scanning electron microscopy
SNMP	Sensory neuron membrane protein
SSR	Single sensillum recording
TREx	Tetracycline-inducible repressor
Ityp	*Ips typographus*
Dpon	*Dendroctonus ponderosae*
Habi	*Hylobius abietis*
Tlin	*Trypodendron lineatum*

Aims and objectives

The antenna of an insect is an organ that is specifically designed for the purpose to sense stimuli from the surroundings, including odors. These antennae are covered with several types of sensilla innervated by sensory neurons: many of these express odorant receptors (ORs) or other chemoreceptors. The chemoreceptor gene families evolve rapidly, however, little is known about the evolution of functionalities in chemoreceptors within Coleoptera (beetles).

In this thesis work, the overall objective is to investigate how the evolution and function of chemoreceptors, primarily ORs, and olfactory sensory neurons (OSNs) relate to the ecological specializations in different beetle species. To address this question, I used a study system that included closely related bark and ambrosia beetles, and weevils, namely Eurasian spruce bark beetle *Ips typographus*, mountain pine beetle *Dendroctonus ponderosae*, striped ambrosia beetle *Trypodendron lineatum,* and large pine weevil *Hylobius abietis*. Using different techniques, including heterologous expression systems, electrophysiology and gene annotation this thesis work had the following main aims:

1) Investigate the functions of evolutionary related ORs, both within and between species (**paper I and II**)
2) Identify pheromone receptors and investigate their evolutionary origins in beetles (**paper II**)
3) Understand the ligand binding mechanisms in beetle pheromone receptors (**paper II**)
4) Characterise olfactory sensory neuron (OSN) classes in the ambrosia beetle *T. lineatum* and compare with the bark beetle *I. typographus* OSNs (**paper III**)
5) Identify the chemoreceptor genes in *T. lineatum* and their phylogenetic relationships with receptors in closely and more distantly related beetles (**paper IV**)

Ips typographus

Trypodendron lineatum

Dendroctonus Ponderosae

Hylobius Abietis

Introduction

All organisms including bacteria, fungi, plants, and animals have the ability to detect and respond to chemical signals. In the case of insects, chemical signals are used to engage with their surroundings for fundamental purposes, including locating food, hosts, mates and evading predators. Chemical communication using odors (i.e., olfaction) is an efficient means to exchange information over long distances. The scientific field of insect chemical ecology was sparked by the identification of the *Bombyx mori* sex pheromone (Karlson and Lüscher 1959; Butenandt 1959). In beetles, the chemical communication using pheromones was first investigated in *Ips paraconfusus*. It was found that while boring into the bark male *I. paraconfusus* were producing pheromone which attracted the females of same species into the newly bored galleries. (Wood 1962; Wood and Bushing 1963). Over time, many studies have been conducted on chemical communication of a lot of insects to understand the evolution of olfaction, and also to target the chemical communication system for pest control.

The peripheral process of insect olfaction

An adult insect has dedicated organs, namely antennae and maxillary palps, to detect the odor molecules from their surroundings (Sato and Touhara 2009). In most insects, the antenna is covered with a large number of sensory hairs called sensilla. These sensilla can be divided into different types based on their morphology (wall structure and shape). Some of the common olfactory sensilla types are trichoidea, basiconica, and coeloconica (Keil 1999; Schneider 1964). The peripheral process of insect olfaction begins when a chemical cue enters the sensillum lymph through the porous wall of the sensilla. Upon entering, the chemical cue is thought to either bind with odorant binding proteins (OBPs) or chemosensory proteins (CSPs) or pass through pore tubules (Leal 2013; Xu et al. 2009; Larter et al. 2016). Odorant binding

proteins and CSPs may act as a stabiliser for hydrophobic odor cues and carry them to the transmembrane domain chemoreceptor proteins that could be either odorant receptors (ORs), gustatory receptors (GRs) or ionotropic receptors (IRs) which are present in the dendrites of olfactory sensory neurons (OSNs) (Sachse and Krieger 2011; Vosshall et al. 1999; Rihani et al. 2021) (Figure 1). The binding of odor molecule to the receptor protein activates the receptor complex which leads to the transduction of the chemical signal into an electric signal generating action potentials (APs) in the neuron. The signals are then transmitted to the brain and finally potentially translated into behaviour (Nakagawa et al. 2005; Ha and Smith 2022). After signal transduction, odor molecules in the lymph are broken down by odorant degrading enzymes (ODEs) and the OSN resumes its sensitivity (Figure 1) (Leal 2013). In addition, some studies have indicated that sensory neuron membrane proteins (SNMPs) may also play a role in the odor detection process (Cassau and Krieger 2021; Liu et al. 2020).

The main focus of this thesis revolves around the proteins that are directly involved in odor recognition, i.e., ORs, GRs, and IRs.

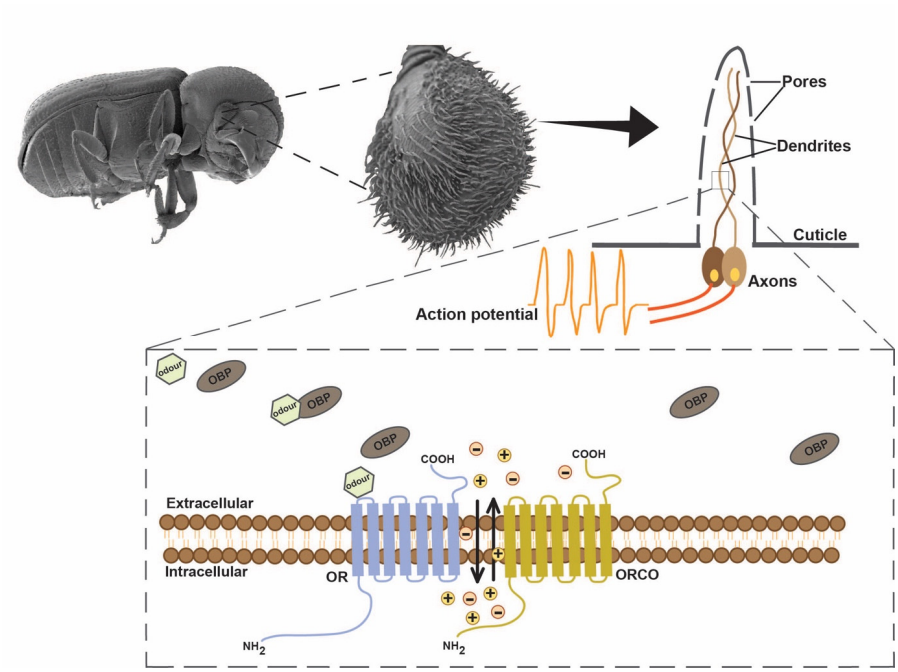

Figure 1. Schematic representation of insect olfactory system.

Chemoreceptors involved in the peripheral process of insect olfaction

Odorant receptors

Odorant receptors (ORs) are seven transmembrane domain proteins. In insects they were discovered for the first time in *Drosophila melanogaster* (Clyne et al. 1999 ; Vosshall et al. 1999). It is interesting that unlike vertebrate ORs, insect ORs do not show homology to G protein coupled receptors (GPCRs), instead insect ORs have reverse membrane topology to the ORs of vertebrates by having an intracellular N-terminus and extracellular C-terminus (Benton et al. 2006; Wistrand et al. 2006). The ORs determine the specificity and sensitivity of an OSN (Hallem and Carlson 2006). The specificity of ORs in binding with ligands define the range of volatile chemicals transmitted by OSN from the antenna to the olfactory centres in the brain. In many cases, at higher doses the OR can be activated by several compounds but the specificity generally increases with decrease in the dosage of the ligand (Andersson et al. 2009 and 2015). Leal (2013) suggested that insect ORs can be categorised into three different types based on their specificity and sensitivity: 1) ORs finely tuned to either one or a few compounds, 2) ORs primarily stimulated by a few molecules, yet capable of sensing other odors at higher concentrations, 3) ORs with a broad spectrum response to different compounds (Hill et al. 2002; Krieger et al. 2003; Nakagawa et al. 2012).

The ORs generally form a heteromeric complex with an odorant receptor coreceptor (Orco) which is highly conserved in insect species (Pitts et al. 2004; Jones et al. 2005; Hansson and Stensmyr 2011; Harini and Sowdhamini 2012). The Orco was first identified in *D. melanogaster* and named as Or83b (Larsson et al. 2004). All insect's co-receptors play similar role of supporting the OR in signal transduction but not directly in odor recognition, hence to unify the nomenclature and emphasize its role as a co-receptor in all the species, it was renamed as "odorant receptor-coreceptor (Orco)" (Vosshall and Hansson 2011). The Orco is distinctive from OR in multiple ways for example, it is the only member of the OR family with a primarily intracellular domain that is substantially conserved across various insect species (Larsson et al. 2004). Second, it is essential for the dendritic membrane targeting of ORs and lacks the ability to bind volatile odorants (Stengl and Funk

2013). While we still do not know much about the subunit structure of OR channels, cryo-electron microscopy (cryo-EM) has revealed an Orco channel composition for the wasp *Apocrypta bakeri*. The Orco creates a non-selective Ca^{2+}-permeable cation channel which is involved in signal transduction (Larsson et al. 2004; Sato et al. 2008; Wicher et al. 2008; Stengl and Funk 2013; Butterwick et al. 2018). The Orco proteins can also create functional ion channels on their own, and they respond to the agonist to VUAA1 (Jones et al. 2011). Several studies have also showed that knockdown of Orco destruct the olfactory recognition process in insect but exactly how the OR-Orco complex function together is poorly understood (Zhao et al. 2011; Li et al. 2016; Liu et al. 2016; Ma et al. 2020; Zhang et al. 2023).

The number of ORs present in an insect differs from species to species, it can vary from as few as 4 to as many as 400, for example 62 ORs are found in common fruit fly *D. melanogaster* (Robertson et al. 2003), 4 ORs in banded demoiselle damselfly *Calopteryx splendens* (Ioannidis et al. 2017), 381 ORs in common Mormon butterfly *Six papilio* (Yin et al. 2022) and ~400 in Indian jumping ant *Harpegnathos saltator* (Zhou et al. 2012). Within coleopterans the number of ORs can also vary significantly. Odorant receptors in beetles range from 39 in skiff beetle *Hydroscapha redfordi* to 341 in red flour beetle *Tribolium castaneum* (Engsontia et al. 2008; Mitchell et al. 2020).

Gustatory receptors

Gustatory receptors are a more ancient and divergent family of seven-transmembrane domain proteins and are able to detect non-volatile as well as volatile chemical cues. The GRs are able to detect some pheromones that are cuticular hydrocarbons (Kohl et al. 2015), carbon dioxide (Kwon et al. 2007; Robertson and Kent 2009; Xu et al 2015; Anderson et al. 2015) and non-volatile compounds like sugars, amino acids, bitter compounds and secondary metabolites of plants upon direct contact (Agnihotri et al. 2016). They also act as an internal nutrition sensor in the brain by monitoring haemolymph fructose levels (Miyamoto et al. 2012) and sense egg production (Hoshino et al. 2023). Hence, GRs are expressed in antennae, legs, wings, ovipositors as well as in mouthparts (Warr, and Carlson 2000; Dahanukar et al. 2005; Vosshall and Stocker 2007; Clyne et al. 2022; Wu et al. 2022). The GRs form a heteromeric functional receptor unit which is made

up of GRX and GRY subunits which exhibit a similar structure and membrane topology to ORs (Jones et al. 2007; Kwon et al. 2007). Insect GRs were first discovered from the *D. melanogaster* genome (Clyne et al. 2000). The GRs are extremely diverse, with typically just 15-25% sequence identity within and between species. The ORs evolved >400 million years ago from GRs and formed another large family of chemoreceptors. ORs and GRs have a similar amino acid residue pattern at the C-terminal domain, implying that they have arisen from the same ancestral chemoreceptor gene family found in arthropods (Robertson et al. 2003; Robertson 2019; Benton and Himmel 2023). A recent cryo-EM study also revealed the similar structural resemblance of OR and GR, but the GR contains an additional beta-hairpin and a longer helix (Frank et al. 2023).

Ionotropic receptors

Ionotropic receptors also form ligand-gated ion channels that are involved in chemical cue recognition but differ in evolutionary history from those of ORs and GRs. Ionotropic receptors are evolved from the ionotropic glutamate receptors (iGluRs) that are present at synapses between neurons. Ionotropic receptors are not only involved in olfaction but also play a role in sensing humidity, salt, temperature and even hearing in *Drosophila* (Benton et al. 2009; Croset et al. 2010; Koh et al. 2014; Rimal and Lee 2018; Sánchez-Alcañiz et al. 2018). The IRs are more ancient than ORs as evidenced by their existence across protostome lineages such as arthropods and nematodes. Generally, IRs are divided into two sub-categories, conserved antennal IRs (which mainly function as olfactory-, humidity- and thermoreceptors) and species-specific divergent IRs (which are involved in taste and food assessment) (Croset et al. 2010). Ionotropic receptors are expressed in a combined manner and seem primarily tuned to short chain organic acids, aldehydes, and amines (Benton et al. 2009; Abuin et al. 2011; Zhang et al. 2022). They contain three transmembrane domains and function together with co-receptors by forming a heteromeric structures. The functional unit of an olfactory IR heteromer generally to be made of four subunits consisting of receptors (IRx) and coreceptor (IRcoY) subunits (Abuin et al. 2019; Ni 2021; Wicher and Miazzi 2021).

Evolutionary model of chemoreceptor gene families

Insect chemoreceptor gene families evolve according to a 'birth and death' model. In this model a new gene is 'born' during a duplication event and losing a functional gene during deletion or pseudogenisation is represented as 'death'. Accordingly, in insects, chromosomes frequently contain tandem arrays of chemoreceptor genes, with large differences in the size of chemoreceptor repertoires between species (Nei et al. 2008; Andersson et al. 2015; Benton 2015; Eyun et al. 2017). Accordingly, the majority of chemoreceptors in a particular species are usually present within species- or taxon-specific phylogenetic receptor lineage radiations and within such radiations new functions may emerge (neo-functionalisation) due to relaxed constraints or positive selection if the duplicated gene is preserved and still expressed (Andersson et al. 2015; Mitchell et al. 2020; Hou et al. 2021). In some cases within species- or taxon-specific phylogenetic lineage radiations, the pre-duplication gene and the duplicated gene retains some of the original function (sub-functionalisation) (Pitts et al. 2017). Following duplication events, the most common outcome for the duplicated copy is, however, pseudogenization due to functional redundancy (non-functionalisation) (Moleirinho et al. 2011). Despite the divergent nature of chemoreceptor, some receptors are conserved as single-copy orthologs primarily in closely related species while this may be very rare or absent in the case of distantly related species (Mitchell et al. 2020; Mitchell and Andersson 2021). Nearly all the chemoreceptors follow the birth-and-death model except for co-receptors, conserved antennal IRs and some conserved GRs like CO_2, fructose receptors. In case of co-receptors, conserved antennal IRs and conserved GRs there is high conservation among various insects which is evident from clear orthologous relationships (Eyun et al. 2017).

Study organisms

The studies that are involved in this thesis mainly focus on bark- and ambrosia beetles in the Curculionidae; Scolytinae, specifically the Eurasian spruce bark beetle *Ips typographus* and the striped ambrosia beetle *Trypodendron lineatum*, respectively. However, the mountain pine beetle *Dendroctonus ponderosae* (Curculionidae; Scolytinae) and the non-scolytine pine weevil *Hylobius abietis*

(Curculionidae; Molytinae) were included for studying orthologous ORs across the curculionids. The species that are included in this thesis have well characterised chemical ecology and are economically important pests on conifers. Even though the studied beetles differ in their ecology, for example: fungal mutualism, pheromone communication system and choice of host tree, they are all phylogenetically related (Byers 2007). Hence, this makes them an ideal study system for comparative studies of the evolution of ORs and other chemoreceptors in relation to their respective ecology.

Several insects that are classified under the order Coleoptera (beetles) are considered as borers, miners or scavengers and predators. The earliest evidence of plant-feeding wood borer beetles is found in a fossil of fungus-decayed wood from mid- to upper Permian period which is roughly ~250 million years ago (Kirejtshuk et al. 2014; Feng et al. 2017; McKenna et al. 2019). The order Coleoptera currently accounts for 25% of all animals making it the most species-rich order, with approximately 400,000 identified species (Hammond 1992; Nielsen and Mound 2000; Foottit and Adler 2009). Bark and ambrosia beetles are snout less weevils and live their adult lives mostly inside decaying wood or other plant tissues (Wood 1982). The majority of these beetles can only invade dead or stressed trees, but few species are also capable of killing healthy trees. Generally, wood boring beetles are forest scavengers and work as decomposers, by promoting forest regeneration, creating snags and rich patchiness in the canopies of forest. But too high populations of these beetles make some of them aggressive species which causes mass attack, harming forests and forest related industries (McCullough et al. 1998; Fleming et al. 2002; Jonášová and Prach 2004; Biedermann et al. 2019).

Bark beetles breed under the bark and use sapwood or phloem as their main source of food. Bark beetles are also associated with fungi which may serve as an additional source of nutrition by adding vitamins and more nitrogen in their food and help in breaking host defences (Beaver et al. 1989; Ayres et al. 2000; Kirisits 2004; Bentz and Six 2006; Linnakoski et al. 2012; Kandasamy et al. 2019; Agbulu et al. 2022; Kandasamy et al. 2023; Zaman et al. 2023). There are more than 6000 species of bark beetles, but only a handful of these species have the ability to fatally harm healthy trees (Kirkendall, Biedermann, and Jordal 2015). The Eurasian spruce bark beetle*, I. typographus* (Coleoptera: Scolytinae), lives primarily under the bark of Norway spruce, *Picea abies* and feed on the phloem tissue. During endemic stage

(low population), *I. typographus* only invades stressed or wind-felled trees while during epidemic stage (high population density) it can also attack and kill healthy spruce trees (Wermelinger 2004; Hlásny et al. 2019). The males of this species find the host and start making mating galleries to attract females. The mated females then make tunnels for oviposition. The parent beetles then depart the tree after a few weeks and occasionally go to another tree to carry on reproducing (Raffa et al. 2015). These beetles carry symbiotic fungi which mainly belong to the genera *Endoconidiophora*, *Ophiostoma*, and *Grosmannia* (Kandasamy et al. 2019; Kirisits 2004; Linnakoski et al. 2012; Six 2003a). The mountain pine beetle, *D. ponderosae* (Coleoptera: Scolytinae), is currently one of the most damaging species of bark beetles which attacks pine (several species) forests in North America (Safranyik et al. 2010; Negrón and Fettig 2014; Audley et al. 2020). Once a host has been found, the female bores through the bark, starts feeding, forming vertical galleries in the outer sapwood or phloem, where they mate and deposit eggs (Reid 1962; Langor 1989). Similar to numerous other bark beetles, *D. ponderosae* shares a close relationship with three symbiotic fungi in particular *Ophiostoma montium*, *G. clavigerum* and *Leptographium longiclavatum* (Six 2003b; Lee et al. 2005; Lee et al. 2006).

Ambrosia beetles are a polyphyletic group of beetles with over 3400 species in the Scolytinae and Platypodinae subfamilies. The fungus-farming lifestyle characteristic of ambrosia beetles has evolved independently at least 11 times the wood-boring weevil lineages (Wood 1982; Farrell et al. 2001; Hulcr and Stelinski 2017). Unlike bark beetles, ambrosia beetles bore into the xylem where they grow galleries of obligate mutualistic fungi which serve as their main source of food required for beetle development and survival (Bakshi 1950; Funk 1970; Kok et al. 1970; Beaver et al. 1989; Mueller et al. 2005). The striped ambrosia beetle *T. lineatum* (Coleoptera: Scolytinae) attacks unhealthy and recently dead conifers and make galleries in which they inoculate the fungus *Phialophoropsis ferruginea* (Lehenberger et al. 2019; Mayers et al. 2020) which is so far the only known fungus that serves as primary source of food for the adult beetles and their larvae (Biedermann et al. 2013). *Phialophoropsis ferruginea* is primarily carried by females (pioneering sex) in a special carrying sac called mycangia and females inoculate the fungus during the excavating phase prior to oviposition (Francke-Grosmann 1956; Batra 1963; Mayers et al. 2015).

The pine weevil, *H. abietis* (Coleoptera: Molytinae) in northern and central Europe is also an extremely damaging pest to coniferous regeneration. This species adults feed on the seedlings' bark, which causes the seedlings to die as weevil damage occurs to the bark close to the seedlings' root collar (Leather et al. 1999; Långström and Day 2004). Typically, *H. abietis* feed on the entire bark ring surrounding the stem which disrupts the flow of nutrients in the growing trees and ultimately causing the plant to die. The females of these beetles often lay eggs in favourable conditions during the summer, while the larvae grow beneath the bark of conifer tree roots and recently cut stumps (Nordlander et al. 1997; Leather et al. 1999; Moore et al. 2004). These beetles have a close symbiotic association with several fungi but *L. procerum* and *O. quercus* are the most common of them (Jankowiak and Bilański 2013).

Olfactory communication in the study species

Chemical communication occurs when a sender releases a cue and a receiver show behavioural/physiological responses accordingly. Olfactory chemical communication is essential for bark and ambrosia beetles and weevils as these chemical cues play a crucial role in the interaction of these beetles with their specific hosts, mates, microbes and also in the interactions where more than one trophic level may be involved (Davis and Landolt 2013; Beck and Vannette 2017; Kandasamy et al. 2019; Pham et al. 2020).

Ips typographus, D. ponderosae, *H. abietis* and *T. lineatum* use volatiles to find a suitable habitat with host species by responding to host and nonhost volatiles. Pine and spruce trees produce several terpene compounds like *α*-pinene, *β*-pinene, (±)-3-carene, myrcene, terpinolene, (-)-bornyl acetate etc which are potential attractants. The non-host produces more green leaf volatiles like 1-hexanol, *E*2-hexen-1-ol, *Z*3-hexen-1-ol, *Z*3-hexenyl acetate, (±)-3-octanol, (±)-linalool etc which also pushes these beetles away towards the more pine and spruce dominated areas (Schroeder 1992; Persson et al. 1996; Zhang and Schlyter 2004; Eriksson et al. 2008; Lehmanski et al. 2023). In response to the dying or stress process, trees produce ethanol due to anaerobic respiration. Some of these beetles can also get attracted to ethanol or a combination of ethanol with other host volatile during this stress

response helping them to select a suitable host. (Graham 1968; Moeck 1970; Vité 1979; Borden et al. 1980; Kelsey and Joseph 2003; Ranger et al. 2015). Similarly, these beetles choose symbiotic fungi and avoid the non-symbiotic fungi with the help of odor blends produced by their specific symbiotic fungi (Jankowiak and Bilański 2013; Kirkendall 2015; Kandasamy et al. 2016; Kandasamy et al. 2019; Lehenberger et al. 2019).

The chemical cues which elicit the physiological or behavioural response within members of the same species are called pheromones. Many beetles use pheromones extensively as a part of their communication system. Depending on what pheromones are used for, they can be classified into several types, e.g. aggregation, anti-aggregation, and sex pheromones. To initiate the mass attack both bark and ambrosia beetles often use aggregation pheromone which attract the individuals of both sexes. Females of *T. lineatum* produce (+)-lineatin as their aggregation pheromone (Macconnell et al. 1977). In the spruce bark beetle, male of *I. typographus* produces aggregation pheromone, consisting of 2-methyl-3-buten-2-ol and (4*S*)-*cis*-verbenol. 2-methyl-3-buten-2-ol is synthesized de novo from mevalonate while (4*S*)-(-)-*cis*-verbenol is synthesized from host monoterpene hydrocarbon *α*-pinene (Bakke 1970; Bakke, Frøyen, and Skattebøl 1977; Birgersson et al. 1984; Erbilgin et al. 2007; Ramakrishnan et al. 2022). In *D. ponderosae*, *trans*-verbenol produced by females works as a powerful aggregation pheromone for both sexes which is synthesized from the host monoterpene *α*-pinene (Pitman and Vité 1969; Billings et al. 1976). Additionally, males also release *exo*-brevicomin which also helps in further colonization of the tree (Conn et al. 1983; Libbeyet al. 1985). The resources present in the tree are limited and overcrowding leads to intraspecific competition. In many of these species of beetles, individuals also produce anti-aggregation (or aggregation stopping) pheromones which assist the spread of beetles to new trees and avoid the intraspecific competition, hence help in maintaining the density of population in a tree (Raffa 2001). *Ips typographus* produces (*S*)-(−)-ipsenol and verbenone during later stages (2-6 days) of an attack which reduces the aggregation (Francke et al. 1980; Bakke 1981; Birgersson et al. 1984; Leufvén et al. 1984; Schlyter and Birgersson 1999). In *D. ponderosae* the process of aggregation is stopped by the production of high concentrations of compounds *exo*-brevicomin and frontalin which are produced by males, and verbenone which is produced by both sexes, formed by intestinal and symbiotic microorganisms (Hunt et al. 1989;

Hunt and Borden 1990). In *H. abietis* not much is known about pheromone communication and their identification. However, Hylodor is reported as potential aggregation attractant (Skrzecz 2003; Kuźmiński and Bilon 2003) and females may produce compounds that acts as sex pheromone over short distances (Selander 1978; Tilles et al. 1988).

General methodology

Heterologous expression systems for functional characterisation of ORs

The term "heterologous expression" refers to the expression of a gene or a part of a gene in a host organism that does not natively possess the gene or the relevant gene fragment (Frommer and Ninnemann 1995). To identify the function of a specific chemoreceptor several heterologous systems can be used including mammalian cell lines (human embryonic kidney 293 cells (HEK293)) and *Xenopus laevis* oocytes. Each of these expression systems vary greatly in terms of their experimental plan, time consumption, ease of use and the opportunity for high throughput analyses (Wang et al. 2016). This thesis primarily involves a HEK293 cell assay for functional characterisation of several ORs and *X. laevis* oocytes was used to characterise one OR **(paper I and II)**.

HEK cell expression system

HEK293 cells are an immortalized mammalian cell line (Graham et al. 1977) commonly utilized in heterologous expression in the biotechnology and pharmaceutical sectors to investigate ligand-induced response. It is also possible to genetically modify HEK293 cells to produce heterologous recombinant proteins with regulated and isogenic expression (Jones et al. 2005a; Thomas, and Smart 2005; Große-Wilde et al. 2006; Forstner et al. 2009; Miller et al. 2011). The first use of HEK cells in chemosensory research was done in 2006 to functionally characterise *B. mori* pheromone receptor co-expressing Gα proteins, and calcium imaging was used to trace the ligand-induced changes in ORs (Große-Wilde et al. 2006). Afterwards, a stable and inducible HEK cell line that co-express OR with the Orco was created by introducing a modified expression vector (Corcoran et al. 2014).

To functionally characterise the ORs (**paper I and II**), the method developed by Corcoran et al. (2014) was used in this thesis. Initially, the ORs and ItypOrco were amplified with the gene specific primers with tag (Myc for Orco, V5 for ORs) and ligated into the expression vector. The HEK293 cells were transfected using Lipofectamin 2000 (Thermo Fisher Scientific) with linearised expression vector containing the targeted genes. Some OR genes require codon optimization for better functional expression in HEK293 cells, in that case the nucleotide sequences codon-optimized for *Homo sapiens* were synthesized and ligated to the expression vector which were then transfected into HEK293 cells (Roberts et al. 2021). After that, the receptors are often able to being transcribed, translated, folded, and placed into the cell membrane. After few weeks, a stable HEK cell line containing the targeted gene is produced which is regularly screened with blasticidin, zeocin, and hygromycin (Gold Biotech) selection antibiotics. The cells are then plated in the poly-D-lysine-coated black-walled 96 well plate (Corning Costar) and incubated overnight. The following day, 16 hrs prior the final analysis half of the wells containing cells were induced with doxycycline to express the exogenous OR and Orco genes, while the other half were left non-induced, which is used as control. The change in calcium fluorescence upon adding chemical compounds is used as indicator for ligand-induced OR activation (Figure 2) which is achieved by incubating the plated cells for approximately half an hour before the experiment with calcium sensitive indicator fluro4-AM (Corcoran et al. 2014). In test odor panel, the Orco agonist VUAA1 is always included and used as control for functional expression of Orco (Jones et al. 2011).

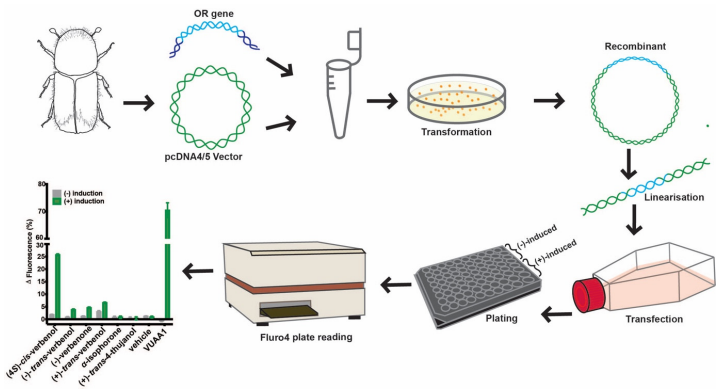

Figure 2. Illustration of HEK293 cell expression system workflow, showing the process from the collection OR genes to data generation.

To confirm the presence of protein expression in HEK cells western blotting was performed for each tested cell line. The tagged proteins are extracted from transfected cells which were cultured in two separate flasks. On the following day cells in one of the flasks were induced with doxycycline while other flask was left uninduced to serve as a control. After 16 hours of incubation, the cells were centrifuged, and total protein was extracted from the cells to perform western blot with primary antibodies (rabbit anti-Myc for Orco, rabbit anti-V5 for ORs) and secondary antibodies (anti-rabbit +IgG) (**paper I** and **II**).

Xenopus laevis oocyte expression system

Xenopus oocyte expression system has been extensively used to functionally characterise ion channels in different animals including insects in past two decades (Krieger et al. 2004; Sakurai et al. 2004; Zhang and Löfstedt 2015). *Xenopus* oocytes have a high capacity of protein synthesis; they are able to correctly assemble the protein products into their membrane and translate the injected genetic information with high efficiency (Markovich et al. 1999). In this study **(paper II)**, fully developed surgically collected *X. laevis* oocytes, preferably stage VI with diameter of more than 1 mm were used. Full length OR and Orco genes were first amplified using gene-specific primers and then ligated into the expression vector (pCS2+). The OR and Orco clones were linearized after generating large quantities of plasmids containing the target genes. The linearized genes were in-vitro transcribed into complementary RNAs (cRNAs) using the SP6 mMESSAGE mMACHINE® kit (Invitrogen, Carlsbad, CA, USA). The cRNAs of OR and Orco genes were mixed at ratio of 1:1 and this mixture was microinjected into the oocytes with 50ng of total RNA from each gene and were incubated for 2-3 days at 16°C in Ringer's buffer. This gives proper time and environment for OR and Orco genes to translate to receptor protein and incorporate into membrane. On the day of experiment, the injected oocytes were introduced to two-electrode voltage clamp coupled with a TEC-03BF amplifier (npi electronic GmbH, Tamm, Germany) and a computer-controlled circulation system was used to add test compounds. If the test compound elicits a response, the ion channel opens up which allows the inward current flow which get detected under the two-electrode voltage clamp (Figure 3).

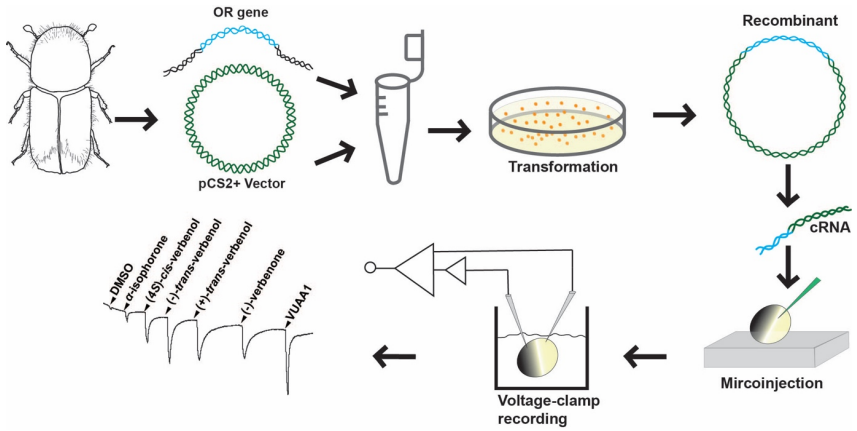

Figure 3. Illustration of *X. laevis* expression system workflow, showing the process from the collection OR genes to data generation.

Electrophysiology for identification of OSNs in *T. lineatum* antenna

Single sensillum recording (SSR) is a quantitative method for measuring action potentials generated when the chemoreceptor in an OSN binds to an odor, and each neuron type is characterised according to the most potent compound and response spectrum. The basic setup for SSR consists of electrodes, an amplifier, an anti-vibrational table, a microscope with high-resolution power, and a Faraday cage to reduce external electrical noise (Figure 4). Ligands from different ecological origins and concentrations elicit different neural responses, by showing different firing rates and temporal patterns. The firing rate of the OSNs can be measured by inserting a recording electrode into the sensillar lymph and a reference electrode into the insect body (usually in the eyes or pronotum). When an active odor stimulus is presented to the sensilla, the neuron in the sensillum generate electrical signals (Figure 4). The recording electrode measures these signals relative to the reference electrode. In this way, a thorough examination of the sensitivity and selectivity of particular OSN can be obtained (Den Otter et al. 1980).

In this thesis SSR was performed on *T. lineatum* antennae to gain more information about the olfactory responses in this ambrosia beetle (**paper III**). Before the recording, the mounting was performed by fixing the beetle with dental wax inside

a 1 cm cut section of a 200 mL pipette tip, so that its head and antenna became accessible for the experiment. The antenna was secured on a microscope slide in such a way that the recording electrode could be inserted and light penetrating from below for visualisation through microscope. Tungsten electrodes were sharpened electronically and were inserted into the sensillum (recording electrode) and pronotum (reference electrode) (Figure 4). The recording electrode was linked to a micromanipulator (PM 10, Märzhäuser, Wetzlar-Steindorf, Germany) for finely controlled movement over the antenna. A charcoal-filtered, humidified airflow at 1.2 L/min was continually applied to the beetle and the stimulus was also added through an air pipe for 0.5 sec after some intervals of time. The data were digitalized using an IDAC4 (Syntech), and the data was visualized and analysed in AutoSpike v. 3.9 (Syntech). Further, to thoroughly examine *T. lineatum* antennal morphology and olfactory sensilla distribution, scanning electron microscopy (SEM) was performed. A detailed methodology of the SEM procedure is available in **paper III**.

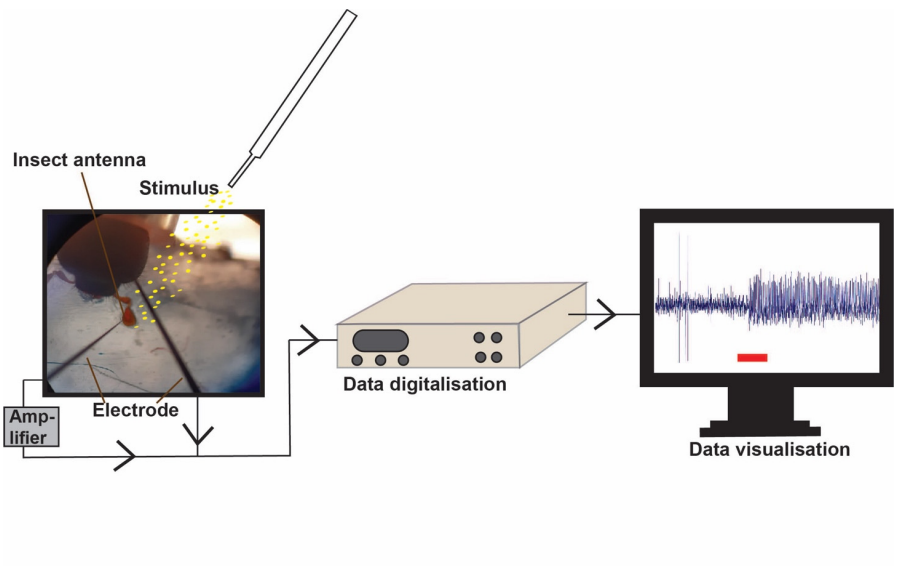

Figure 4. Schematic overview of single sensillum recording (SSR) setup.

Analysis of chemoreceptors from *T. lineatum* genome

DNA sequencing is a technique that is used to identify the sequence of nucleotides or bases present in a DNA molecule. In this thesis, this technique was used to identify chemosensory receptors from genomic DNA (gDNA) of *T. lineatum* (**paper IV)**. gDNA was extracted from *T. lineatum* beetle specimens using the Nano bind Big DNA kit (Circulomics, Baltimore, MD, USA). Sequencing of gDNA was done on a MinION platform (Oxford Nanopore Technologies). Following the sequencing process, an optimized version of Guppy v6.0.1 (Oxford Nanopore Technologies) was used to do high-accuracy base calling of the raw reads (dna_r9.4.1_450bps_hac.cfg model). A total of 25 Gb of sequence data was produced (~190x genome coverage). The clean reads were assembled *de novo* using Flye v2.7.1 (Kolmogorov et al. 2020), followed by four rounds of polishing with Racon v1.3.3 (Vaser et al. 2017). After the polishing, haplotype redundancies were merged using Purge haplotigs v1.0.4 (Roach et al. 2018) and duplicated haplotigs collapsed using Haplomerger2 v2.01 (Huang et al. 2017).

The chemosensory genes were manually annotated from assemble sequence by performing BLAST searches using protein sequences of previously identified chemosensory genes from closely related beetles (*I. typographus* and *D. ponderosae*). Maximum likelihood phylogenetic trees were constructed using FastTree 2.1.11 which was based on MAFT alignments of chemoreceptor protein models from different species. A more detailed description of the protocol used for the analysis of chemosensory receptors in *T. lineatum* is shown in the material and method section of **paper IV**.

Result and discussion

To identify the functional role of an OR, genomic or transcriptomic data is needed in order to identify the sequences of chemoreceptor (in this case OR) proteins. Electrophysiological data is also needed to get information on which ligands an insect responds to. Since this information was available for *I. typographus*, and *D. ponderosae* (Andersson et al. 2013; Andersson et al. 2019; Yuvaraj et al. 2021), they were directly subjected to the next step (functional characterisation of ORs) while in *T. lineatum*, electrophysiological data and genomic chemoreceptor data were gathered, as a prerequisite for future studies of its chemoreceptor proteins in heterologous expression systems. For *H. abietis*, an antennal transcriptome was annotated and 79 ORs were identified in this species before functional studies were performed.

Functional conservation of OR responses amongst curculionids (**paper I**)

Coleoptera is the largest order in the class Insecta but only few ORs have been functionally characterised. The deorphanized ORs from beetles, including the ones characterised in this thesis, include one OR from each of the dark black chafer *Holotrichia parallela*, the Adonis ladybird *Hippodamia variegata* Goeze, the rice water weevil *Lissorhoptrus oryzophilus* and the copper green chafer *Anomala corpulenta* (Wang et al. 2020; Xie et al. 2022; Zhang et al. 2023b; Qu et al. 2024), two ORs from each of the mountain pine beetle *D. ponderosae* and the pine weevil *H. abietis* (**paper I**), three ORs from each of the hickory borer *Megacyllene caryae* and the red palm weevil *Rhynchophorus ferrugineus* (Mitchell et al. 2012; Antony et al. 2021; Ji et al. 2021; Antony et al. 2023) and twelve ORs from the Eurasian spruce bark beetle *I. typographus* including those characterised in **paper I** and **II** (Hou et al. 2021; Roberts et al. 2021; Yuvaraj et al. 2021; Yuvaraj et al. 2023).

Simple (1:1) orthologs of some ORs are conserved in closely related beetle species, however due to lack of functional data nothing was known whether orthologs respond to the same or different compounds in different species of Coleoptera. With the aim to investigate the functional similarities or differences between the orthologous ORs, two different clades comprised of one OR from each of *I. typographus* (Ityp), *D. ponderosae* (Dpon) and *H. abietis* (Habi) were targeted for functional study.

The candidate ORs in **paper I** were selected based on the response of an OR from Cerambycidae beetle, *M. caryae* (McarOR5). *Megacyllene caryae* OR5 belongs to beetle OR subfamily named Group 2B which has several ORs from different beetle families including orthologous ORs in Curculionidae. This McarOR responds strongly to the male produced pheromone 2-phenylethanol in this species (Mitchell et al. 2012; Mitchell et al. 2020). 2-phenylethanol is also an ecologically relevant compound for several Curculionidae beetles as in *D. ponderosae* it reduces the attraction to the aggregation pheromone when the tree is over-colonised (Pureswaran et al. 2000), a fungal symbiont of *I. typographus* releases 2-phenylethanol (Kandasamy et al. 2019), and in case of pine weevil *H. abietis*, 2-phenylethanol is a strong anti-feedant present in deterrent non-host plants (Eriksson et al. 2008). Since 2-phenylethanol is widely used among beetles mentioned above, the hypothesis was that the curculionid ORs that are related to McarOR5 may also respond to the same or structurally similar compounds. Hence, two sets of OR orthologs which were phylogenetically close to McarOR5 from each of *I. typographus*, *D. ponderosae*, and *H. abietis* were selected for functional characterisation (Figure 5).

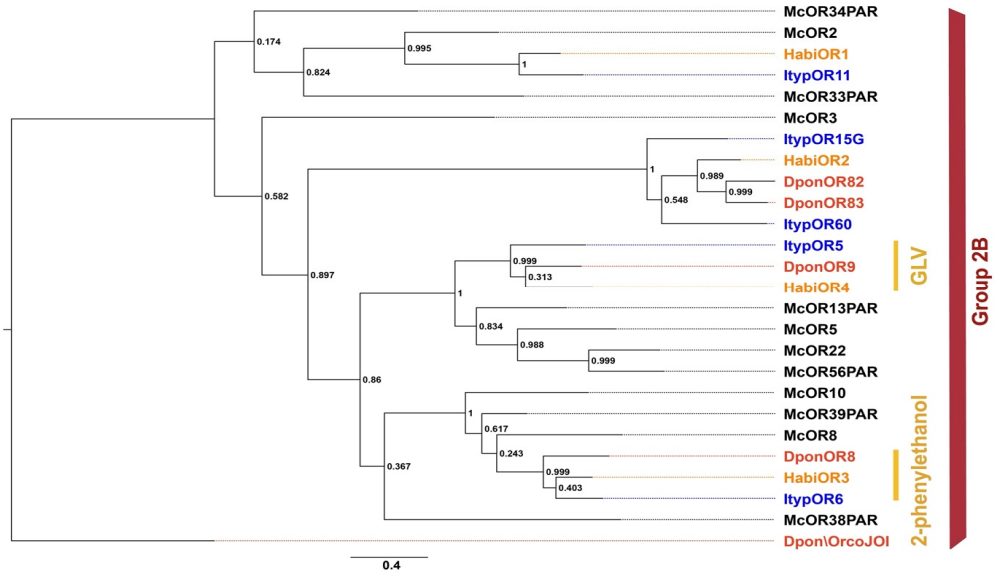

Figure 5. Maximum likelihood tree of odorant receptors (ORs) from beetles of Curculionidae and Cerambycidae from group 2B. The tree was made from OR amino acid sequences from the curculionids *H. abietis* (Habi: orange), *I. typographus* (Ityp: blue), *D. ponderosae* (Dpon: red), and the cerambycid *M. caryae* (Mcar: black). The numbers at nodes indicate aLRT (approximate Likelihood Ratio Test) SH (Shimodaira-Hasegawa)-like branch support. The thick red line indicates the Group of ORs and yellow lines shows the OR orthologues in this study (from **paper I**).

A phylogenetic OR tree was constructed which was rooted with the conserved Orco lineage (Figure 5). HEK cell assay was performed on the six curculionid OR orthologs after their relatedness to McarOR5 had been established. The results of this study showed that the conserved orthologous receptors show highly conserved response specificities. HabiOR3, ItypOR6 and DponOR8 responded only to 2-phenylethanol (Figure 6), while the other group of HabiOR4, ItypOR5 and DponOR9 responded to C_6 green leaf volatiles (GLVs) which are widespread among angiosperm plants and are generally avoided by conifer-feeding beetles. With only slight variation in response profiles, HabiOR4 responded to five GLV compound including 1-hexanol, *Z*3-hexenol, *E*2-hexenol, *Z*2-hexenol and *E*3-hexenol. ItypOR5 and DponOR9 responded to 1-hexanol, *Z*3-hexenol, *E*2-hexenol and *Z*2-hexenol (Figure 7).

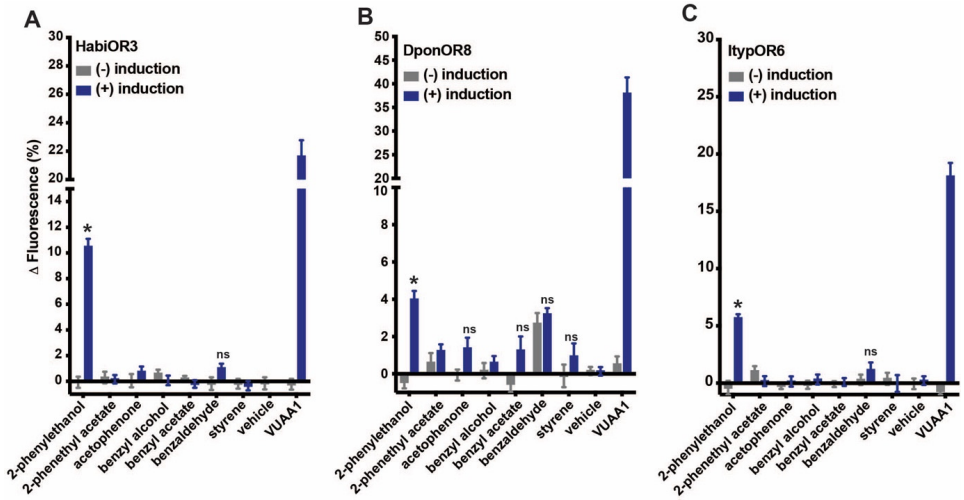

Figure 6. The conserved response for 2-phenylethanol by HabiOR3 (A), DponOR8 (B) and ItypOR6 (C) co-expressed with ItypOrco in screening test with 30 μM stimuli concentration. Th grey bars shows response from uninduced cells ((-)-induction), and the blue bars show the response from doxycycline induced cell line ((+)-induction). VUAA1 was tested as a functional Orco expression control. Significantly ($p < .001$) stronger responses in induced compared to non-induced cells are indicated by asterisks and ns represent non-significant response (from **paper I**).

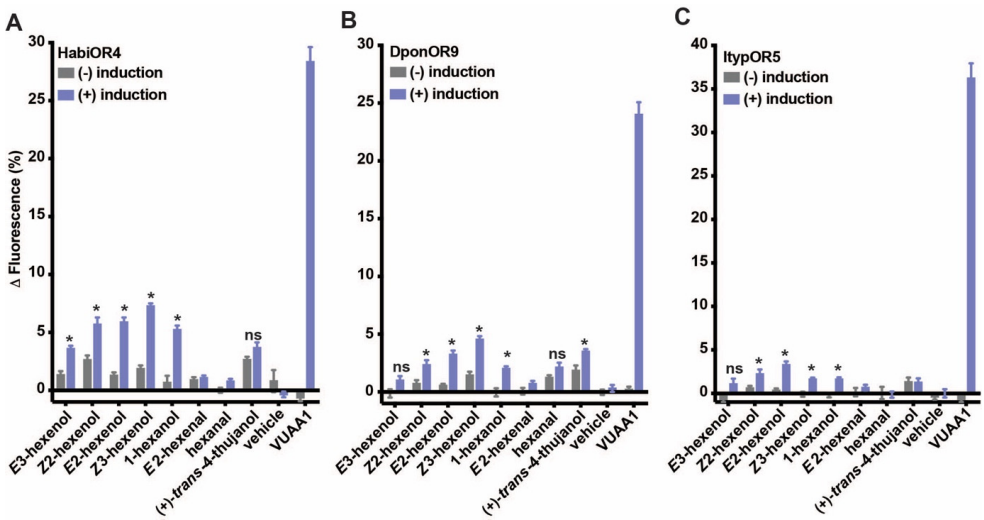

Figure 7. The conserved responses of HabiOR4 (A), DponOR9 (B) and ItypOR5 (C) co-expressed with ItypOrco for GLV compounds in screening test with 30 μM stimuli concentration. The grey bars show response from uninduced cells ((-)-induction), and the violet bars show the response from doxycycline induced cell line ((+)-induction). VUAA1 was tested as a functional Orco expression control. Significantly ($p < .001$) stronger responses in induced compared to non-induced cells are indicated by asterisks and ns represent the non-significant response (from **paper I**).

For the first time conserved OR responses in three difference species of beetles were revealed through this study. The phylogenetic analysis of the orthologous ORs also indicates that receptors for detecting 2-phenylethanol and GLVs share a common ancestor among the curculionids (Figure 5). Both, *D. ponderosae* and *I. typographus* belong to subfamily Scolytinae; however also the non-scolytine curculionid (*H. abietis*: Molytinae) showed similar responses. It is interesting to note that the receptors most closely related to McarOR5 are responding to GLV compounds, while the receptors responding to 2-phenylethanol are present in the sister clade (Figure 5). This indicates that the response for 2-phenylethanol may be ancestral and the new function to detect GLVs may have evolved later in this receptor lineage. Another possibility could be that the ancestral receptor was broadly tuned to both 2-phenylethanol and GLVs and after gene duplication higher specificity for either GLVs or 2-phenylethanol may have evolved. The strong functional conservation of the ORs highlights the importance of 2-phyenylethanol and GLVs in all the beetles compared in **paper I**.

Ips typographus aggregation pheromone receptor and its paralogue (**paper II**)

Several studies including **paper I** have suggested that phylogenetically closely related ORs may show similar responses. This study aims for the characterisation of *I. typographus* aggregation pheromone receptor and its paralogue, understanding their ligand binding mechanism and finally investigating the evolutionary origins of pheromone receptors in curculionids. The aggregation pheromone of *I. typographus* consist of (4*S*)-*cis*-verbenol and 2-methyl-3-buten-2-ol (Birgersson et al. 1984; Schlyter et al. 1987). Specificity of an OSN is determined by the OR(s) it expresses, and previously it have been shown that (4*S*)-*cis*-verbenol responsive OSNs are highly abundant on *I. typographus* antenna (Andersson et al. 2009). Also a few ORs were previously shown to be highly expressed in the antennae of *I. typographus,* including the one involved in this study (Yuvaraj et al. 2021). The most highly expressed OR in *I. typographus* responded to lanierone, a pheromone component in some North American *Ips* spp. (Birgersson et al. 2012; Yuvaraj et al. 2023). Hence, the second highest expressed OR (ItypOR41) and its paralogues were targeted for

this study with the prediction that the receptors may show responses to the components of the aggregation pheromone.

In the HEK cell assay ItypOR41 showed a strong response to the aggregation pheromone component (4*S*)-*cis*-verbenol and secondary response to structurally similar compounds (-)-*trans*-verbenol, (+)-*trans*-verbenol, (-)-verbenone (Figure 8).

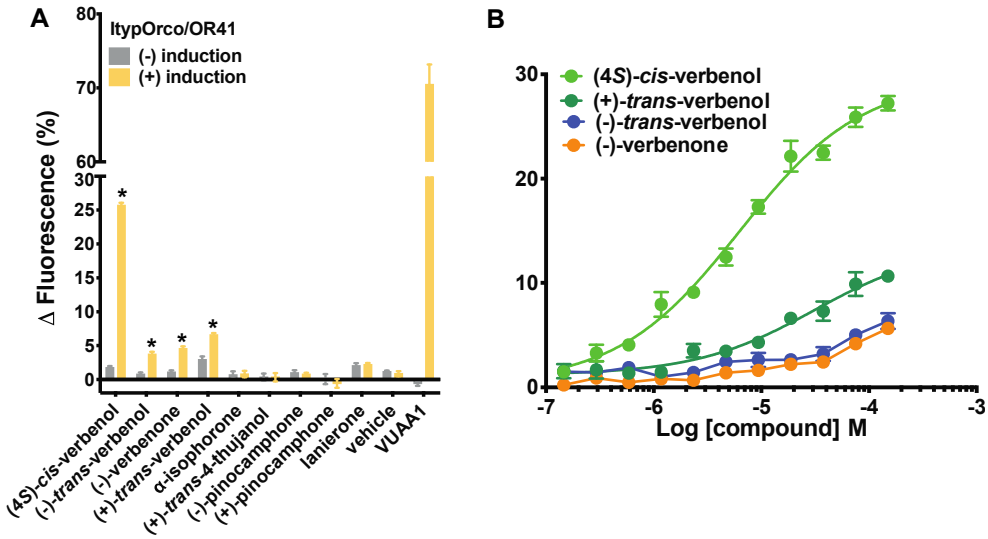

Figure 8. (A) The screening response of ItypOR41 co-expressed with ItypOrco for (4*S*)-*cis*-verbenol and for structurally similar compounds, (-)-verbenone, (+)-*trans*-verbenol, (-)-*trans*-verbenol at a concentration of 30 μM. As a positive control for functional Orco expression 50 μM VUAA1 was tested. The (+)-induction cells were treated with doxycycline while the (-)-induced cells were not and served as a negative control (from paper I). Significantly ($p < .001$) stronger responses in induced compared to non-induced cells are indicated by asterisks (B) Dose response of ItypOR41 to the four most active compounds. Error bars show SEM (from **paper II**).

After deorphanisation of ItypOR41, to investigate the function in ORs that are closely related to ItyOR41 in *I. typographus*, the paralogous ItypOR40 and ItypOR45 were also subjected to the functional characterisation in HEK cells. *Ips typographus* OR40 and ItypOR45, exhibited no response in HEK cell assay despite both were detected in Western blot. They were therefore further tested in *X. leavis* oocytes. In the *X. leavis* oocyte expression system only a few numbers of compounds could be tested, hence, (-)-verbenone, (+)-*trans*-verbenol, (-)-*trans*-verbenol, (4*S*)-*cis*-verbenol, *α*-isophorone and *α*-pinene were selected to test ItypOR40 and ItypOR45. In the oocyte system ItypOR45 showed strong response to (-)-verbenone, (+)-*trans*-verbenol, (-)-*trans*-verbenol and comparatively lower

secondary response to (4*S*)-*cis*-verbenol and *α*-isophorone while *α*-pinene did not elicit any response (Figure 9). However, ItypOR40 did not respond to the any tested compounds. The results revealed that ItypOR45 is more of a generalist or broadly tuned OR compared to ItypOR41 which could be the result from sub-functionalization. The responses from ItypOR41 is nearly identical to the corresponding *cis*-verbenol responsive OSN class reported from *I. typographus* in a previous SSR study but ItypOR45 responses do not exhibit high resemblance with any described OSN class, although it shows some similarities to the verbenone responsive OSN class (Andersson et al. 2009; Kandasamy et al. 2019).

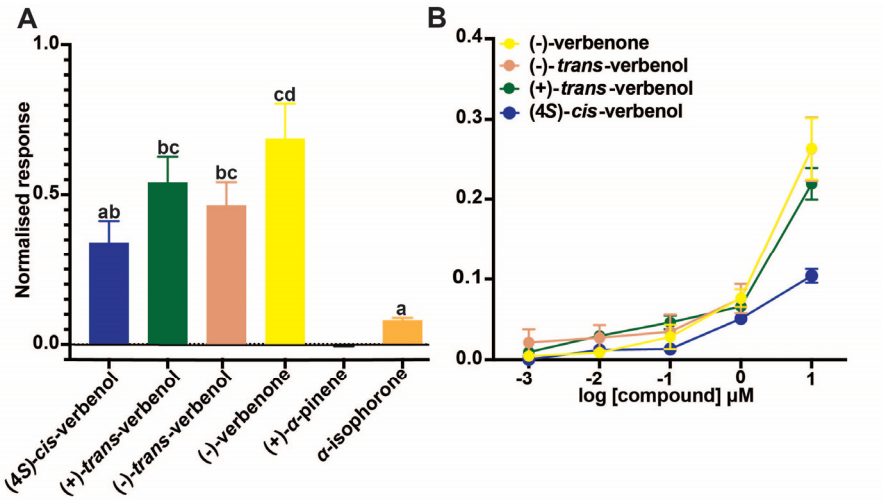

Figure 9. (A) The response of ItypOR45 co-expressed with ItypOrco in *X. laevis* oocytes to 100 µM Stimuli. Different lowercase letters indicate significant differences in response between compounds at $p < 0.05$. (B) Dose response of ItypOR45 to the four most active compounds. Error bars show SEM (from **paper II**).

Pheromone receptors are known to often be highly specific, and the ecological importance of (4*S*)-*cis*-verbenol may have also led to the high specificity ItypOR41. Within group 7 of beetle OR phylogeny, five other ORs that reside in two separate and distantly related OR clades have previously been identified to respond for pheromone compounds including, RferOR1 responding to ferrugineol and ferrugineone, ItypOR28, ItypOR36, ItypOR46 and ItypOR49 responding to *E*-myrcenol, lanierone, (-)-ipsenol and (-)-ipsdienol, respectively (Antony et al. 2021; Hou et al. 2021; Yuvaraj et al. 2021; Yuvaraj et al. 2023). The data from ItypOR41 and ItypOR45 provides additional support that unlike lepidopterans which have

specific PR clades, coleopterans pheromone receptors appear to have multiple evolutionary origins (Figure 10).

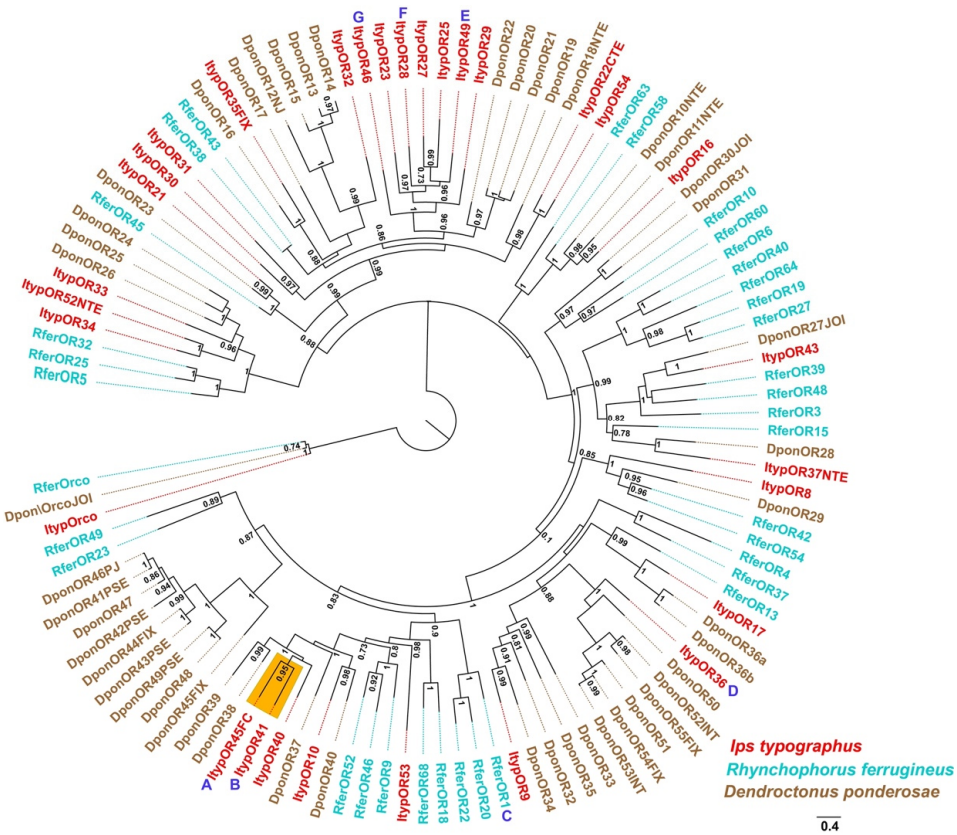

Figure 10. Maximum likelihood tree showing phylogenetic relationships among the selected group 7 odorant receptors (ORs). The ORs included are from *I. typographus* (Ityp: red), *D. ponderosae* (Dpon: brown), and *R. ferrugineus (Rfer: cyan).* The small clade containing the tested ORs are highlighted in orange and the local support values shown at the node of branches. The capital letters show the pheromone receptors that have been functionally characterised. The compounds for which receptors responding for: (A) (-)-verbenone, (+)-*trans*-verbenol, (-)-*trans*-verbenol, (B) (4*S*)-*cis*-verbenol, (C) ferrugineol and ferrugineone (D) lanierone, (E) (*R*)-(-)-ipsdienol, (F) *E*-myrcenol, (G) (*S*)-(-)-ipsenol (from **paper II**).

Ips typographus OR41 and ItypOR45 were subjected to further analysis in order to understand whether these receptors show structural similarity and their potential ligand binding mechanism. Thanks to recent advances in protein structure (Jumper et al. 2021) and cryo-EM structural analysis of insect ORs (Butterwick et al. 2018; del Mármol et al. 2021), protein models and molecular docking of responding OR

could be predicted with AlphaFold using ColabFold. The predicted models of both receptors revealed high similarity between ItypOR41 and ItypOR45, several of the residues lining the central binding cavity were conserved. However, two notable differences between the structures of ItypOR41 and ItypOR45 was predicted. In one site (Site I) ItypOR41 amino acid residue Gln179 was able to form hydrogen bond with (4*S*)-*cis*-verbenol while that was unlikely in case of ItypOR45 (Figure 11 A and B). Additionally, in ItypOR41 Trp310 displayed to have π-π interactions with (4*S*)-*cis*-verbenol, and Phe313 exhibited a significant movement for the flexible docking (Figure 11 A and C). Another deeper binding site (Site II) was found in ItypOR45 which was not present in ItypOR41 (Figure 11 D). The compounds that elicited secondary responses in ItypOR41 (+)-*trans*-verbenol, (−)-*trans*-verbenol, and (−)-verbenone were also analysed with molecular docking. In case of ItypOR41, all compounds eliciting secondary responses also clustered in Site I, forming π-π interactions (Figure 11 C). In case of ItypOR45, all the compounds were clustered in both Site I and Site II. This difference in predicted hydrogen bonding may explain the difference in functional selectivity between narrowly tuned ItypOR41 and broadly tuned ItypOR45, hence indicating the importance of hydrogen bonding for the specificity of an ORs as suggested previously (Yuvaraj et al. 2021).

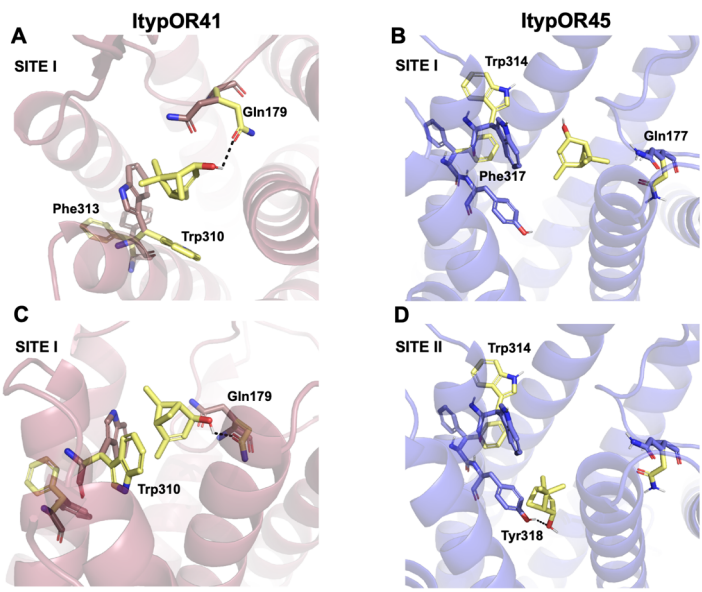

Figure 11. Molecular docking results for ItypOR41 and ItypOR45 showing the predicted binding sites. Predicted binding of (4*S*)-*cis*-verbenol (yellow) (A) to ItypOR41 at Site I, (B) to ItypOR45 at Site I (C) to ItypOR41 at Site I from a different angle, and (D) to ItypOR45 at Site II (from **paper II**).

Based on molecular docking results for ItypOR41, three putative binding residues (Gln179, Trp310 and Phe313) were subjected to site-directed mutations to gain support for the computational predictions of ligand binding residues. In total, four mutations to ItypOR41were made: the amino acid residue was mutated from glutamine to alanine at amino acid position 179 (ItypOR41^{Gln179Ala}) and to glutamic acid (ItypOR41^{Gln179Glu}), the tryptophan at position 310 was changed to alanine (ItypOR41^{Trp310Ala}) and the phenylalanine at position 313 to alanine (ItypOR41^{Phe313Ala}). Since glutamine and alanine are very different in structure, glutamic acid was also introduced by mutation in the same position to observe the effect of mutation in comparatively structurally similar amino acid. The mutated receptors were tested in HEK cell expression system and were screened against the same set of compounds as the cell line with non-mutated ItypOR41. The response to (4*S*)-*cis*-verbenol of the cell lines expressing ItypOR41^{Gln179Ala} or ItypOR41^{Gln179Glu} were both highly reduced in comparison to the non-mutated receptor with no responses elicited by compounds that elicited secondary responses in the non-mutant ItypOR41 (Figure 12 A and B). In case of ItypOR41^{Trp310Ala}, approximately half of the response to the primary ligand remained in comparison to non-mutated receptor while secondary response was only observed for (+)-*trans*-verbenol (Figure 12 C). The last predicted residue, ItypOR41^{Phe313Ala}, showed no difference in response in comparison to the non-mutated receptor (Figure 12D). The absence of difference in the responses between the non-mutated ItypOR41 and ItypOR41^{Phe313Ala} suggests this residue is not directly involved in the ligand binding/receptor activation mechanism. This work demonstrates a high sensitivity of ItypOR41 to small structural changes in the binding site and further confirm the importance of hydrogen bonding for the specificity of an OR.

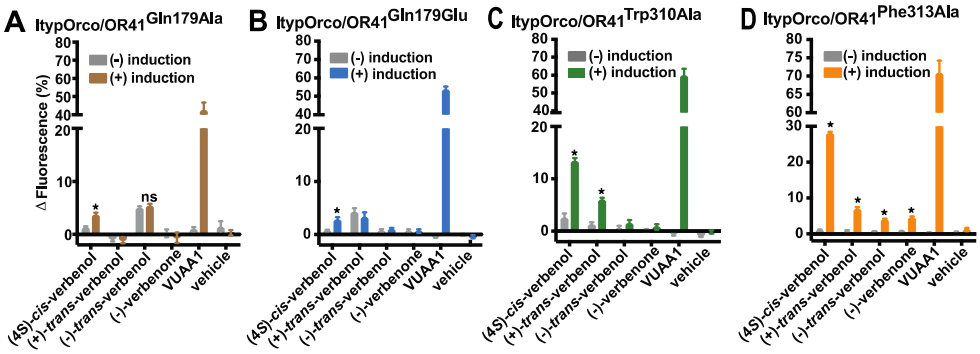

Figure 12. The screening responses of cells co-expressing ItypOrco and (A) ItypOR41^{Gln179Ala} (B) ItypOR41^{Gln179Glu} (C) ItypOR41^{Trp310Ala} (D) ItypOR41^{Phe313Ala} in HEK cells to 30 μM Stimuli. As a positive control for functional Orco expression 50 μM VUAA1 was tested. The (+)-induction cells were treated by doxycycline while the (-)-induced were not and served as a negative control. Significantly stronger responses in induced compared to non-induced cells are indicated by asterisks (from **paper II**).

Electrophysiological responses from *T. lineatum* OSNs (**paper III**)

Electrophysiological studies have been extensively done in recent times and extensive data are available for some species like *I. typographus* (Andersson et al 2009; Kandasamy et al. 2019), but much less in known for *T. lineatum*. Previously SSR was performed on *T. lineatum* by Tømmerås and Mustaparta (1989) with common bark- and ambrosia beetle pheromone compounds, short aliphatic alcohols, vapours and extracts from host and non-host trees (Tømmerås and Mustaparta 1989). But since then, more ecologically significant compounds for *T. lineatum* have been discovered; in particular, the volatiles from the fungal mutualist have been found. With the aim to fill this missing gap and to compare the olfactory responses of ambrosia beetles with bark beetles which differ in their ecology, I performed SSR on *T. lineatum* antennae. A total of 170 sensilla were screened using 57 ecologically relevant compounds. Thirteen separate OSN classes of "strongly responding (response: ≥80 Hz)" neurons were identified from a total of 90 (female: 46, male: 44) out of 119 responsive neurons using randomized screening. The remaining 29 responsive neurons showed only low responses, hence could not be assigned to any class. Several sensilla contained more than one OSN, namely A neuron and B neuron, which were differentiated based on their large and small spike amplitudes, respectively. Each OSN class was assigned a number. Olfactory sensory

neuron class 1 responded to *T. lineatum* aggregation pheromone, lineatin which was the most abundant OSN class on the antenna with 37 neurons in total number (Figure 13). Olfactory sensory neuron class 2 specifically detects lanierone which is a pheromone component produced by some North American *Ips* species (Teale et al. 1991; Seybold et al. 1992). The second most abundant OSN class 3 responds to 2-methyl-3-buten-2-ol which is an aggregation pheromone component of *I. typographus* but also produced by *I. typographus* fungal symbionts (Schlyter et al. 1987; Zhao et al. 2015). Olfactory sensory neuron class 4, 5, 6, and 7 all respond to the fungal mutualist odors (±)-2-methyl-1-butanol, 3-methyl-1-butanol, short-chained esters (e.g., ethyl acetate, ethyl isobutyrate, ethyl butyrate, propyl acetate, isopropyl acetate) and geranylacetone, respectively. Interestingly, *T. lineatum* was shown to have two different OSN classes for structurally very similar compounds, (±)-2-methyl-1-butanol and 3-methyl-1-butanol. The OSN class 4 and 5 were differentiated based on the different rank orders between the most active compounds and variation in their secondary responses. Olfactory sensory neuron class 8 responds to 6-methyl-5-hepten-2-one which is produced by non-host trees, beetles as well as fungi. OSN class 9 responds to (+)-*trans*-4-thujanol an odor from bark beetle fungi and spruce trees. OSN class 10 responds best to 1,2-dimethoxybenzene which is produced by angiosperm non-host trees. Olfactory sensory neuron class 11 responds to acetophenone which is an anti-attractant for several bark beetle species in the *Dendroctonus* genus (Erbilgin et al. 2007; Erbilgin et al. 2008). OSN class 12 responds to C8-alcohols emitted by angiosperm plants and fungi, primarily (±)-1-octen-3-ol. Olfactory sensory neuron class 13 responded to C_6 GLV compounds with primary response to 1-hexanol. Most of the OSNs classes were present in both male and female beetles but some OSN were either present in males (OSN classes 5, 8 and 11) or in females (OSN class 7). But since the numbers of OSNs found of these classes were very low they cannot be assigned as sex specific neurons.

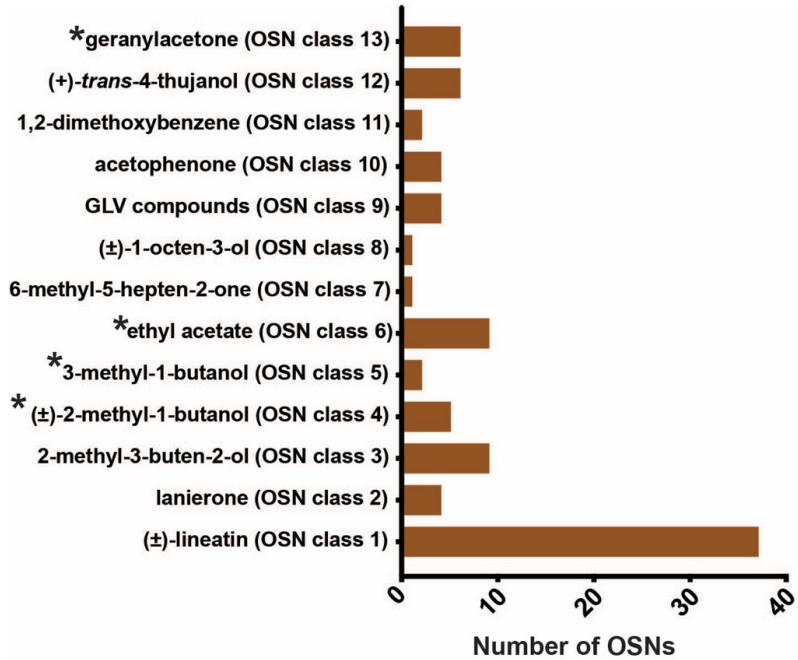

Figure 13. Number of OSNs found in *T. lineatum* antenna during the screening at dose of 10 µg concentration. Asterisks indicate OSN classes responding to volatiles from the mutualistic fungus *P. ferruginea* (from **paper III**).

Additionally, 33 OSN were subjected to dose response trials from six of the strongly responding OSN classes (OSN classes 1, 3, 4, 5, 6, and 12). Some of the dose response data is shown in Figure 14. A detailed OSN class responses and more does-tests are shown in **paper III.** All these neurons were found in the ventral side of antenna except of OSN class 1 (Figure 15 A and B).

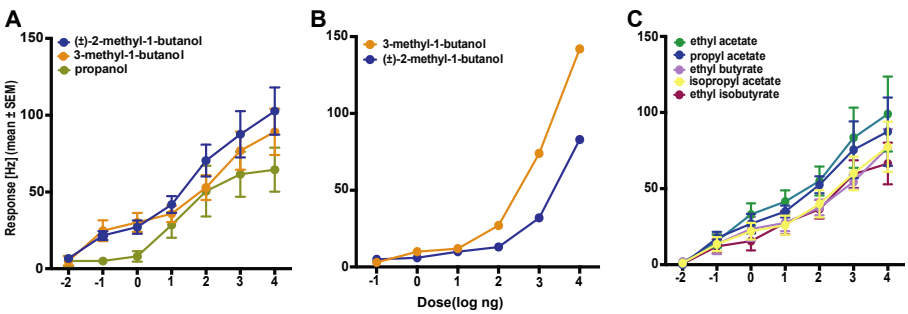

Figure 14. Dose–response curves of (A) 2-methyl-1-butanol (B) 3-methyl-1-butanol (C) ethyl acetate olfactory sensory neuron (OSN) classes from *T. lineatum*, which is volatiles of *P. ferruginea* (from **paper III**).

As OR underlie the response of an OSN, it is feasible that conserved ORs in closely related species could mediate the responses of functionally conserved OSN classes. Upon comparison, 5 OSN classes in *T. lineatum* showing similar response profiles with OSNs in *I. typographus* were revealed. Two of these OSN classes in *T. lineatum* (OSN class 3 and 12) showed very similar response profiles with *I. typographus* (OSN classes responding to 2-methyl-3-buten-2-ol and 1-octen-3-ol, respectively). While the other three OSN classes, i.e., OSN classes 7, 9, 13 in *T. lineatum* corresponds to geranyl acetone, (+)-*trans*-4-thujanol, and GLV alcohols in *I. typographus*, respectively, as they have same responses to primary compound but moderately different in their secondary responses (Andersson et al. 2009; Kandasamy et al. 2019; Schiebe et al. 2019). Several fungal-odor responsive neurons in *T. lineatum* were not found in *I. typographus*, and these differences are likely due to the differences in their ecology. As *T. lineatum* is so far reported to have comparatively restricted diet by being dependent on a single fungal species that release odors different from those of the *I. typographus* associated fungi (Biedermann et al. 2013; Kandasamy et al. 2019). The presence of several specific OSNs tuned to *P. ferruginea* odors (*T. lineatum* fungal symbiont) may help to identify their specific fungus and suggesting that olfactory adaptations in *T. lineatum* are likely important for the maintenance of the beetle-fungus interaction. The identification of these new OSNs from this study will be helpful in future research on ORs since we now have a better understanding of the active ligands for this species.

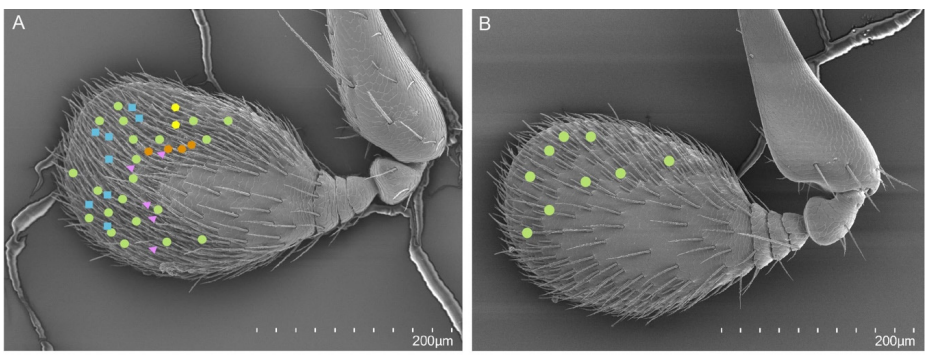

Figure 15. Scanning electron micrographs of the antennal club of female *T. lineatum* showing ventral (A) and dorsal (B) view of antenna with the approximate positions of six OSN classes. Green circles: lineatin OSNs (OSN class 1), blue squares: 2-methyl-3-buten-2-ol OSNs (OSN class 3), orange hexagons: 2-methyl-1-butanol OSNs (OSN class 4), Yellow hexagons: 3- methyl-1-butanol OSNs (OSN class 5), pink triangles: ethyl acetate OSNs (OSN class 6) (from **paper III**).

Chemosensory receptor genes from *T. lineatum* (**paper IV**)

Identification of chemoreceptor genes is needed for functional characterisation studies. This study was performed with the aim to identify chemoreceptors (ORs, GRs and IRs) from *T. lineatum*, and to investigate the phylogenetic relationship of identified chemoreceptors with those of related species with different ecology. To obtain the results, chemosensory receptor genes were identified by performing BLAST searches with previously identified putative chemoreceptor proteins from *D. ponderosae* and *I. typographus* against the *T. lineatum* genome, which was sequenced and assembled in this study. To investigate the phylogenetic relationship of chemoreceptors and correlate the identified chemoreceptors with ecology, chemoreceptors from three different beetle species were used, which had their chemoreceptors previously identified from their respective genomes: the scolytines *D. ponderosae*, which attacks several species of pines, and the coffee berry borer *Hypothenemus hampei*, which attacks coffee berries by boring into the bean, destroying it in the process. The third beetle species was chosen from the Cerambycidae subfamily, the Asian long-horned beetle *Anoplophora glabripennis*, which is polyphagous and attacks many different types of plants. Functionally characterised ORs from *I. typographus* was also included in the phylogenetic analysis in order to find potential orthologues from *T. lineatum* which could be targeted for future functional studies.

From the genome of *T. lineatum*, a total of 67 ORs were identified including Orco. Among the identified ORs, 4 were identified as putative pseudogenes while another 4 were partial genes as they were lacking one or two N-terminal exons. The total number of ORs in *T. lineatum* was the same as in *H. hampei,* however, it was lower than in *D. ponderosae* and much lower than in the polyphagous species *A. glabripennis* (table 1). Out of 9 major OR subfamilies (Group 1, 2A, 2B, 3, 4, 5A, 5B, 6, and 7) in the beetle OR phylogeny, ORs from *T. lineatum* were present in Group 1, 2A, 2B, 5A and 7 (Mitchell et al. 2020) and 19 clear orthologs to either *D. ponderosae* or *H. hampei* or both were found in *T. lineatum* (Figure 16). The pattern of OR distribution of *T. lineatum* in the phylogenetic tree was similar to other curculionids but the distribution of *T. lineatum* ORs among the 9 groups was different from the OR distribution of *A. glabripennis* (Figure 16). Only two larger

Trypodendron-specific OR radiations were found, of which one contain 12 ORs in Group 5A (TlinOR50-61) and the other contains 11 ORs in Group 7 (TlinOR28-36). Further, two clear TlinOR orthologues of functionally characterised ORs from *I. typographus* and *D. ponderosae* were also revealed in phylogenetic analysis. TlinOR9 was orthologous to ItypOR6 and DponOR8 which were previously reported to respond for 2-pheynylethanol, and TlinOR10 was orthologous to ItypOR5 and DponOR9 which respond to GLV compounds (**paper I,** Figure 16), suggesting that these *T. lineatum* ORs could also respond to 2-pheynylethanol and GLV compounds.

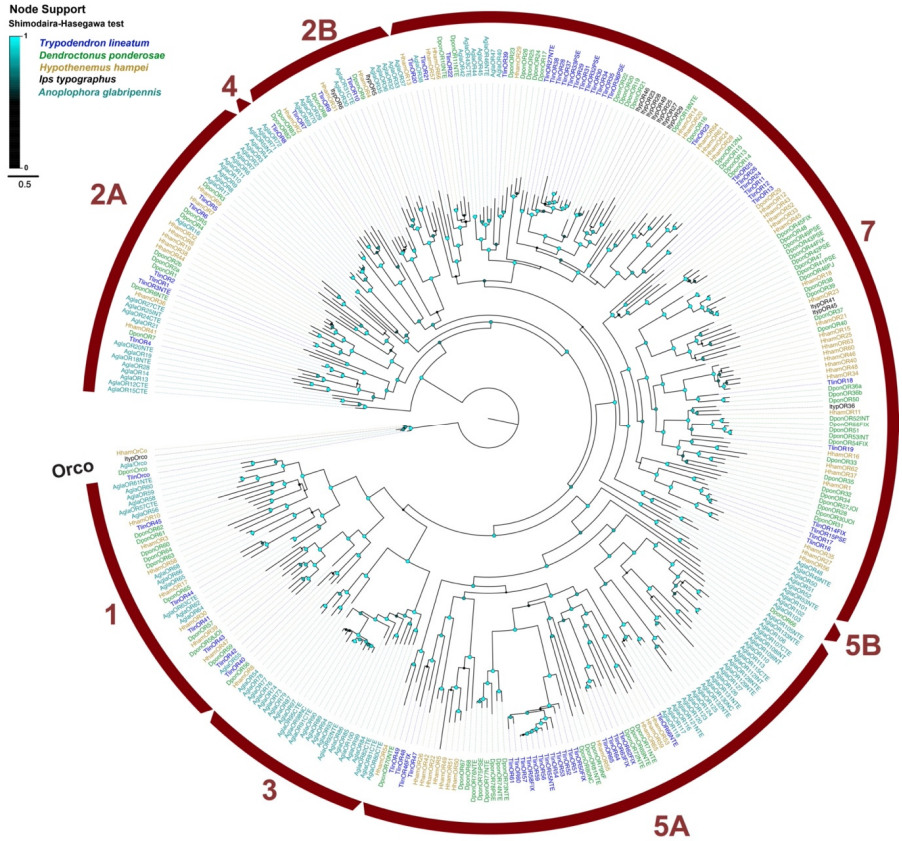

Figure 16. Maximum likelihood phylogenetic tree of ORs from *T. lineatum* (Tlin: blue), *D. ponderosae* (Dpon: green), *H. hampei* (Hham: yellow), *A. glabripennis* (Agla: sea green) and functionally characterised *I. typograph* (Ityp: black). The colours at nodes indicate aLRT (approximate Likelihood Ratio Test) SH (Shimodaira-Hasegawa)-like branch support. The thick maroon arc indicates the different Groups of OR subfamilies (from **paper IV**).

In case of GRs, 38 genes were annotated from the *T. lineatum* genome, of which 4 were considered fragmented pseudogenes and 5 could only be annotated as partial genes. The total number of GRs in *T. lineatum* was lower than *D. ponderosae* and *H. hampei* and like ORs, much lower than in the polyphagous species *A. glabripennis* (Table 1). In the phylogenetic analysis, the distribution of *T. lineatum* GRs was observed to be a bit different than other beetles in comparison. *Trypodendron lineatum* did not show any large GR expansion, especially compared to *H. hampei* and *A. glabripennis* whose species-specific expansions consists of more than 30 GRs. The biggest species-specific GRs expansion in *T. lineatum* consist of eight GRs (TlinGR31- TlinGR38PSE), which included all the four GR pseudogenes found in *T. lineatum*. Clear simple (1:1) orthologs were also observed for several receptors including CO_2, receptors, fructose and non-fructose sugar receptors as well as among the bitter taste GRs (Figure 17). One of the orthologous groups of bitter taste GRs included a receptor also from the cerambycid *A. glabripennis*. No GR from *T. lineatum* was found in the previously identified highly conserved clade named 'GR215' (Andersson et al. 2019). Similarly, in the sugar receptor clade, there was just one GR (TlinGR4), while in the other beetles in the comparison, there are four or six receptors present in that clade (Figure 17).

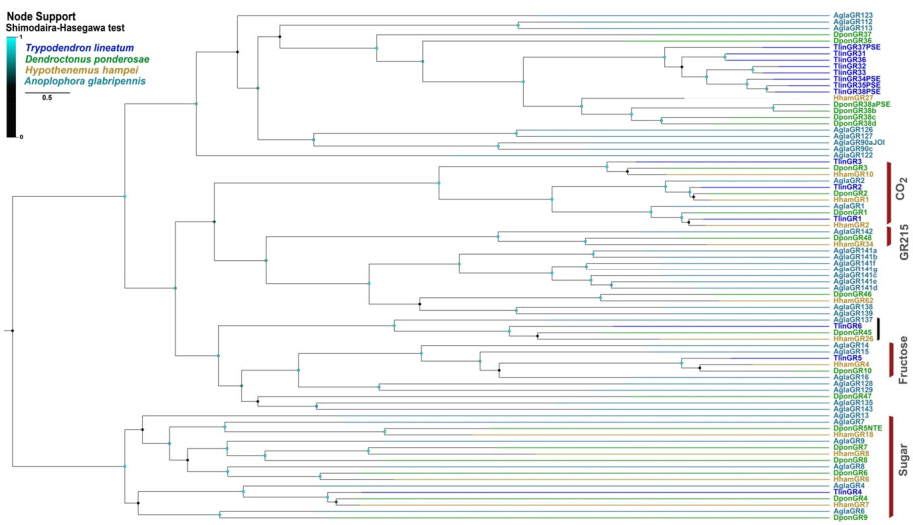

Figure 17. Maximum likelihood phylogenetic tree of gustatory receptors (GRs) showing some of the conserved receptors from *T.lineatum* (Tlin: blue), *D. ponderosae* (Dpon: green), *H. hampei* (Hham: yellow) and *A. glabripennis* (Agla: sea green). The black line indicates the new conserved clade that includes a receptor also from *A. glabripennis* and the thick maroon lines indicate well established GR clades (from **paper IV**).

In case of IRs, 44 genes were annotated from *T. lineatum* genome including one partial gene. The number of IRs in *T. lineatum* was higher than in *H. hampei* but lower than in *D. ponderosae* and *A. glabripennis*. Phylogenetic analysis showed that all the members of conserved antennal IRs (IR8a, IR21a, IR25a, IR40a, IR41a, IR68a, IR76b, IR93a, and eight IR75 members) were present. All TlinIR75 members either had orthologs in both scolytines species or only in *D. ponderosae* or *H. hampei*. Among the divergent IRs, single ortholog of IR60a and three paralogs in the IR100a clade were found in *T. lineatum* which was similar to *D. ponderosae*. Unlike *A. glabripennis* and similar to other two curculionids no large species-specific IR expansion from *T. lineatum* was observed.

Table 1. The number of chemoreceptor genes present in *T. lineatum* and other species used for comparison.

Species	Odorant receptors	Gustatory receptors	Ionotropic receptors
T. lineatum	67	38	44
D. ponderosae	86	60	57
H. hampei	67	66	33
A. glabripennis	132	234	72

In the overall comparison, the total number of chemoreceptors in *T. lineatum* was lower than in the other beetles (Table 1). The total number of chemoreceptors varies between different species, this could also be linked to the different chemical ecology of different species as previously suggested (Nei et al. 2008; Smadja et al. 2009; Benton, 2015; Andersson et al. 2019). The number of ORs in *T. lineatum* was similar to *H. hampei* which also has specialised ecology as they spend majority of lifetime inside the coffee berry. However, the OR number was clearly lower than the species which attacks several different host species, such as *D. ponderosae* and especially *A. glabripennis*. Also *I. typographus* has more ORs (73) annotated from an antennal transcriptome (Yuvaraj et al. 2021) than the number of ORs in the *T. lineatum* genome. In case of GRs, a large reduction in sugar receptor was observed in *T. lineatum* in comparison to other beetles. The conserved orthologous clade from GR family 'GR215' was previously found across several beetle families and was named based on the GR representative in the red flour beetle *Tribolium castaneum* (TcasGR215) (Andersson et al. 2019), but it was surprisingly missing in *T. lineatum*. Based on the phylogenetic data, it is evident that at least the IR and GR families in

T. lineatum lack major lineage expansions. The specialised ecology of *T. lineatum*, which is reported to exclusively feed on the fungus *P. ferruginea*, may be correlated with its reduced number of chemoreceptors, thus reflecting adaptations to its specific ecological niche.

Conclusion and future perspective

Six orthologous ORs from three species of the family Curculionidae were deorphanized in **paper I**. This is the first study to functionally characterise orthologous ORs in beetles and to report high functional conservation, in this case in two orthologous groups of OR responding to 2-phenylethanol and GLVs, respectively. The characetrised ORs were found to share evolutionary history with the 2-phenylethanol receptor in *M. caryae*. The conserved responses also indicates the importance of 2-phenylethanol and GLVs in the ecology of these beetles. In **paper II** an OR detecting the *I. typographus* aggregation pheromone component (4*S*)-*cis*-verbenol was functionally characterised. Subsequently, the characterisation of its paralogous OR revealed a distinct specificity in responding to pheromone compounds yet sharing some olfactory functions which was predicted to be partly due to the absence of hydrogen bonding of the amino acid residue with ligand in ItypOR45. The prediction of ligand binding was further confirmed by testing the ItypOR41 mutant receptors which revealed two amino acid that are likely to be directly involved in ligand binding mechanism. Further, in phylogenetic analysis, the position of these ORs suggested multiple origins of pheromone receptors in bark beetles. In total eight functionally characterised ORs are reported from both studies, accounting for a significant fraction of the overall number of functionally characterised ORs in beetles, which is just above 20 in total.

Single sensillum recordings on *T. lineatum* antennae revealed several new OSN classes (**paper III**), hence, enhancing the information about the odors that are detected by *T. lineatum*. Further, the comparison between *T. lineatum* and *I. typographus* OSN showed that some OSN classes in both the species are similar, which suggest that some olfactory functions within the Scolytinae subfamily are conserved. However, several differences were also discovered, which could relate with the difference in their ecology. Several *T. lineatum* OSNs specifically responded to fungal symbiont volatiles showing the ability of *T. lineatum* to detect its crucial fungal mutualist. Few OSN responded to the pheromone compounds of

other scolytines, indicating a restricted ability to detect and interact with other scolytine beetles.

A total of 149 chemoreceptor genes were annotated from the genome of *T. lineatum* (**paper IV**), which represents a reduced repertoire of chemoreceptors compared to related species. The total number of chemoreceptors in *T. lineatum* was lower than all the analysed beetles, however the chemoreceptors were only slightly lower than *H. hampei* which also has high specialised ecology. *T. lineatum* has ORs orthologous to functionally characterised curculionid ORs responding to 2-phenylethanol and GLVs, suggesting that *T. lineatum* ORs may also be able to detect the same compounds. Future functional characterisation is needed to confirm this hypothesis. No GRs fall in widely conserved beetle GR clade for putative bitter taste receptors "GR215" and only one sugar receptor was observed which may relate to restricted fungal diet. The decrease in repertoires of chemoreceptor genes suggests that specialised ecology of *T. lineatum* has led to deletion of some chemoreceptor genes and a low extent of gene lineage expansions.

Overall, this thesis sheds light on the functional evolution of beetle chemoreceptors and reveals adaptations in the peripheral olfactory system that likely connect to ecological specializations. However, more functional characterisation studies are needed for better understanding of evolution of ORs in Coleopterans. Expanding the investigation of OR orthologues amongst more beetle species would provide more precise insight into their ancestral relations. This includes for example the orthologues of 2-phenylethanol and GLV receptors present *T. lineatum*. The electrophysiological studies help discovering new active ligands which could be used for tests including both OR deorphanization and behavioural studies. The identification of chemoreceptor genes provides a framework for several novel studies including functional characterisation. Specifically, the function of GRs and IRs in beetles is still completely unknown and because several GRs and IRs are conserved across beetles, it would be intriguing to find out their function. The functional characterisation of receptors in this thesis opens up a promising field of applications such as the development of biosensors and the search for antagonists that may interfere with chemical communication of pest species. Hence, this study also provides a window help develop better semiochemical-based management strategies for these pest insects.

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List of papers

I. Roberts R. E., Biswas T., Yuvaraj J. K., Grosse-Wilde E., Powell D., Hansson B. S., Löfstedt C., & Andersson M. N. (2022). Odorant receptor orthologues in conifer-feeding beetles display conserved responses to ecologically relevant odours. *Molecular ecology*, 31(13), 3693-3707.

II. Biswas T., Sims C., Yuvaraj J. K., Roberts R. E., Löfstedt C., & Andersson M. N. Functional characterisation supports multiple evolutionary origins of pheromone receptors in bark beetles. *Manuscript*.

III. Biswas T., Yuvaraj J. K., Hansson B. S., Löfstedt C., Anderbrant O., & Andersson M. N. (2023). Characterization of olfactory sensory neurons in the striped ambrosia beetle *Trypodendron lineatum*. *Frontiers in Physiology*, 14, 1155129.

IV. Biswas T., Vogel H., Beidermann P., Lehenberger M., & Andersson M. N. Reduced chemoreceptor gene repertoires in the ambrosia beetle *Trypodendron lineatum* may reflect its specialized ecology. *Manuscript*.

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