Evolution of Olfaction in Bark Beetles and Termites:

Gene families and odorant receptor characterization

by

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Doctor of Philosophy

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Objectives of thesis

The current research explores the evolution of olfaction in bark beetles (Coleoptera: Curculionidae: Scolytinae) and termites (Blattodea: Isoptera), two wood-boring insects having evolutionary conservation in pheromone composition and differ in eusociality. Both critically impact forests and wood structures as ecosystem engineers and use volatile cues of remarkable diversity for their survival and host selection (Biedermann et al., 2019; Bordereau and Pasteels, 2010). (Biedermann et al., 2019). In bark beetles, a rapid 'switching on and switching off' of specific aggregation pheromone components was observed across Scolytinae (Symonds and Elgar, 2004, 2008; Raffa et al., 2016). Similarly, in termites, a shift from branched primary alcohols and aldehydes to unsaturated primary alcohols was observed in trail-following pheromones from basal to derived lineages (Bordereau and Pasteels, 2010). On the chemoreception side, the evolution of olfaction is also a key research area (Hansson and Stensmyr, 2011; Hansson and Wicher, 2016; Thoma et al., 2019). As changes in pheromone composition were highly conserved across species, I hypothesize rapid heritable changes in their olfactory perception at the molecular level, i.e., the expression and function of odorant receptors.

As the current knowledge of chemosensory gene families in genus Ips is limited to I. typographus, similar to the understudied order Blattodea, the first objective of this research was aimed at the identification of chemosensory gene families and expand the gene trees (Objective 1). This was a prerequisite for studying the functional evolution of odorant receptors. Three species from the Ips genus (including the available I. typographus) and three from the Blattodea order were used for generating antennal transcriptomes, followed by gene annotations and phylogenetic analysis. I hypothesize a multi-step origin of odorant receptors in both bark beetle and termite odorant receptors and used phylogenetic analysis to test their monophyletic or polyphyletic origin (Objective 2).

Further, receptors with minimal sequence variations were selected for functional characterization to address the hypothesized rapid/adaptive changes in odorant receptors (Objective 3). Here, the aim is to understand the molecular basis of olfactory adaptations as random genetic variations or standing genetic polymorphisms.

The results from the functional characterization studies could indicate the functional evolution of odorant receptors in terms of pheromone diversity and evolution. This is one of the promising research areas, and the current research contributes to that (Objective 4).

Finally, for developing practical pest management solutions, the functional studies were narrowed down to the amino acid level, where key amino acids in the ligand binding site of the receptors were identified (Objective 5). Here the minimal sequence variations that are required for functional changes in odorant receptors were evaluated.
The following objectives were designed to address these hypotheses.
1. Antennal transcriptome analysis and identification of chemosensory gene families using next-generation sequencing technologies
2. Phylogenetic analysis of olfactory-related genes in bark beetles and termites.
3. Analysis of variations in odorant receptors
4. Functional characterization of selected ORs using the transgenic expression in Drosophila empty-neuron system.
5. Analysis of ligand binding sites for future applications in pest management

Methodology

The current research methodology uses cutting-edge research methods in the fields of transcriptomics, population genomics, molecular biology, fly genetics, evolution, bioinformatics, electrophysiology, and behavioral assays to understand the evolution of olfaction in bark beetles and termites at the molecular and physiological levels. The research falls under the evolutionary theme in insect olfaction with the aim of understanding the ecological adaptations occurring in the bark beetle and termites in response to the rapid changes in their pheromone diversity. The methodology was designed to address the key objectives of this study, i.e., 1) extending the current knowledge on chemosensory gene families in Ips genus and termites; 2) exploring the olfactory adaptations in selected insects by studying the functional evolution of receptors.

The study organisms were,

Bark beetles (Coleoptera: Scolytinae): Ips typographus, Ips duplicatus and Ips acuminatus

Termites: Neotermes cubanus (Kalotermitidae), Prorhinotermes simplex (Rhinotermitidae) and Inquilinitermes inquilinus (Termitidae)

The methodology, in general, includes the following techniques

1) Antennal transcriptome sequencing and annotation of chemosensory gene families

Insect collection and antennal tissue dissection, RNA extraction and sequencing, Transcriptome assembly and gene annotation

2) Phylogenetic analyses to study the gene family evolution

Sequence retrieval and multiple sequence alignments, Prediction of amino acid substitution models, Phylogenetic tree reconstruction and visualization and Classification of chemosensory gene families.

3) Functional characterization of odorant receptors

Insect collection, RNA extraction, and cDNA synthesis, Phylogenetic analysis for the selection of candidates, PCR amplification, Gateway cloning, and LR recombination of ORs: Identification of variants, Population genomics for the confirmation of variant, Drosophila embryo injection and transgenic expression of ORs, Single sensillum recordings

4) in silico approaches to study the variations at ligand binding sites

Protein structural predictions and molecular docking, Binding site analysis

5) Evaluating the significane of identified receptor and ligands: Expression quantification by RT-qPCR, Behavioural assays and Electroantennography recordings.

The proposed extent of the thesis

100 SP

Keywords

bark beetles, termites, olfactory adaptation, functional evolution, pheromone receptors, forest pest, functional polymorphisms

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Recommended information sources

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in Bark Beetles and Termites:

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by

Jibin Johny

Dedication

To Almighty God

To my parents, family, and friends

To my teachers

To the scientific community

Certificate of Authorship

I hereby declare that this dissertation, titled "*Evolution of Olfaction in Bark Beetles and Termites: Gene families and odorant receptor characterization*", was created independently and ethically. I declare that all the information sources and literature have been indicated accordingly, and the Thesis was prepared under the direct supervision of my scientific supervisors.

I agree with the disclosure of this Ph.D. Thesis according to Czech Law (Act No. 111/1998 Coll. Sb.) regardless of the Defence of Thesis results.

Jibin Johny

30th September 2024
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Abstract

Evolution is the central theme of biology, representing organismal variations in various contexts, from the molecular level to social behavior. The current research explores the variations in the context of the sense of smell, i.e., the evolution of olfaction in two wood-boring insects, bark beetles (Coleoptera: Curculionidae: Scolytinae) and termites (Blattodea: Isoptera). Both have a critical impact on forests and wood structures (Biedermann et al., 2019; Bordereau and Pasteels, 2010) as engineers of the ecosystem and use volatile cues of remarkable diversity for their survival and host selection (Biedermann et al., 2019). In bark beetles, a rapid 'switching on and switching off' of specific aggregation pheromone components was observed across Scolytinae (Symonds and Elgar, 2004, 2008; Raffa et al., 2016). Similarly, in termites, a shift from branched primary alcohols and aldehydes to unsaturated primary alcohols was observed in trail-following pheromones from basal to derived lineages (Bordereau and Pasteels, 2010).

As such changes in pheromone composition were highly conserved across species, the current research hypothesizes rapid heritable changes in their olfactory perception at the molecular level, i.e., in the expression and function of odorant receptors (Hansson and Stensmyr, 2011; Hansson and Wicher, 2016; Thoma et al., 2019). To test this hypothesis, the current knowledge on bark beetle and termite chemosensory genes was extended by identifying the same from three termites and two bark beetle species. The gene families were identified by using antennal transcriptome sequencing and annotations. The phylogenetic analysis revealed the evolution and diversification of these gene families. The Drosophila transgenic expression system was used for the functional characterization of odorant receptors. The study reports the multi-step origin of odorant receptors in insects and highlights the standing genetic variations in bark beetle odorant receptors as a possible source of olfactory adaptation. Further, the research reports population-level functional polymorphism in insects other than Drosophila by characterizing two variants of a bark beetle pheromone receptor. The

structural predictions conclude that mutations outside the ligand binding sites could alter the ligand selectivity of the receptor. Further research is needed to extend the identified olfactory adaptations at the molecular level to population and behavioral levels.

Keywords: bark beetles, termites, olfactory adaptation, functional evolution, pheromone receptors, forest pest, functional polymorphisms

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List of Publications

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Included in the dissertation:

1. **Johny, J.,** Große-Wilde, E., Kalinová, B., Roy, A., 2024. Antennal Transcriptome Screening and Identification of Chemosensory Proteins in the Double-Spine European Spruce Bark Beetle, *Ips duplicatus* (Coleoptera: Scolytinae). *Int. J. Mol. Sci.* 25, 9513. doi:10.3390/ijms25179513
2. **Johny, J.,** Große-Wilde, E., Bláha. J., Modlinger, R., Synek, J., Kalinová, B., Roy, A., 2024. Antennal Transcriptome Analysis and Identification of Chemosensory Gene Families in *Ips acuminatus*. *For. Ecosyst.* (in submission)
3. **Johny, J.**, Diallo, S., Lukšan, O., Shewale, M., Kalinová, B., Hanus, R., Große-Wilde, E., 2023. Conserved orthology in termite chemosensory gene families. *Front. Ecol. Evol.* 10. doi:10.3389/fevo.2022.1065947
4. **Johny, J.,** Diallo, S., Nadachowska-Brzyska K., Moliterno, A.A.C., Roy, A., Kalinová, B., Große-Wilde, E., Schlyter, F. 2024. Olfactory adaptations in bark beetle populations: Functional polymorphisms in *Ips typographus* pheromone receptor, ItypOR33. *Mol. Ecol.* (submitted)
5. Diallo, S., Kašparová, K., Šulc, J., **Johny, J.,** Křivánek, J., Nebesářová, J., Sillam-Dussès, D., Kyjaková, P., Vondrášek, J., Machara, A., Lukšan, O., Grosse-Wilde, E., Hanus, R., 2024. Identification of the trail-following pheromone receptor in termites. *bioRxiv* 2024.07.24.605012. doi:10.1101/2024.07.24.605012 (submitted)

Authors contribution to the listed publications

1. The study was conceived by J.J. and B.K.; J.J. performed the RNA extraction, assembly, annotation, and bioinformatics analysis, and wrote the first draft; E.G.-W. guided the assembly and annotation; B.K. and A.R. provided the resources and supervision; J.J. was responsible for conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, funding acquisition, writing—original draft, writing—review and editing, and project administration; E.G.-W. was responsible for conceptualization, methodology, validation, and supervision; B.K. was responsible for conceptualization, resources, and supervision; A.R. was responsible for supervision, validation, resources, writing—review and editing. All authors have read and agreed to the published version of the manuscript.
2. The study was conceived by J.J. B.K. and A.R.; R.M., J.S. and J.B. performed the insect collection; J.J. performed the RNA extraction, assembly, annotation, and bioinformatics analysis, and wrote the first draft; E.G.-W. guided the assembly and annotation; B.K. and A.R. provided the resources and supervision; J.J. was responsible for conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, funding acquisition, writing—original draft, writing—review and editing, and project administration; E.G.-W. was responsible for conceptualization, methodology, validation, and supervision; B.K. was responsible for conceptualization, resources, and supervision; R.M., J.S and J.B. provided the resources; A.R. was responsible for supervision, validation, resources, writing—review and editing. All authors have read and agreed to the published version of the manuscript.
3. OL and RH performed initial sequencing. Bioinformatic analysis was performed mainly by JJ, with assistance from SD, OL, MS, and EG-W. The study was conceived by RH and EG-W. Data interpretation and

manuscript drafting were shared by all authors. All authors contributed to the article and approved the submitted version.

4. J.J.: conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, resources, funding acquisition, writing-original draft, writing-review and editing; S.D.: formal analysis, investigation, resources, visualization, writing-review and editing; K.N.B.: population genomic data and formal analysis; A.A.C.M.: formal analysis, investigation, writing-review, and editing; A.R.: resources, supervision, writing-review and editing; B.K.: conceptualization, resources, validation, supervision; E.G.W.: conceptualization, methodology, validation, supervision, writing-review and editing, project administration; F.S.: conceptualization, methodology, validation, project administration, funding acquisition, writing-review, and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.
5. JJ – RNA extraction, cloning and vector constructions, fly transgenesis; SD – SSR, fly transgenesis; OL – bioinformatics; KK – EAG; JK – insect culture, SEM; JN – SEM, HR-SEM; DSD – advising; PK – chemical analysis; JV, JS' – molecular dynamics; RH, EGW, OL, SD – conception, supervision, statistics, writing. All authors contributed to the manuscript writing and approved its final version.

Abbreviations

ABP	Antennal binding protein
ACN	Genbank accession number
AF	Antenna (female)
AIC	Akaike information criterion
AM	Antenna (male)
AMPA	Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BIC	Bayesian information criterion
BLAST	Basic Local Alignment Search Tool
BUSCO	Benchmarking universal single-copy orthologs
CD	Conserved domains
CRLBP	Chemical-sense-related lipophilic-ligand-binding protein
CSP	Chemosensory protein
cV	*cis*-verbenol
DNS	Drosophila empty-neuron system
d_N	non-synonymous substitutions
d_S	synonymous substitutions
EAG	Electroantennography
EM	*E*-myrcenol
GC-MS	Gas chromatography-mass spectrometry
GLV	Green leaf volatiles
GR/Gr	Gustatory receptor
GW	Gateway
HEK	Human embryonic kidney cells
Hex	Hexane
IAC	*Ips acuminatus*
ID	Ipsdienol
IDUP	*Ips duplicatus*
iGluR	Ionotropic glutamate receptor
ipTM	Interface predicted template modelling

IR	Ionotropic receptor
IUPAC	International Union of Pure and Applied Chemistry
MB	2-methyl-3-buten-2-ol
MEME	Mixed effects model of evolution
NCBI	National Center for Biotechnology Information
NMDA	N-methyl-D-aspartate
nr	non-redundant
OBP	Odorant binding protein
OR	Odorant receptor
ORCo/Orco	Odorant receptor co-receptor
ORF	Open reading frame
OSN	Olfactory sensory neuron
PBP	Pheromone binding protein
PCR	Polymerase chain reaction
Phe	Pheromone
pLDDT	Predicted local-distance difference test
pTM	Predicted template modeling
pUAST	P element-based vector for Gal4-regulated expression of genes in Drosophila
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SNMP	Sensory neuron membrane protein
SNP	Single nucleotide polymorphism
SPP	Sex-pairing pheromones
SRA	Sequence read archive
SSR	Single sensillum recording
TFP	Trail-following pheromones
TM	Transmembrane
TOPO	Topoisomerase-based cloning
TPM	Transcripts per million
UAS	Upstream activation sequence
VCF	Variant call format

VOCs	Volatile organic compounds

Symbols

ω	The ratio of non-synonymous to synonymous substitutions (d_N/d_S)

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Introduction

Evolution is the central theme of biology. It represents the change in the current context in the form and behavior of organisms at the molecular level. The change or variation can be observed at all levels, from DNA sequences to macroscopic morphology and social behavior. The remarkable advances in molecular biology in recent years have made it possible to understand how diverse organisms have evolved from such monotonously similar materials accumulating adaptive variations. Ultimately, organic diversity has evolved in response to the diversity of the environment on the planet Earth, allowing us to explore how the variations at the DNA level might have contributed towards species diversity and adaptations, particularly olfactory adaptations. Here, in the context of the forest ecosystem, two key players that have a critical impact on it were selected for the current research. Beetles represent almost one-fourth of all described species, with an outstanding species richness associated with extreme morphological, ecological, and behavioral diversity (Hunt et al., 2007). Within beetles, bark beetles gain our particular interest due to their socioeconomic impacts on the forest ecosystem, indirectly impacting overall human well-being (Huang et al., 2020). Termites, on the other hand, are ecosystem engineers with high economic importance. Forest ecosystems represent 30% of the Earth's land surface and make an excellent model for evolutionary ecology, acting as a pool of variation. On the other hand, beetles and termites, with species richness attributed to the diversification in various niches, stand out as excellent models for understanding how organisms cope with this diversity in response to the environment. Within the broad aspects of diversity in the forest, bark beetles, and termites, the current research focuses on one of the key common elements, *i.e.,* semiochemical diversity and complementing diversity and evolution of the olfactory system in bark beetles and termites. The semiochemical diversity and the evolutionary aspects of both insect groups are well-reported, whereas olfactory perception is largely understudied. The current research aims to understand how these insects cope with the rapid changes in semiochemical diversity at the olfactory perception

level, mainly focusing on odorant receptors, the key protein that detects signals from the external environment (Biedermann et al., 2019; Mitchell et al., 2020; Netherer et al., 2021; Schiebe et al., 2011; Symonds and Elgar, 2004).

Recent advances in genomic and transcriptomic approaches enable us to identify the olfactory receptors and their expression, which opened new avenues to understand how variation in DNA at the molecular level leads to changes in social behavior. Identification and functional characterization of highly similar receptors within species, or orthologs that are diverging functionally, are considered the best candidates for studying the functional evolution of olfaction in insects and the current scenario in bark beetles and termites (Benton, 2015). The termites and bark beetles are strong candidates to study this evolutionary aspect, with their shared ecosystems, well-studied pheromone diversity, and expected complexity in olfactory communication regarding eusociality. However, the lack of functionally characterized receptors makes such predictions on functional evolution difficult in termites. Moreover, there is a significant gap in our knowledge of chemosensory gene families in both bark beetles and termites. Antennal transcriptome analysis was used to identify the olfactory receptors, and phylogenetic analysis was performed to explore the evolution of chemosensory gene families. Using a well-established heterologous expression system, the research examines the functional differences in receptors carrying minimal sequence variations. While termites and bark beetle pheromone receptors are characterized in this research, most focus is given to the major forest pest, *Ips typographus*, with the identification of population-level variants of a pheromone receptor in this study. Together, the research aims to fill the gap in our understanding of insect olfactory adaptations.

Bark beetles (Coleoptera: Curculionidae, Scolytinae) are distributed worldwide, and one of the native European species, *I. typographus*, is a major pest of Norway spruce (*Picea abies*) forests of Eurasia (Biedermann et al., 2019; Yoritsune and Aonuma, 2012). As a natural part of the conifer forest ecosystems, bark beetles rely on damaged trees but switch to healthy trees

during the outbreak phase (Biedermann et al., 2019). The severity of such outbreaks, fueled by recent climate changes, has led to an annual loss of 18 million m^3 of wood in the Czech Republic and 40 million m3 of wood p.a. in Europe (Hlásny et al., 2021). One of the intriguing questions in understanding bark beetle outbreaks and management is how they adapt to different environments, hosts, and odor spaces. Most phytophagous insects have adaptive potential in populations (Fry, 1996), mediated mainly by chemosensory systems (Yohe and Brand, 2018). Local adaptations such as genetic shifts have proved more effective in host selection than short-term phenotypic plasticity (Olazcuaga et al., 2022). As rapid evolution contributes to a quick adaptation in Drosophila populations (Thompson, 1998), what remains unclear is whether the adaptive potential is driven by standing genetic variations in populations or acquired by new mutations. Interestingly, standing genetic variations are favorable for sensory adaptation within populations, as per the sensory drive hypothesis (Barrett and Schluter, 2008). Further, high genetic diversity has been reported in bark beetle populations (Ellerstrand et al., 2022; Müller et al., 2022). However, no attempts were made to explore sensory adaptations at the population or molecular level. Although species-level olfactory plasticity has been reported in insects (Anton and Rössler, 2021; Zhao and McBride, 2020), population-level plasticity has been reported only in Drosophila (Crowley-Gall et al., 2016; Shaw et al., 2021). Therefore, the search and reporting of olfactory polymorphisms within populations drive new dimensions to our understanding of evolution at the population level.

Termites, on the other hand, are eusocial insects with remarkable pheromone diversity and have acquired many favorable traits in the course of their evolution from lower to higher termites (Bagnères and Hanus, 2015; Chouvenc et al., 2021). The ubiquitous use of trail-following pheromones (TFPs) in termites across the phylogeny indicates the conservation and shift in TFP components, mainly from the branched aldehydes and alcohols in lower termites to unbranched unsaturated C12 fatty alcohols towards higher termites

(Bordereau and Pasteels, 2010; Hanus et al., 2012, 2009). Apart from the chemosensory genes identified in a few species, an evolutionary perspective of termite olfaction is understudied.

At the molecular level, odorants are detected by specific olfactory receptors (ORs) located on the dendrites of the olfactory sensory neurons (OSNs). Generally, each insect OSN expresses only one type of OR, along with the broadly expressed odorant receptor co-receptor (Orco) (Mika and Benton, 2021). ORs have an inverted seven-transmembrane topology compared to the G protein-coupled receptors found in vertebrates (Benton et al., 2007). Generally, in insects, OR and Orco (1:3 ratio) form a heterotetrameric complex of ligand-gated ion channels in the OSN membrane (Wang et al., 2024; Zhao et al., 2024). Variations exist in this ratio as ion channels with homotetrameric OR complexes without Orco have also been found in insects (del Mármol et al., 2021). As a multigene family protein, insect ORs are believed to have evolved from gustatory receptors following a 'birth and death' evolution model, resulting in repertoire sizes ranging from 62 to ~400 (Brand et al., 2018; Hallem and Carlson, 2006; Missbach et al., 2014). The lineage-specific OR expansions fueled by adaptive evolution reported in several insect species resulted in OR sequence similarity as low as ~20% across species (del Mármol et al., 2021). Interestingly, population-level OR variations have been reported in mammals, with very few cases of functional correlations (Keller et al., 2007; Mainland et al., 2014). However, in insects, such population-level OR functional variations are understudied, except for Drosophila (Pellegrino et al., 2011).

In *I. typographus,* ORs have been identified (Yuvaraj et al., 2021) and classified into seven major subfamilies (Mitchell et al., 2020). However, an ItypOR clade that detects structurally similar compounds indicates functional divergence with no signs of positive selection (Hou et al., 2021; Yuvaraj et al., 2021). Interestingly, more than half of ItypORs are found in chromosomal polymorphic inversions (Mykhailenko et al., 2023). Inversion polymorphisms generally indicate signs of adaptation, usually harboring essential genes for intraspecific divergence and speciation (Faria et al., 2019); such

polymorphisms are highly relevant in species with high gene flow, like *I. typographus* (Müller et al., 2022). In the current study, we focus on *ItypOR33*, one of the highly expressed ORs found in the largest inversion, Inv5 (Mykhailenko et al., 2023). We performed a population-level analysis followed by functional characterization to understand the potential role of this receptor in olfactory adaptation in bark beetle *I. typographus.* With the well-reported sex and aggregation pheromones in more than 34 species of bark beetles (Symonds & Gitau-Clarke, 2016) and trail-following pheromones known from 68 termite species, both selected insect groups provide an opportunity to explore the olfactory perception at the molecular level.

The literature review provides more details on the pheromone diversity and the observed evolutionary patterns in the pheromone composition of both insect groups. The current status of odorant receptor characterization in Coleoptera is provided as a table. Following the review, the general methodology provides an overview of methods used in this research, with key objectives being the identification of odorant receptors and functional characterization. The methodology was elaborated with specific datasets and research designs used in each experimental design.

The four major findings were reported and discussed in the four chapters of 'Results' and 'Discussion.' Chapters 1 and 2 address the chemosensory gene families identified, and Chapters 3 and 4 address the functional characterization of odorant receptors. As the functional studies are in progress for both insect groups, the thesis mainly focuses on the most critical forest pest, *Ips typographus,* due to an exciting finding of population-level functional polymorphisms in pheromone receptors. The four chapters, however, discuss the main objectives of this research, and the 'Conclusion' provides the key findings of this study, including the hypothesis tested and the future preceptive.

Objective and hypothesis

The current research explores the evolution of olfaction in bark beetles (Coleoptera: Curculionidae: Scolytinae) and termites (Blattodea: Isoptera), two wood-boring insects having evolutionary conservation in pheromone composition and differ in eusociality. Both critically impact forests and wood structures as ecosystem engineers and use volatile cues of remarkable diversity for their survival and host selection (Biedermann et al., 2019; Bordereau and Pasteels, 2010). (Biedermann et al., 2019). In bark beetles, a rapid 'switching on and switching off' of specific aggregation pheromone components was observed across Scolytinae (Symonds and Elgar, 2004, 2008; Raffa et al., 2016). Similarly, in termites, a shift from branched primary alcohols and aldehydes to unsaturated primary alcohols was observed in trail-following pheromones from basal to derived lineages (Bordereau and Pasteels, 2010). On the chemoreception side, the evolution of olfaction is also a key research area (Hansson and Stensmyr, 2011; Hansson and Wicher, 2016; Thoma et al., 2019). As changes in pheromone composition were highly conserved across species, I hypothesize **rapid heritable changes in their olfactory perception at the molecular level, i.e., the expression and function of odorant receptors**.

As the current knowledge of chemosensory gene families in genus Ips is limited to *I. typographus*, similar to the understudied order Blattodea, the first objective of this research was aimed at the identification of chemosensory gene families and expand the gene trees (Objective 1). This was a prerequisite for studying the functional evolution of odorant receptors. Three species from the *Ips* genus (including the available *I. typographus*) and three from the Blattodea order were used for generating antennal transcriptomes, followed by gene annotations and phylogenetic analysis. I hypothesize a multi-step origin of odorant receptors in both bark beetle and termite odorant receptors (Hypothesis 1) and used phylogenetic analysis to test their monophyletic or polyphyletic origin (Objective 2).

Further, receptors with minimal sequence variations were selected for functional characterization to address the hypothesized rapid/adaptive changes in odorant receptors (Objective 3). Here, the aim is to understand the molecular basis of olfactory adaptations as random genetic variations or standing genetic polymorphisms (Hypothesis 2). The factors contributing the most to olfactory adaptations were evaluated regarding standing genetic variations.

The results from the functional characterization studies could indicate the functional evolution of odorant receptors in terms of pheromone diversity and evolution (Hypothesis 3). This is one of the promising research areas, and the current research contributes to that (Objective 4).

Finally, for developing practical pest management solutions, the functional studies were narrowed down to the amino acid level, where key amino acids in the ligand binding site of the receptors were identified (Objective 5). I hypothesize minimal sequence variations are required for functional changes in odorant receptors (Hypothesis 4)

The following objectives were designed to address these hypotheses.

1. Antennal transcriptome analysis and identification of chemosensory gene families using next-generation sequencing technologies
2. Phylogenetic analysis of olfactory-related genes in bark beetles and termites.
3. Analysis of variations in odorant receptors
4. Functional characterization of selected ORs using the transgenic expression in Drosophila empty-neuron system.
5. Analysis of ligand binding sites for future applications in pest management

Literature Review

'Nothing in biology makes sense except in the light of evolution.'

-Dobzhansky, 1973

Evolution is the central theme of biology; it represents change or variation in different contexts caused mainly by adaptive multi-tropic interactions. One of the severe factors that contribute to the rapid changes in the ecosystem is anthropogenic interactions, with no exception to forest ecosystems (McDowell et al., 2020). As a result, severe direct and indirect damages in the form of drought, fire, insect outbreaks, and various tree diseases have been reported in forests (McDowell et al., 2020). The exponential rise in invasive species is one of the key monitoring factors that explain this variation in the ecosystem (Hansen et al., 2013; Huang et al., 2020; Trumbore et al., 2015). For insects, which depend heavily on environmental and chemical cues for survival, these changes are lethal unless they adapt to the environmental changes, mainly by olfactory adaptation (Boullis et al., 2016; Hughes, 2000).

With clear diversity and complexity in pheromone communication and eusociality, bark beetles and termites provide distinguishable differences in their communication and serve as excellent candidates for a comparative study on olfaction. Beetles represent almost one-fourth of all described species, with an outstanding species richness associated with extreme morphological, ecological, and behavioral diversity (Hunt et al., 2007). In contrast, termites provide an excellent comparison with equal diversity and additional eusociality. Both have a critical impact on forests and wood structures (Biedermann et al., 2019; Bordereau and Pasteels, 2010) as ecosystem engineers and use volatile cues of remarkable diversity for their survival and host selection (Biedermann et al., 2019; Bordereau and Pasteels, 2010).

The current review will, therefore, focus on two aspects: first, the well-studied volatile profiles and pheromone diversity in bark beetles and termites, and second, the understudied olfactory perception and functional evolution of olfaction in bark beetles and termites. The first part of the review deals with

the impact of bark beetle outbreaks on forest ecosystems, bark beetle chemical ecology, and the management and evolution of pheromone diversity. Once the evolutionary aspects of pheromone diversity are defined, the second part addresses the complementary olfactory perception explaining the role of olfaction in insects, followed by the molecular basis of olfaction, the role of odorant receptors, the evolution of chemosensory proteins, the current state of bark beetles odorant receptor studies including the research gaps and advantages and limitations of current odorant receptor characterization methods. Although the basic theme of this research addresses the evolutionary adaptations at the molecular level, the specific theme of forest ecosystems and bark beetle and termite olfaction enables us to explore the applications of this research to save ecosystems. One of the key applications of pheromone receptor research is the development of olfaction-based pest control or early monitoring strategies, like biosensors, which have been applied in the agriculture sector. Ultimately, this research will support the development of innovative olfaction-based pest control strategies against bark beetles and termites, mainly aimed at early detection of bark beetle infestations in forests. Moreover, understanding the olfactory adaptations in species with a critical impact on ecosystems will aid in our understanding of evolving forest ecosystems in the Anthropocene era.

Impact of bark beetle outbreaks on forest ecosystem

Forests, a vital part of the ecosystem, are key in maintaining and regulating climate and biodiversity. Increased anthropogenic interactions in recent years have caused a direct and indirect impact on our ecosystem, and forests, being the most affected, are facing damages such as drought, fire, insect outbreaks, and various tree diseases (McDowell et al., 2020). Currently, the anthropogenic stressors have reached a point where forest health is under deep threat. The exponential rise in invasive species is a key monitoring factor that explains this phenomenon (Hansen et al., 2013; Huang et al., 2020; Trumbore et al., 2015). Within Europe, canopy mortality has doubled in temperate forests at an alarming rate of +2.40% per year, with 1.17% per year

in Czechia and 4.14% per year in Slovakia (Senf et al., 2018) as monitored in the last three decades. Although forests are evolving constantly and have natural adaptive strategies against disturbances, the current rate is far too high for the ecosystem to manage (McDowell et al., 2020). Thus, the future of the forest ecosystem depends on how quickly they manage the adaptations to the stressors at global and local scales (Trumbore et al., 2015). Recent comparisons in tree mortality rates between managed and unmanaged forest ecosystems further conclude the need for better forest management and innovative strategies (Senf et al., 2018). Although insects are a natural part of the forest ecosystem, recent reports highlight that 8% of all European tree mortality was caused by native European spruce bark beetle, *Ips typographus* (Hlásny et al., 2021; Senf et al., 2018). As a natural part of the forest ecosystem, beetles rely on damaged trees but can switch to healthy hosts during the 'aggressive' outbreak phase (Biedermann et al., 2019). The transition of beetle densities from endemic to irruptive was explored in a recent population ecology study using mountain pine beetles as an example, and the results highlighted the significance of pheromone signaling in aggressive outbreaks (Howe et al., 2022). Within the Czech Republic, such outbreaks fuelled by long dry spells and high temperatures have resulted in an annual loss of 18 million m^3 of wood, accounting for the total loss of 40 million m^3 of wood p.a. in Europe (Biedermann et al., 2019). The need for better control systems is evident from recent research, and the evidence has already created social unrest and political instability in parts of Europe (Hlásny et al., 2021).

Bark beetle ecology and management

Bark beetles have a worldwide distribution and belong to a diverse subfamily of weevils (Coleoptera: Curculionidae, Scolytinae). These are natural parts of the forest ecosystem and play a crucial role in nutrient recycling by using dead trees and tree parts as natural habitats (Huang et al., 2020; Raffa et al., 2015). Olfactory communication plays a ubiquitous role in all insects, and semiochemical diversity and olfaction are important aspects of the biology of scolytine bark beetles (Borden, 1989). Semiochemicals used by these

beetles are unique mixtures of insect or plant origin. Sex sex-specific and aggregation pheromone compounds unique within bark beetles have been identified early on and used in various pest control strategies (Vité et al., 1972). Considerable importance has been given to the aggregation pheromone research as the species-specific cues determining successful mass attacks and colonization of healthy trees (Biedermann et al., 2019; Gitau et al., 2013). It is not unusual that breaking the tree defense or successful colonization of bark beetles may take a few generations (Kausrud et al., 2012). In most cases, additional colonization support was provided by fungi, which in this case is also carried by beetles (Aukema et al., 2010; Raffa et al., 2015). In such secondary attacks, the ethanol produced from the decaying host trees will serve as a kairomone (Gilbert et al., 2001). Within the Ips species, we find both aggressive or primary bark beetles like *Ips typographus* belong to the group of primary beetles, and secondary bark beetles like *I. amitinus* (in Europe) and *Pityogenes chalcographus* (in Europe) and *I. pini* (in North America) which are considered as non-aggressive or semi-aggressive species (Byers, 1989). Both types use different adaptive strategies and semiochemicals using a unique combination of components in aggregation pheromone. Aggregation pheromone-based mass attack is considered a key adaptation of beetles to colonize and survive in a new habitat or host tree (Raffa et al., 2016). Considering the importance of aggregation pheromones, beetles vary their composition and elicit dose-dependent differences in physiological responses. For example, most pheromones are multifunctional, eliciting aggregation in lower concentrations but repulsion in higher concentrations (Raffa, 2001). This helps the beetles to avoid interspecies competition for mating and foraging at a minimal cost. (Raffa, 2001). A study on bark beetle semiochemical diversity also sheds light on specific cues on long-range and short-range attraction to host trees (Saint-Germain et al., 2007). As successful bark beetle colonization demands subsequent attacks and different generations, the current bark beetle outbreak management focuses on immediately clearing freshly windthrown trees, as well as sanitation logging. Apart from that, two main strategies are trap trees and pheromone traps (Faccoli and Stergulc, 2008; Hlásny et al., 2021;

Resnerová et al., 2020; Stadelmann et al., 2013). However, the increasing number of bark beetle outbreaks suggests the limitations of current unidirectional approaches and the role of natural disturbances in the ecosystem dynamics (Hlásny et al., 2021; Singh et al., 2024). However, using the knowledge of semiochemical diversity in bark beetles is more appropriate to initiate olfactory disruption-based forest pest control strategies.

Semiochemical diversity in bark beetles and termites

The sociochemical composition of aggregation pheromone in Ips spp. consists of three components: ipsenol, ipsdienol, and verbenone, which are major pheromone components (Gitau et al., 2013). It was identified that ipsenol and ipsdienol are released by Ips males proportional to the host tree monoterpene release (Byers and Wood, 1981). Except for *I. amitinus*, most of the Ips species use a host plant volatile myrcene, for the synthesis of pheromone components ipsdienol and ipsenol, whereas in *I. amitinus* myrcene is converted to amitinol, which again acts as a pheromone component (Byers and Wood, 1981). So far, our knowledge of the chemical ecology of these species is limited to economically important pests like *Ips typographus*. In this species, a clear difference in the perception of major and minor host plant volatiles as the former for habitat selection and the latter for the host tree selection (Kalinová et al., 2014; Mustaparta et al., 1984; Netherer et al., 2021).

The aggregation pheromone of *I. typographus* consists of (4S)-*cis*-verbenol and 2-methyl-3-buten-2-ol as active components and has been effectively tested, validated, and used in pheromone traps (Dickens, 1981; Schlyter et al., 1987). The semiochemical diversity in Ips species and the major aggregation pheromone components in this genus were classified as highly conserved major components and less conserved minor components. As per the phylogenetic pattern observed in Ips pheromone composition, (4*S*)-*cis*-verbenol is considered as a common component and 2-methyl-3-buten-2-ol as a rare component (Symonds and Elgar, 2008; Matthew R.E. E Symonds and Elgar, 2004). The other compounds released by beetles, like verbenone, ipsenol,

and ipsdienol, are also considered common pheromone components within the Ips genus but are known to reduce the attraction to the aggregation pheromone (Byers et al., 1988; Schlyter et al., 1987). There are also rare or variable components reported from these species and recently reviewed in detail, like amitinol from *Ips amitinus*, 3-Methyl-3-buten-1-ol from *Ips cembrae,* and *E*-myrcenol from *Ips duplicatus* (Sweden) (Cognato, 2015). Verbenone is another compound beetle produces to reduce intraspecific competition in breeding areas (Allison et al., 2012). Interestingly, as verbenone indicates already occupied hosts, it was explored as an inhibitor in managing Scolytines. Apart from the insect-produced pheromones, the host monoterpenes also infer tunneling behavior in Ips species, as higher concentrations of monoterpenes deter the entry or continued tunneling (Wallin and Raffa, 2002). It was also reported that the bark beetles can detect tree stress physiology cues from root infection, defoliation, and fire injury (Lindgren and Raffa, 2013). More host-derived compounds, such as alpha-pinene and frontalin, are combined with the insect pheromone to coordinate the mass attack on host trees (Byers, 1989; Seybold et al., 2000). It is also reported that tree defense compounds like pine monoterpenes can act as a kairomone for bark beetles (Seybold et al., 2006).

Like bark beetles, the pheromone diversity in termites is one of the well-studied areas in chemical ecology. With the caste-based division of labor, termites use additional intra- or inter-caste chemical communications, with the following trail: pheromone composition from 68 species of termites. With the ubiquitous use of trail-following pheromones across species, termites are excellent candidates for studying pheromone diversity and evolution (Dolejšová et al., 2022; MacHáček et al., 2023; Mitaka and Akino, 2021). A clear pattern of TFP evolution can be observed between the taxonomic groups in termites, as a shift from branched primary alcohols and aldehydes in basal lineages to unsaturated primary alcohols in most derived lineages (Bordereau and Pasteels, 2010). The basal lineages were found to be carrying (*E*)-2,6,10-trimethyl-5,9-undecadien-1-ol (TMU), 4,6-dimethyldodecanal (DMD), and 4,6-dimethylundecan-1-ol (DMU) as TFPs; whereas such branched aldehydes and

alcohols disappear and unbranched unsaturated C12 fatty alcohols like (*3Z*)-dodec-3-en-1-ol (DE), (*3Z,6Z*)-dodeca-3,6-dien-1-ol (DDE), and (*3Z,6Z,8E*)-dodeca-3,6,8-trien-1-ol (DTE) appeared as TFPs in most derived termite lineages (Bordereau and Pasteels, 2010).

Evolution of pheromone diversity in bark beetles

Changes in pheromone composition, either as additional or removal of major or minor components or the ratio of these components, could gradually lead to speciation as the sexual attractants are highly species-specific (Symonds and Elgar, 2008). However, these changes will be under strong selection pressure within the genus, as in the case of Ips, as the changes directly affect the behavioral response, like mismating avoidance (Gröning and Hochkirch, 2008). The literature-based analysis has already provided such 'saltational' changes in pheromone composition between closely related Ips species (Matthew R. E. Symonds and Elgar, 2004). As explained earlier, in Ips, the major pheromone components are ipsenol, ipsdienol and cis-verbenol (Cognato, 2015) and the minor or rare components include amitinol (itself closely related to ipsdienol), and 2-methyl- 3-buten-2-ol, lanierone; and *E*-myrcenol is solely used by *I. duplicatus* (Symonds & Gitau-Clarke, 2016). As these rapid changes in pheromone compositions are positively selected, and the Ips have well-defined aggregation pheromone components, it is interesting to study how insects cope with these changes at the reception level, which ultimately explains the behavioral impact of these variations. The current focus on Ips and termites and the broad-scale evolutionary pheromone composition patterns explain that the changes in pheromone composition have been fixed in these insect groups, thus allowing us to hypothesize rapid and heritable changes in the pheromone reception at the molecular level, i.e., at the odorant receptors, the key proteins that detect the external stimuli like pheromones in insects.

Olfactory communication in insects

Insects live in complex odor spaces and use chemosensory systems to detect and discriminate thousands of odors. The chemosensory system is essential for ecological adaptability in host plant recognition, mating and survival (Hansson and Stensmyr, 2011). A well-defined chemosensory organ, antennae covered with hair-like olfactory sensilla, underlines the pivotal role of olfaction in insects. Each olfactory sensilla has multiple cuticular pores and houses the olfactory sensory neurons (OSNs) (Schmidt and Benton, 2020). Odorant receptors (ORs) are located on the membrane of OSN's dendrite (Schmidt and Benton, 2020), and axons are projected to the antennal lobe, the primary olfaction center in the insect brain. Neurons expressing the same olfactory receptor converge onto specific glomeruli, synapsing with projection neurons that carry sensory information to higher brain centers like mushroom bodies (Grabe and Sachse, 2018; Schlegel et al., 2021).

Molecular basis of olfaction

The odor molecules enter the sensillum lymph through the sensilla pores and initially bind to odorant-binding proteins (OBPs) (Dobritsa et al., 2003; Vogt and Riddiford, 1981), which can transport the odor to the receptors in the dendritic membrane of the OSNs (Antony et al., 2018; Leal, 2013). ORs are seven-transmembrane proteins with an inverted topology compared to the G protein-coupled receptors of vertebrates (Benton et al., 2007). Recent advances in cryo-electron microscopy provided the structure of odorant co-receptors, explaining their role as ligand-gated ion channels in OSNs for signal transduction (Butterwick et al., 2018; del Mármol et al., 2021; Hansson and Stensmyr, 2011; Trona et al., 2010). Once the odorant molecules bind to the specific receptor, the signals are generated and passed to the brain to process the information. Thus, the key protein that determines the specificity and sensitivity of OSN response is the odorant receptors. The tuning specificity of ORs varies based on the physiological relevance of the odor. For example, the odorants constituting intraspecific signals, such as sex pheromone

constituents, generally bind to narrowly tuned ORs, whereas broadly tuned ORs are involved in detecting general odorants (Andersson et al., 2015; Auer et al., 2020; Su et al., 2009). The chemical specificity in odorant detection can go up to the enantiomeric level and is determined by the original architecture of odorant-gated ion channels created by a conserved co-receptor (Orco) and a divergent odorant receptor. The ultrastructural studies of the receptor complex reveal that the sequence conservation of the OR-Orco complex (1:3 ratio) largely accounts for the pore and anchor domain of Orco but not with ORs, highlighting the remarkable sequence diversity of ORs and probably facilitating the evolution of the odor tuning (Butterwick et al., 2018; Johny et al., 2024b; Wang et al., 2024; Zhao et al., 2024).

The peripheral olfactory communication occurring in the sensillar hairs of the insect antenna involves three major processes: odorant reception, signaling, and odorant degradation, each carried out mainly by odorant-binding proteins (OBPs), odorant receptors (ORs) and odorant degrading enzymes (ODEs) respectively (Antony et al., 2018; Breer, 2011; Leal, 2013). Other proteins involved in olfaction are odorant co-receptors (Orco) and sensory neuron membrane proteins (SNMPs) (Benton et al., 2007; Hansson and Stensmyr, 2011; Johny et al., 2024b; Leal, 2013). ORs play a crucial role in the detection and discrimination of odors and elicit specific responses to higher brain centers for a behavioral output. Characterizing each OR and finding their tuning specificities towards multiple odorants are crucial for understanding insect behavior. Specifically, individual odorants can activate specific groups of receptors, while individual receptors can also respond to overlapping groups of odorants. Some receptors broadly respond to many odorants as 'generalists' while others are considered 'specialist' ORs responding to small sets of odorants like pheromones (Carey and Carlson, 2011). Such ORs also show novel interaction with other membrane components, including the SNMPs (Vogt et al., 2009).

Evolution of chemosensory gene families

Odorant receptors are large multigene family proteins capable of detecting various environmental odors. This remarkable diversity in odorant receptors, to a large extent, accounted for the gene duplication events, ultimately leading to the birth of new OR genes (Ramdya and Benton, 2010). This also demands that the duplicated gene is free of selective pressures and maintains a redundant function. Studies from the drosophilids and moths have demonstrated the functional divergence of recently duplicated genes (Guo and Kim, 2007; Li et al., 2023; Robertson et al., 2003). The OR genes mainly exist in tandem arrays in the genome, indicating the role of gene duplication by non-allelic homologous recombination in the OR multi-gene family (Robertson et al., 2003). The functional evolution of these genes through other measures like random mutations cannot be ignored as genes closely related by function were often found at distant sites within the same chromosome or on other chromosomes (Robertson et al., 2003; Sánchez-Gracia et al., 2009). The large repertoires of GRs and ORs in insects thus explain the evolution of these genes, and the presence of pseudogenes explains the selective pressures. In insects, the recent review compares the number of this gene family and specifies ~100 in drosophilids, mosquitoes, and bark beetles *I. typographus* and ~400 in ants (Benton, 2015). In the case of ORs in insects, the evolution of this gene family is clearly defined by the demand for a wide range of chemical recognition specificities. These multigene families are excellent for studying 'reverse-genetic' models where the evolutionary properties– their birth, expansion, and diversification in sequence, expression, and function – can be studied within and between species to relate genetic changes to adaptive phenotypes (Benton, 2015; Robertson et al., 2003). The current study addresses the evolution of pheromone receptors concerning the number of genes, phylogenetic origin, and functional diversification. The evolutionary origin of OR gene family in insects is believed to be from the ancestral gustatory receptor (GR) gene family (Scott et al., 2001). There are different hypotheses on the origin of ORs in insects, one concomitant with the evolution of terrestriality in insects and the

other one as an adaptation to winged flight in insects and most recently as a multi-step origin of these receptors in terms of identified 'primitive ORs' clade (Brand et al., 2018; Robertson et al., 2003; Thoma et al., 2019). Comparative genomics and transcriptomic analyses revealed several related but highly divergent ORs in many insect genomes, and in coleopterans, seven such subfamilies were identified (Andersson et al., 2013; Antony et al., 2016; Gonzalez et al., 2021; Grosse-Wilde et al., 2011; Yuvaraj et al., 2021). However, the available data on the evolution of bark beetles based on the BEAST (Bayesian evolutionary analysis by sampling trees) analyses within the stem group Scolytinae falls within the timeline of 90–115 Ma, and therefore, the predicted timeline of evolution within Ips will be ~40 Ma compared to the emergence time reported from other species (Hulcr et al., 2015). This expansion has been hypothesized to be linked to the strong diversification of pheromones and other behaviourally active volatiles from the host and non-host sources.

ORs as a reverse-genetic model for functional adaptation studies

Chemoreceptor families are excellent models for studying how selection acts over organisms in a changing environment because they show rapid adaptation over short timescales, which seems to be a function of relaxed constraints, as reported in Drosophila ssp. (Arguello et al., 2016). Recently, an olfactory preference shift in *D. sechellia* to hexanoic acid has been reported to be associated with a single amino acid change in the IR75b protein, together with some changes in the promoter and trans-acting loci (Prieto-Godino et al., 2017). This is an example of natural minimal variations occurring at the molecular level acting as an adaptive variation for its specific ecological niche. However, considering ORs and a reverse genetic model for the adaptive functional evolution of proteins demands more experimental validation. Highly similar receptors within the repertoire of a species, or variants of receptors that are diverging functionally between closely related species, provide an excellent solution to address this issue. The advantage of studying ORs within species is that they exhibit minimal sequence variations with possible rapid adaptations at the sequence level to address the pheromone

diversity. Studying sequence variation in insect chemoreceptors over short evolutionary timescales has substantial promise to provide insights into microevolution. Similar studies in drosophilids and *Aedes agypti* mosquitoes have reported ORs with minimal sequence differences and distinct functionality (Dekker et al., 2006; Linz et al., 2013; McBride et al., 2014). In Lepidoptera, the functional evolution of ORs has been reported recently (De Fouchier et al., 2017). The current research aims to identify such functionally important amino acid residues in bark beetle and termite ORs, possibly from the ligand-binding sites of ORs. The rich diversity of species and knowledge of chemical ecology make both insect groups ideal candidates for exploring the functional adaptation of ORs. However, functional characterization studies are very limited due to technical difficulties.

Current status of bark beetle odorant receptor characterization

The sophisticated methods limit the number of OR deorphanizations, as the most widely used methods are *in vitro* expression systems like HEK293 cells and *Xenopus oocytes* (Hou et al., 2021; Roberts et al., 2022; Yuvaraj et al., 2021). Here, we used an *in vivo* expression system, *Drosophila* empty-neuron system (DNS), that offers better true-to-insect odorant detection as transgenic ORs are expressed in an 'empty' OSN in an ab3 antennal sensilla, replacing its native OR (Dobritsa et al., 2003). This method has been efficient in deorphanizing lepidopteran (De Fouchier et al., 2017), dipteran (Carey et al., 2010), and coleopteran ORs (Antony et al., 2024, 2021). Regarding OR functional characterization studies, moths, flies, and mosquitos are on the research frontline (Carey et al., 2010; De Fouchier et al., 2017; Grosse-Wilde et al., 2007; Hughes et al., 2014). So far, in Coleoptera, functional characterization of at least one odorant receptor was performed the following species: *Megacyllene caryae* (Mitchell et al., 2012), *I. typographus* (Hou et al., 2021; Yuvaraj et al., 2021), *Holotrichia parallela* (Wang et al., 2020), *Rhynchophorus ferrugineus* (Antony et al., 2021), *Rhynchophorus palmarum* (Brajon et al., 2024), *Dendroctonus ponderosae* (Roberts et al., 2022) and *Hylobius abietis L.* (Roberts et al., 2022). However, in termites, no ORs have been characterized so far, but in order, Blattodea

pheromone receptors have been reported from *Periplaneta americana* (Li et al., 2024; Tateishi et al., 2024). The complete list of functionally characterized coleopteran ORs and their respective ligands are provided in Table 1.

Table 1. Functionally characterized coleopteran odorant receptors and their respective ligands

Species	OR	Ligands	Method	References
Megacyllene caryae	McarOR3	(S)-2-methylbutan-1-ol	*Xenopus* oocytes	(Mitchell et al., 2012)
Megacyllene caryae	McarOR5	2-phenyl ethanol	*Xenopus* oocytes	(Mitchell et al., 2012)
Megacyllene caryae	McarOR20	(2S,3R)-2,3-hexanediol	*Xenopus* oocytes	(Mitchell et al., 2012)
Ips typographus	ItypOR46	(*S*)-(−)-ipsenol	HEK293 cells; Drosophila empty-neuron system	(Yuvaraj et al., 2021); **Current research**
Ips typographus	ItypOR49	(*R*)-(−)-ipsdienol	HEK293 cells	(Yuvaraj et al., 2021)
Ips typographus	ItypOR29	(+)-Isopinocamphone	*Xenopus* oocytes	(Hou et al., 2021)
Ips typographus	ItypOR23	(+)-trans-4-Thujanol	*Xenopus* oocytes	(Hou et al., 2021)
Ips typographus	ItypOR27	p-Cymene	*Xenopus* oocytes	(Hou et al., 2021)
Ips typographus	ItypOR25	(+)-3-Carene	*Xenopus* oocytes	(Hou et al., 2021)
Ips typographus	ItypOR28	E-Myrcenol	*Xenopus* oocytes	(Hou et al., 2021)
Ips typographus	ItypOR5	Angiosperm green leaf volatiles (GLVs)	*Xenopus* oocytes	(Roberts et al., 2022)
Ips typographus	ItypOR6	2-phenylethanol (2-PE)	*Xenopus* oocytes	(Roberts et al., 2022)
Ips typographus	ItypOR41	(*4S*)-cis-verbenol	*Xenopus* oocytes	(Biswas et al., 2024a)
Ips typographus	ItypOR33a	(S)-(−)-ipsenol	Drosophila empty-neuron system	**Current research**

Ips typographus	ItypOR33b	amitinol	Drosophila empty-neuron system	**Current research**
Dendroctonus ponderosae	DponOR8	2-phenylethanol (2-PE)	*Xenopus* oocytes	(Roberts et al., 2022)
Dendroctonus ponderosae	DponOR9	Angiosperm green leaf volatiles (GLVs)	*Xenopus* oocytes	(Roberts et al., 2022)
Hylobius abietis L.	HabiOR3	2-phenylethanol (2-PE)	*Xenopus* oocytes	(Roberts et al., 2022)
Hylobius abietis L.	HabiOR4	Angiosperm green leaf volatiles (GLVs)	*Xenopus* oocytes	(Roberts et al., 2022)
Rhynchophorus ferrugineus	RferOR1	(4RS, 5RS)-4-methyl nonan-5-ol 4(RS)-methyl nonan-5-one	Drosophila empty-neuron system	(Antony et al., 2021)
Rhynchophorus ferrugineus	RferOR6	α-Pinene	*Xenopus* oocytes	(Ji et al., 2021)
Rhynchophorus palmarum	RpalOR1	Aggregation pheromone and host plant volatiles	Drosophila empty-neuron system	(Brajon et al., 2024)
Holotrichia parallela	HparOR27	Hexanal Lauric Acid Tetradecane	*Xenopus* oocytes	(Wang et al., 2020)

Methodology

The current research methodology uses cutting-edge research methods in the fields of transcriptomics, population genomics, molecular biology, fly genetics, evolution, bioinformatics, electrophysiology, and behavioral assays to understand the evolution of olfaction in bark beetles and termites at the molecular and physiological levels. The research falls under the evolutionary theme in insect olfaction with the aim of understanding the ecological adaptations occurring in the bark beetle and termites in response to the rapid changes in their pheromone diversity. The methodology was designed to address the key objectives of this study, i.e., 1) extending the current knowledge on chemosensory gene families in *Ips* genus and termites; 2) exploring the olfactory adaptations in selected insects by studying the functional evolution of receptors. A representative figure is provided as Figure M1.

The study organisms were,

Bark beetles (Coleoptera: Scolytinae):

1. *Ips typographus*
2. *Ips duplicatus*
3. *Ips acuminatus*

Termites:

1. *Neotermes cubanus* (Kalotermitidae)
2. *Prorhinotermes simplex* (Rhinotermitidae)
3. *Inquilinitermes inquilinus* (Termitidae)

The methodology, in general, includes the following techniques

1) Antennal transcriptome sequencing and annotation of chemosensory gene families
    i. Insect collection and antennal tissue dissection
    ii. RNA extraction and sequencing

iii. Transcriptome assembly and gene annotation

2) Phylogenetic analyses to study the gene family evolution
   i. Sequence retrieval and multiple sequence alignments
   ii. Prediction of amino acid substitution models
   iii. Phylogenetic tree reconstruction and visualization
   iv. Classification of chemosensory gene families.
3) Functional characterization of odorant receptors
   i. Insect collection, RNA extraction, and cDNA synthesis
   ii. Phylogenetic analysis for the selection of candidates
   iii. PCR amplification, Gateway cloning, and LR recombination of ORs:
   iv. Identification of variants.
   v. Population genomics for the confirmation of variants
   vi. *Drosophila* embryo injection and transgenic expression of ORs
   vii. Single sensillum recordings
4) *in silico* approaches to study the variations at ligand binding sites
   i. Protein structural predictions and molecular docking
   ii. Binding site analysis
5) Validation of results in *I. typographus*
   i. Expression quantification by RT-qPCR
   ii. Behavioural assays to interpret the results
   iii. Electroantennography recordings

Generation of antennal transcriptomes

Insect collection and rearing:

Bark Beetles: Norway spruce logs with *I. typographus, I. duplicatus and I. acuminatus* adults were collected from Kostelec nad Cernými lesy (50° 00′ 07.2″ N 14° 50′ 56.3″ E) located in the Central Bohemia region in the Czech Republic. Beetles were reared on Norway spruce logs in the laboratory under conditions:

70% humidity, 24 °C,16:8 h day/night period. Logs were debarked, and adult beetles were collected and stored at 4 °C in collection bottles for sex separation. Cold anesthetized adult beetles were sex separated under a light microscope and stored at 4 °C in collection bottles. For total RNA extraction, antennae were dissected from ~500 cold-anesthetized *I. duplicatus* adults, generating four pools (2x 500 males and 2x 500 females separately) under a light microscope. The dissected antennal pools were stored in RNA*later* (Themo-Fisher Scientific, WA, USA) until extraction.

Termites: We used the following termite species for RNA sequencing and *de novo* assembly of antennal transcriptome: *N. cubanus*, *P. simplex* and *I. inquilinus*. Multiple colonies of *N. cubanus* (Snyder) and *P. simplex* (Hagen), originating from field collections in Cuba, are kept in laboratory at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences. Colonies live in glass vivaria at 27°C and 80% relative humidity in clusters of spruce wood slices. Mature colony of *I. inquilinus* (Emerson) was collected by the authors during the field mission to French Guiana along the Road to Petit Saut (N05 03.975 W053 02.764) in 2019 with the consent of Office National des Forêts (Cayenne). Ninety workers from one colony per species were cold-anesthetized, quickly washed in cold ethanol and decapitated under a stereomicroscope. Heads were transferred into RNase free collection tubes and kept at 4°C overnight in 1 ml of RNA*later* (Thermo Fisher Scientific). Antennae were dissected the next day, collected in a droplet of 96% ethanol, snap-frozen, and stored at -80°C until RNA extraction.

RNA extraction and sequencing

Bark Beetles: Total RNA was extracted from the four pools of ~500 adult beetle antennae using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) as described earlier (Antony et al., 2021). In brief, each dissected pool of antennae was freeze-dried using liquid nitrogen and ground using a pestle and mortar maintained at low temperatures. Freeze-dried, powdered samples were then homogenized using lysis buffer, and total RNA was extracted using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA). The total RNA was quantified

using a NanoDrop spectrophotometer (Thermo, Delaware, USA) and sequenced at Novogene (HK) Co., Ltd., Cambridge, United Kingdom. After the RNA sample quality check, mRNA library preparation with poly A enrichment, followed by Illumina NovaSeq Paired End 150 sequencing, was performed at Novogene (HK) Co., Ltd., Cambridge, United Kingdom.

Termites: Total RNA from pools of 180 worker antennae from each species was extracted using acid guanidinium thiocyanate-phenol-chloroform extraction. Deep frozen samples were transferred on liquid nitrogen, ground using a PP pestle directly in the collection tube, and homogenized at room temperature after adding TRI reagent solution (Thermo Fisher Scientific). Extraction steps included vortexing and centrifugation (15,000 g, 15 min at 4°C), RNA precipitation using isopropanol (1:1, followed by centrifugation 15,000 g, 15 min at 4°C), washing in 75% ethanol (centrifugation 5,000 g, 5 min at 4°C), drying at room temperature in laminar flow box and resuspension in 10mM Tris-HCl, pH 8.0 with 0.1mM EDTA. The quality and quantity of isolated RNA was inspected on Nanodrop ND-1000 UV/VIS spectrophotometer and Qubit 4 fluorometer using the RNA HS Assay Kit (all Thermo Fisher Scientific), the integrity was evaluated on 1% agarose gel after staining with ethidium bromide. Library preparations of all three antennal poly(A)-selected strand-specific cDNA libraries and high throughput sequencing analysis on Illumina HiSeq with 30 millions of 2 x 150 paired end reads was conducted at Eurofins Genomics (Ebersberg, Germany).

Transcriptome assembly and gene annotation

Quality checks for the RAW sequencing reads were performed using MultiQC (Ewels et al., 2016). The de novo transcriptome assembly was performed individually for each sample paired-end reads using Trinity-v2.15.0 with default settings for strand-specific reads (Grabherr et al., 2011). The representation of reads to the assembly was checked by mapping all reads back to *I. duplicatus* genome (unpublished) using HISAT2 (Kim et al., 2019). A combined assembly of all four sets of transcriptomes was performed using Trinity-v2.15.0 with default settings (Grabherr et al., 2011), and finally,

redundant sequences were removed by clustering approach using CD-HIT (Fu et al., 2012). The candidate coding regions were identified upon predicting open reading frames with Transdecoder v5.5.0.2.

To check the completeness of assembly, the combined assembly was subjected to BUSCO v5.3.2 analysis (Simão et al., 2015) using insecta_odb10 with *E*-value 1.0E-3. The raw data used for transcriptome assembly are deposited in the NCBI SRA repository as BioSample 1: SAMN42904001 (IDUP_AM1: SRR30040105 and IDUP_AM2: SRR30040104) and BioSample 2: SAMN42917254 (IDUP_AF1: SRR30041954 and IDUP-AF2: SRR30041953).

For manual annotation, we created databases based on the longest assembled isoform of each transcript. tBLASTn searches (Camacho et al., 2009) were performed on these local databases using reference datasets of each multigene family: ORs, IRs, GRs, OBPs, CSPs, and SNMPs as queries with an e-value cut-off of 0.001. The retrieved sequences were used for BLASTx searches (Camacho et al., 2009) performed on these local databases.

Reference sequences used for annotation

Bark beetles: The OR reference dataset included protein sequences (Refseq NCBI or published) from *I. typographus* (Yuvaraj et al., 2021), *Megacyllene caryae* (Mitchell et al., 2012), *D. ponderosae* (Andersson et al., 2019), *Agrilus planipennis* (Andersson et al., 2019), *Anoplophora glabripennis* (Andersson et al., 2019), *Tribolium castaneum* (Dippel et al., 2016), *Leptinotarsa decemlineata* (Liu et al., 2015), *Drosophila melanogaster* (Refseq-NCBI), *Rhynchophorus palmarum* (Gonzalez et al., 2021). Finally, all sequences with insufficient similarities compared to the reference dataset were manually filtered out based on an all-against-all BLAST analysis and subsequent clustering in CLANS (Frickey and Lupas, 2004). To identify all orthologs of *I. typographus* ORs sequences, combined and individual transcriptome assemblies were performed. All IdupORs and IacuORs are named based on their similarities with ItypORs. Finally, candidate protein sequences were checked for transmembrane domains using DeepTMHMM (Hallgren et al., 2022) and TOPCONS (Tsirigos et al., 2015) before phylogenetic analysis.

For IRs, the reference dataset included, sequences reported from *D. ponderosae* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), *Inquilinitermes inquilinus* (Johny et al., 2023), *R. palmarum* (Gonzalez et al., 2021) and insect iGluR amino acid sequences (Croset et al., 2010). The GR candidates reference dataset contained amino acid sequences from *D. ponderosae* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), *A. glabripennis* (Andersson et al., 2019), *I. inquilinus* (Johny et al., 2023), *D. melanogaster* (NCBI RefSeq) and *T. castaneum* (NCBI RefSeq).

For OBPs and CSPs, the reference data set included amino acid sequences from *D. ponderosae* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), *A. glabripennis* (Andersson et al., 2019), *D. melanogaster*, and the dataset used in (Vogt et al., 2015) and (Guo et al., 2018). The SNMP dataset was created using sequences from *D. melanogaster* (nr), *Aethina tumida* (nr), *Manduca sexta* (nr), *T. castaneum* (nr), *D. ponderosae* (Andersson et al., 2019), *R. palmarum* (Gonzalez et al., 2021) and *Sitophilus oryzae* (nr). Finally, the predicted amino acid sequences of each multigene protein family were retrieved manually from the transcriptome assemblies based on the blastx search results.

Termites: For ORs, reference datasets included amino acid sequences of *Z. nevadensis* (nr), *C. secundus* (nr) and *D. melanogaster* (Refseq NCBI) as well as OR sequences from Obiero et al. (2021). OR candidates were further subject to analysis for presence of the correct transmembrane domains in the predicted proteins using TMHMM 2.0 (Krogh et al., 2001). In the final step, prior to multiple sequence alignment and phylogenetic analyses, all sequences with insufficient sequence similarities comparing to the reference dataset were manually filtered out based on an all-against-all BLAST analysis and subsequent clustering in CLANS (Frickey and Lupas, 2004).

For IRs, we used the iGluR amino acid sequences from Croset et al. (2010). For OBPs and CSPs the reference data set included amino acid sequences from *Z. nevadensis* (nr), *C. secundus* (nr), *D. melanogaster*, and *Locusta migratoria,* as well as the dataset used in Vogt et al. (2015) and Guo et al. (2018). The SNMP dataset was created using sequences from the termites *Z. nevadensis* (nr), *C. secundus*

(nr), the fruit fly *D. melanogaster* (nr), the beetle *Aethina tumida* (nr), the moth *Manduca sexta* and SNMPs from other selected Coleopteran species (nr). Finally, the predicted amino acid sequences of each multigene protein family were retrieved manually from the transcriptome assemblies based on the blastx search results. Additionally, in the odorant binding proteins, signal peptides were predicted using SignalP v6.0 (Teufel et al., 2022).

Multiple sequence alignment

Multiple sequence alignment was performed for each chemosensory protein family using MAFFT v.7 (Katoh et al., 2017) under the E-INS-*i* iterative refinement method and trimmed by trimAl v1.4 (Capella-Gutiérrez et al., 2009). The best-fit amino acid substitution model was determined using ProtTest v.3.4.2 (Darriba et al., 2017) and used for the maximum likelihood phylogenetic reconstruction using IQ-TREE v1.6.12 with 1000 bootstrap replications (Minh et al., 2020). The local node support values were calculated using the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999).

Additionally, the signal peptides in the odorant binding proteins were predicted using SignalP v6.0 (Teufel et al., 2022). For the classification, multiple sequence alignment was performed using MAFFT v.7 (Katoh et al., 2017) under the E-INS-*i* iterative refinement method. For the identified tetramer-OBP, the domain architecture was predicted using the NCBI conserved domain database (Wang et al., 2023), the structural predictions were made using AlphaFold3 (Abramson et al., 2024) and visualized using USCF ChimeraX v1.6.1 (Meng et al., 2023).

Phylogenetic analysis of candidate chemosensory proteins

The phylogenetic reconstruction of each protein family was performed using the Maximum Likelihood method (Felsenstein, 1981). We retrieved relevant chemoreceptor protein sequences from the GenBank nr database to compare and predict phylogenetic relationships.

For bark beetles OR phylogeny, amino acid sequences from *I. typographus* (Yuvaraj et al., 2021), *D. ponderosae* (Andersson et al., 2019) *Trypodendron*

lineatum (Biswas et al., 2024b), and a GR from *I. duplicatus* was used as an outgroup. For IR phylogeny, protein sequences were retrieved from *D. ponderosae* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), *T. castaneum* (Dippel et al., 2016), *D. melanogaster* (Refseq-NCBI), *R. palmarum* (Gonzalez et al., 2021) , and *Daphnia pulex* (Saina et al., 2015). For GR phylogeny, *D. ponderosae* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), and *D. melanogaster* (Refseq-NCBI) GR amino acid sequences were used. For OBP phylogeny, amino acid sequences from *I. typographus* (Andersson et al., 2013), *D. ponderosae* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), *T. castaneum* (Dippel et al., 2016), *D. melanogaster* (NCBI), *Colaphellus bowringi* (NCBI) and *Tomicus yunnanensis* (Liu et al., 2018), *R. ferrugineus* (Antony et al., 2016)and *R. palmarum* (Gonzalez et al., 2021) were used in the analysis. Reported pheromone binding proteins from *Popilio japonica* (Maïbèche-Coisne et al., 2004) and *Anomala corpulenta*, *Anomala cuprea, Anomala octiescostata* PBPs from NCBI were included for analysis, and *Lepismachilis y-signata* OBPs (Missbach et al., 2015) were used as outgroup. For CSPs phylogeny, sequences from *D. melanogaster* (Refseq-NCBI), *T. castaneum* (NCBInr), *D. ponderosae* (Andersson et al., 2019), *R. palmarum* (Gonzalez et al., 2021), *Anoplophora glabripennis* (Andersson et al., 2019), *Bombyx mori* (Zhou et al., 2009), *Camponotus japonicus* (NCBI), *Clunio marinus* (NCBI), *Apis mellifera* (NCBI) were included and *D. pulex* (Saina et al., 2015) was used as outgroup. For SNMPs, protein sequences from *D. melanogaster* (Refseq-NCBI), *T. castaneum* (NCBI-nr), *D. ponderosae* (Andersson et al., 2019), *R. palmarum* (Gonzalez et al., 2021), *A. glabripennis* (Andersson et al., 2019), *A. planipennis* (Andersson et al., 2019), *Aethina tumida* (NCBI-nr), *Manduca sexta* (NCBI-nr), *R. palmarum* (Gonzalez et al., 2021), *R. ferrugineus* (Johny et al., 2024b), *Popilio japonica* (NCBI-nr) and Scarabaeidae specific SNMPs from (Zhao et al., 2020). A non-SNMP protein, croquemort (crq) from *D. melanogaster* was used as an outgroup.

For the termite odorant receptor phylogeny, OR sequences from the termite species *Z. nevadensis*, *C. secundus*, *N. cubanus*, *P. simplex*, *R. speratus* and *I. inquilinus*, as well as the termite relative, the cockroach *Blattella germanica* were

used. We further included *Bombyx mori* and *Manduca sexta* (Lepidoptera), *Drosophila melanogaster* (Diptera), *Ips typographus* and *Tribolium castaneum* (Coleoptera), *Forficula auricularia* (Dermaptera), *Athalia rosae* and *Apis mellifera* (Hymenoptera). As an outgroup, the crustacean *Daphnia pulex* Gr 42, 43, and 44 (Saina et al., 2015) sequences were used. The larger number of datasets was required to reach a predicted phylogeny with sufficient support, likely due to the high sequence diversity of ORs. The ionotropic receptor phylogeny was reconstructed using LG+F+R as the best-fit amino acid substitution model and was rooted with non-NMDA iGluRs as an outgroup, using 1000 replicates to calculate bootstrap support. The amino acid sequences from the following species were added to study the phylogenetic relationship: the termites *Z. nevadensis*, *C. secundus, N. cubanus*, *P. simplex*, *I. inquilinus*, the cockroach *B. germanica*, the fruit fly *D. melanogaster,* and the beetles *Dendroctonus ponderosae* and *Rhynchophorus palmarum*. Non-blattodean species were added to allow for better determination of the correct iGluR-subclades of novel candidates. The SNMP phylogeny was reconstructed using LG+R as the best-fit amino acid substitution model under Bayesian information criterion with 1000 bootstrap replications. Species compared in the phylogeny were the termites *Z. nevadensis*, *C. secundus, N. cubanus*, *P. simplex* and *I. inquilinus*, the cockroach *B. germanica*, the fruit fly *D. melanogaster*, the beetles *T. castaneum*, *Sitophilus oryzae* and *R. palmarum*, the moths *B. mori* and *M. sexta,* and the ant *Harpegnathos saltator.* Coleopteran SNMPs are included in the phylogeny as additional SNMP groups are reported in this insect order (Dippel et al., 2016; Zhao et al., 2020). We used *D. melanogaster* Croquemort (crq) protein, a member of the CD36 family but not an SNMP, as an outgroup. The maximum likelihood phylogeny of termite OBPs was reconstructed using LG+R as the best-fit amino acid substitution model under AIC with 1000 bootstrap replications. Bristletail *Lepismachilis y-signata* OBPs were used as outgroup. The species included in the analysis were the termites *Z. nevadensis*, *C. secundus, N. cubanus*, *P. simplex* and *I. inquilinus*, the beetles *T. castaneum* and *R. palmarum*, the moth *M. sexta* and the fruit fly *D. melanogaster.* The Maximum likelihood phylogeny of termite CSPs was constructed using LG+R as amino acid substitution model

and rooted with *D. pulex* CSP sequences as outgroup. The other species included in the analysis were the termite *R. speratus*, the beetles *T. castaneum* and *Rhynchophorus palmarum*, the moth *B. mori*, the honey bee *Apis mellifera,* the fruit fly *D. melanogaster*, the chironomid *Clunio marinus*, and the ant *Camponotus japonicus*. Inclusion of CSPs from the listed species allowed a better comparison of termite CSPs across insect orders.

Functional characterization of odorant receptors

Insect collection, RNA extraction, and cDNA synthesis

I. typographus adults were collected from Kostelec nad Cernými lesy (50° 00′ 07.2″ N 14° 50′ 56.3″ E) located in the Central Bohemia region in Czech Republic and reared on Norway spruce logs in the laboratory under conditions: 70% humidity, 24 °C,16:8 h day/night period. For RNA extraction, antennae were dissected from ~500 cold anesthetized adult beetles (males and females in 1:1 ratio) under a light microscope and stored in RNA*later* (Themo-Fisher Scientific, WA, USA). Total RNA was extracted using PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) using 2µg of total RNA as described earlier (Antony et al., 2021). The cDNA was quantified using a NanoDrop spectrophotometer (Thermo, Delaware, USA), and the quality was checked by the amplification of *ItypOrco*. The list of primers is provided in Table 3.1. For termites, multiple laboratory colonies of *P. simplex* are held in the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences. Colonies are reared in glass vivaria at 27°C and 80% relative humidity in clusters of spruce wood slices. These colonies were used for RNA extraction, single sensillum recordings (SSR) and electroantennogram recordings (EAG).

Phylogenetic analysis

Full-length OR nucleotide sequences of *I. typographus* were retrieved from previous publication (Yuvaraj et al., 2021) and our own-lab antennal transcriptomes (Johny et al., unpublished). Multiple sequence alignment was performed using MAFFT v.7 (Katoh et al., 2017) under the E-INS-i iterative

refinement method and trimmed by trimAl v1.4 (Capella-Gutiérrez et al., 2009). The best-fit amino acid substitution model, JTT+G+F was determined under AIC criteria and used for the maximum likelihood phylogenetic reconstruction using IQ-TREE version 1.6.12 with 1000 bootstrap replications (Minh et al., 2020). The local node support values were calculated using the Shimodaira-Hasegawa (SH) test (Shimodaira and Hasegawa, 1999). For the test for positive selection, we used the Codeml program in the PamlX v1.3.8 package (Xu and Yang, 2013).

PCR amplification, Gateway cloning, and LR recombination of ORs

Full-length open reading frames (ORFs) of each OR were amplified from the cDNA using custom primers designed using PrimerQuest Tool (Integrated DNA Technologies, Belgium). The PCR reactions were prepared using DreamTaq Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) following Touch-down PCR with the conditions as 94^{o}C for 3 min, followed by five cycles of denaturation at 94^{o}C for 1 min, annealing at 60^{o}C for 1 min with 1^{o}C decreasing per cycle, and extension at 72^{o}C for 1:30 min; followed by 25 cycles of denaturation at 94^{o}C for 1 min, annealing at 55^{o}C for 1 min and extension at 72^{o}C for 1:30 min, followed by 72^{o}C for 10 min final extension. The Advantage 2 PCR Kit (Takara Bio, USA) was used for proofreading PCRs following the manufacturer's protocols. The amplicons were purified by QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). For TOPO Gateway Cloning, amplicons of each OR were ligated into pCR8/GW/TOPO vector using pCR8/GW/TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and transformed into OneShot TOP10 *Escherichia coli* competent cells (Invitrogen, Carlsbad, CA, USA). Resulting colonies were screened by colony PCR using Dream-taq Green Master Mix (Invitrogen, Carlsbad, CA, USA), and recombinant plasmids were isolated using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and verified by Sanger sequencing (Eurofins Genomics, Germany). The expression vector constructs were prepared by LR recombination using Gateway LR Clonase II Enzyme Mix (Invitrogen, Carlsbad, CA, USA) using pCR8/GW/TOPO vector with *ItypORX/PsimORX*

as entry clone and in-lab prepared pUASg.attb as destination vector (stock prepared using the resources from Drosophila Genomics Resource Center, Bloomington IN, USA and Belgian Coordinated Collections of Microorganisms, Ghent, Belgium). Colony PCRs screened the resulting colonies, and recombined expression vector constructs vectors were isolated, quantified, and verified by the Sanger Cycle sequencing.

Population genomics analysis

Recently generated whole genome resequencing data (Mykhailenko et al., 2023) was screened for variation in *ItypOR33*. To obtain the *ItypOR33* genome coordinates, the *ItypOR33's* published transcript sequence (Yuvaraj et al., 2021) was mapped to the spruce bark beetle reference genome using minimap2 (Li, 2018). The coordinates were used to extract polymorphic positions (SNPs) from a VCF file containing information on SNP variation identified in 240 spruce bark beetle individuals from 18 European populations using GATK (McKenna et al., 2010) (for details, see (Mykhailenko et al., 2023) (Table 3.2). For each individual, the ItypOR33 SNP variation was transformed into a fasta file using bcftools: consensus command (Danecek et al., 2021). Heterozygous positions were coded using the IUPAC ambiguity code, and missing genotypes were coded as 'N's. The *Fasta* sequences were combined with *ItypOR33* and *ItypOR33a* sequences obtained in this study and aligned using MAFFT v.7 (Katoh et al., 2017). Allele frequencies for non-synonymous polymorphic positions were calculated using tables generated by GATK; *VariantsToTable* option (McKenna et al., 2010). Pearson correlation coefficients were calculated between allele frequencies and latitude to determine whether these variants differed in frequency along the species distribution. Finally, as *ItypOR33* is located within one of the polymorphic inversions identified in spruce bark beetle populations (Mykhailenko et al., 2023), we tested whether specific alleles were associated with specific inversion haplotypes. The differences in *ItypOR33* nucleotide polymorphism and heterozygosity were also measured as Tajima's D (Tajima, 1989). We further tested whether any polymorphic sites are under positive selection using MEME (Murrell et al., 2012).

Drosophila embryo injection and transgenic expression of ORs

The *I. typographus* and *P. simplex* ORs were expressed in the *Drosophila* empty-neuron system for functional screening. Transgenic *D. melanogaster UAS-OR* lines were generated by BestGene Inc. (Chino Hills, CA, USA) injecting *pUASg.attB-OR* plasmids into fly embryos expressing the integrase *PhiC31* and carrying an *attP* landing site, resulting in flies with genotype *w−; +; UAS-ItypOR-X/PsimOR-x (w+)/+*. We used CRISPR-cas9-engineered empty-neuron lines (Chahda et al., 2019) with *ΔHalo* genetic background to express ItypORs in *Drosophila* ab3 sensilla. The fly crossing scheme was adapted (Gonzalez et al., 2016) with the following modifications in F3 crossing: *w-; ΔHalo/Cyo; UAS-OR(w+)/TM6B* x *w-; DsRed; Or22ab-GAL4*. The genotype of each test fly, and control lines were confirmed by single-wing PCRs (Carvalho et al., 2009). *DmelOR22a* and gene-specific primers were used for PCR with control and test flies, respectively (Table 3.1). All fly lines were reared at 24±2 ˚C with a relative humidity of 50 ± 5%, fed with in-house prepared standard cornmeal media.

Using the fly line *w; DsRed-Gal4; +*, we first generated a rebalanced line *w; DsRed-Gal4; TM2/TM6b*, which was used to drive the expression of OR-X in *Drosophila* ab3. In brief, *w; +/+; UAS-OR(w+)/UAS-OR(w+)* was crossed to *w-; Bl/Cyo; TM2/TM6b* for the F1 generation. Progeny with curly wings and tubby phenotypes was selected (*w; +/Cyo; UAS-OR(w+)/TM6B*) for the next cross. In F2; *w; +/Cyo; UAS-OR(w+)/TM6B* was crossed to *w-; Bl/Cyo; TM2/TM6B* and progeny with bristles, curly wings and tubby phenotypes were selected for the F3 cross. In F3, *w; Bl/Cyo; UAS-OR(w+)/TM6B* was crossed to *w-; DsRed-Gal4/ DsRed-Gal4; TM2/TM6B* for the expression cross. Progeny with *w; DsRed-Gal4 /Cyo; UAS-OR(w+)/TM6B* selected and self-crossed to generate test-fly. The test fly is a viable homozygote, and they were kept for single sensillum screening (SSRs). SSRs were performed on *w; DsRed-Gal4; UAS-OR-X* homozygotes by targeting the ab3 sensillum.

Single sensillum recordings

The single sensillum recording (SSR) was performed using 2-7 days-old female flies as described previously (Olsson and Hansson, 2013; Pellegrino et al., 2010). A list of 88 synthetic chemicals is provided in Table 3.4. Except for bark

beetle pheromones, all the odorants were purchased from the commercial provider at the highest purity. The purity of bark beetle pheromones resourced from the scientific collaborators was checked by GC-MS. The dose-response curves were plotted using GraphPad Prism v5 (GraphPad, La Jolla, CA, USA). Standard protocols were followed for SSR recording (Benton and Dahanukar, 2023; Olsson and Hansson, 2013). In brief, the flies were mounted in a cut pipette tip (yellow) with the head protruding, and a small amount of cotton roll was placed at the back of the tip to immobilize the fly. The pipette was then fixed onto a microscope slide with wax, and the antennae were fixed on a coverslip with a glass electrode, positioning the arista down to expose the ab3 sensilla. A sharpened tungsten electrode was placed in the eye for grounding, a second recording electrode was brought into contact with the base of the sensillum using a Kleindiek Nanotechnik MM3A micromanipulator connected to a cubic micromanipulator device. The electrodes were sharpened using a saturated potassium nitrite 10% (KNO_2) solution. Only 1-3 recordings were performed from a single fly to avoid neuronal adaptations from multiple stimulations. A single fly per recording was used for the dose-response curves. The dose-response experiments used six concentrations ranging from 10-3 ng to 1000ng. The sensilla were observed under the Nikon FN1 eclipse microscope at 60x magnification. Odorants were diluted in paraffin oil at 10-3 v/v, except for pheromone compounds, which were diluted at 10ng in hexane. From each diluted odorant, 10µl were pipetted on a 1 cm diameter filter paper disk placed in glass Pasteur pipettes. The stimulation was done by placing the tip of a cartridge into a tube connected to a stream of humidified air (0.4 L/min). The odors were delivered by puffing using the Syntech stimulus delivery system (Ockenfels Syntech GmbH, Buchenbach, Germany). The odor stimulus was administered as a 0.3 s pulse by placing the tip of the glass Pasteur pipette through a hole in a tube carrying a purified air stream. The distance between the antenna and the odor delivery system was approximately 4 cm. The signal was amplified (Syntech UN-06, http://www.syntech.nl) and digitally converted using IDAC4 (Syntech IDAC-4). The responses (spikes/s) were analyzed by counting the number of spikes,

0.5 seconds during stimulation minus 0.5 seconds before stimulation offline, using the software AutoSpike v3.9 (Benton and Dahanukar, 2023). For spike count, neurons were sorted based on their amplitude. The final generated response of individual OSNs was multiplied by two to generate total spikes per second (delta spikes/s) (Benton and Dahanukar, 2023). All the spiked sorting and counting were done using the software, AutoSpike v3.9 (Syntech Ockenfels, Germany).

Protein structural predictions and molecular docking

For ItypOR33, the protein 3D modeling was performed using AlphaFold2 (Jumper et al., 2021) with multiple sequence alignments generated by MMseqs2 and HHsearch (Mirdita et al., 2022). For convenience, both ItypOR33 and variant ItypOR33a were renamed as ItypOR33^{53Y,226G} and ItypOR33a^{10E,288I,249Q,319R} respectively, indicating variations from the first published version, named ItypOR33^{WT} (Figure 3.2). The structure predictions were repeated with AlphaFold3 (Abramson et al., 2024). The best-ranked model based on the predicted template modeling (pTM) score was used for docking experiments. The models were prepared for docking using the DockPrep tool in UCSF ChimeraX (Pettersen et al., 2021). The coordinates of putative binding pockets were identified using DeepSite (Jiménez et al., 2017) and CASTp 3.0. (Tian et al., 2018). The best ligand binding site was chosen based on the druggability score predicted by Caver Analyst 2.0 (Jurcik et al., 2018; Stourac et al., 2019). The ligands were downloaded from PubChem and prepared for docking using Autodock tool in MGLTools v1.5.6 (Morris et al., 2009). Autodock Vina v1.2.5 (Eberhardt et al., 2021) was used to dock the *in vivo* identified ligands to modeled structures using coordinates obtained from DeepSite, generating nine poses for each ligand. Residues located at <5 Å from the best-ranked pose were considered as putatively interacting with it. The ligand binding activity was calculated using Autodock Vina v1.2.5 and visualized using UCSF ChimeraX (Pettersen et al., 2021) and PyMOL v.2.5.5 (Schrödinger and Warren, 2020). Additionally, Caver Analyst 2.0 (Jurcik et al., 2018; Stourac et al., 2019) was used for the prediction of tunnels within the

modeled structures with 0.09 as the minimum probe radius and the desired radius as 5. Finally, all predicted structural elements were mapped to multimeric structures generated using AlphaFold3 (Abramson et al., 2024). Only the best-ranked AlphaFold3 model based on pTM score was used in visualization. We compared both the 1:1 and 1:3 OR to Orco ratios to model the heteromeric complexes of ItypOR33:ItypOrco and ItypOR33a:ItypOrco based on the ipTM and pTM scores generated by AlphaFold3.

Expression quantification by RT-qPCR

For the relative quantification of *ItypOR33,* primers were designed for *ItypOR33* and *ItypORco* using parameters Tm: 55-60°C; GC content: 40–50%, length 150-200 bp, as mentioned earlier. *β- tubulin* was used as endogenous control specific to head tissues (Sellamuthu et al., 2022) (Table 3.1). Total RNA was extracted from a pool of 10 heads from *I. typographus* males and females separately, generating three biological replicates using the previously mentioned methods. The cDNAs synthesized using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) as described earlier were used for relative quantification with three biological and three technical replicates using Power SYBR Green PCR Master Mix (Thermo Fisher), following manufacturer's instructions. RT-qPCR conditions used were: 50°C for 20 sec; 95°C for 5 min; 40 cycles of 95°C for 15 s and 60°C for 30 s; followed by continuous melting curve stages of 95°C for 15 s, 60°C for 1 min, 95°C for 30 s, and 60°C for 15 s. The relative expression of *ItypOR33* compared to *ItypOrco* in males and females was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The significant difference in expression between the males and females was tested using a *t*-test with α- significance level at $p < 0.05$.

Behavioral assays

As amitinol, the identified ligand of ItypOR33, is a known heterospecific pheromone component in *Ips*, we performed behavioral assays to evaluate its role in *I. typographus* adults. The beetles were sourced and reared, as mentioned earlier. The F1 adults were used for bioassays with three setups: i) pheromone *vs.* hexane, ii) amitinol *vs.* hexane, and iii) pheromone *vs.*

(pheromone + amitinol). The tested aggregation pheromones were 2-methyl-3-buten-2-ol (100 µg/mL) and (*S*)-*cis*-verbenol (10 µg/mL) in a 10:1 ratio, based on the literature (El-Sayed, A, 2023). The bioactivity of amitinol in adult beetles was tested at three doses (1, 10, and 100 µg/mL). For the assay design, we conducted pilot assays using established setups that match the size and flying behavior of *I. typographus*. The pilot studies using a Y-tube olfactometer were unsuccessful due to the vertical beetle movements as an initial response. While the trails using Petri dish-based bioassays limited the insect behavior to walking, the wind tunnels were found inappropriate due to the vertical movements and wind speed affecting the flight behavior. We, therefore, used a cage-based two-choice bioassay design modified from (Lyu et al., 2021). The assay was performed in a controlled air flow with a slow-release capillary method to release compounds (Anbesse and Ehlers, 2013; Hiltpold et al., 2010). The assays were performed in four replicates (n = 4), testing 50 adult beetles per replicate under controlled conditions. After testing different doses of amitinol, the most significant dose was used for checking sex-specific behavior in beetles (n = 4 of each sex). The preference index of beetles towards the test zone *vs.* control zone was calculated as, the number of beetles preferred in the test zone (T) – the number of beetles preferred in the control zone (C) / total number of beetles responded (T + C). The significance of beetle preferences to test *vs.* control zones was analyzed using the Chi-square (χ^2) test with the α level 0.05 using the R program v4.1.0 (R Core Team, 2023).

Electroantennography Recordings

The behavioral responses elicited by amitinol were then further checked by EAG recordings. We tested the combinations and concentrations that elicited significant behavioral activity, *i.e.* MB:cV (10:1) as Pheromone, amitinol (10ug/mL), and Pheromone MB:cV +Amitinol (10:1:10) for the EAG recordings. Standard protocols were followed for insect preparations and EAG recordings, as described earlier (Olsson and Hansson, 2013). Measurements were made using EagProV2.1.0 (Ockenfels Syntech GmbH, Buchenbach, Germany). Six concentrations of amitinol ranging from 1ng/mL to 100µg/mL were used for the dose-response analysis. One-way ANOVA with Turkey's

HSD was used to test the statistical significance between each tested compound and between males and females at *a* level 0.05 using SPSS v24 (IBM, SPSS, USA).

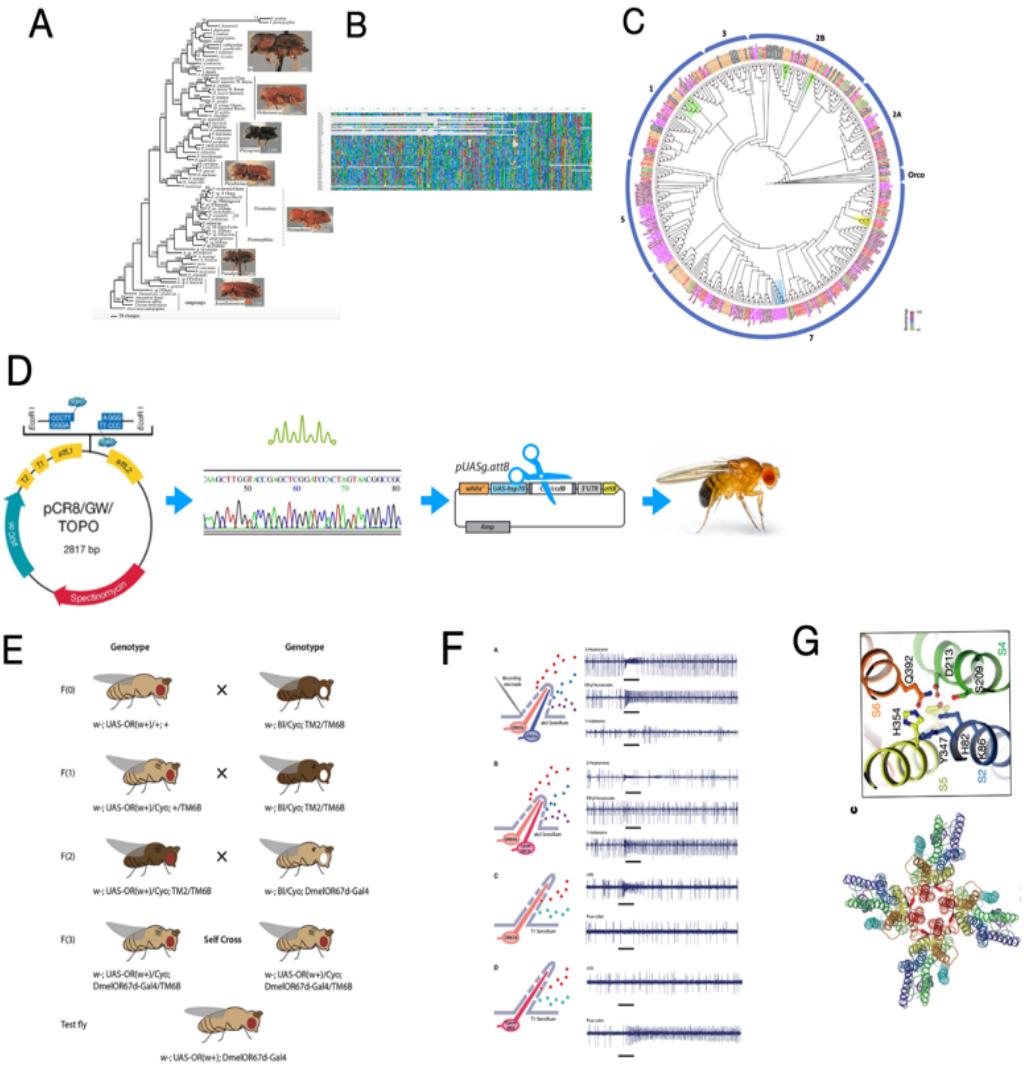

Figure M1 : General steps in the identification and functional characterization of ORs: A to G; from selection of Ips species (A), transcriptome analysis (B), phylogenetic analysis (C), cloning and expression in Drosophila (D), crossing scheme for the Drosophila lines (E), single sensillum recordings (F), and finally identification of specific amino acid residues involved in odorant detection (G). Part of the image was adapted from (Gonzalez et al., 2016).

Results

The results obtained from this study were presented in four chapters:

Chapter 1: Conserved orthology in bark beetle chemosensory gene families

Chapter 2: Conserved orthology in termite chemosensory gene families

Chapter 3: Population-level functional polymorphisms in *Ips typographus* pheromone receptor, ItypOR33

Chapter 4: Functional evolution of termite chemosensory genes.

Results

Chapter 1: Conserved orthology in bark beetle chemosensory gene families

Partially based on the manuscripts:

1. **Johny, J.,** Große-Wilde, E., Kalinová, B., Roy, A., 2024. Antennal Transcriptome Screening and Identification of Chemosensory Proteins in the Double-Spine European Spruce Bark Beetle, *Ips duplicatus* (Coleoptera: Scolytinae). Int. J. Mol. Sci. 25, 9513. doi:10.3390/ijms25179513
2. **Johny, J.,** Große-Wilde, E., Bláha, J., Modlinger, R., Synek, J., Kalinová, B., Roy, A., 2024. Antennal Transcriptome Analysis and Identification of Chemosensory Gene Families in *Ips acuminatus*. For. Ecosyst. (in submission)

Transcriptome assembly of *I. duplicatus* and *I. acuminatus*

Four *I. duplicatus* and two *I. acuminatus* antennal transcriptomes were generated using Illumina paired-end sequencing and named IDUP_F1: *I. duplicatus* female 1, IDUP_F2: *I. duplicatus* female 2, IDUP_M1: *I. duplicatus* male 1 and IDUP_M2: *I. duplicatus* male 2. The IDUP_M1 generated 20.37 million paired reads and were assembled into 105,416 transcripts with an 88.56% overall reads-to-assembly alignment to the *I. duplicatus* genome. IDUP_M2 generated 23.82 million paired reads and were assembled into 121,285 transcripts with an 88.30% overall alignment rate to the genome. IDUP_F1 generated 19.74 million paired reads and were assembled into 91,822 transcripts with an 87.97% overall alignment rate to the genome. IDUP_F2 generated 20.35 million paired reads, assembled into 98,264 transcripts with a mapping percentage of 88.63% overall alignment to the genome (Kim et al., 2019). A combined assembly of all reads generated from all four IDUP samples generated 204,588 transcripts, whereas the same in two IAC transcriptomes (IAC_AF1 and IAC_AF2) generated 118,579 total transcripts. The BUSCO 5.3.2 analysis (Simão et al., 2015) as a measure for completeness of the transcriptomes revealed 99.71% completeness for the *I. duplicatus* combined assembly using the insecta10 dataset as a reference. For *I. acuminatus* the BUSCO analysis revealed 98.91% completeness with the same database and only 0.58% missing.

Table 1.1. Assembly and mapping statistics of the four *I. duplicatus* antennal transcriptomes and combined assembly of two *I. acuminatus* antennal transcriptomes generated in this study (IAC_AF1 ad IAC_AF2 combined). The *I. duplicatus* transcriptomes are named as male antennal transcriptomes (IDUP_AM1 and IDUP_AM2) and female antennal transcriptomes (IDUP_AF1 and IDUP_AF2).

	IDUP_AM1	**IDUP_AM2**	**IDUP_AF1**	**IDUP_AF2**	**All IDUP Combined**	**All IAC combined**
Total transcripts	105,416	121,285	91,822	98,264	204,588	118,579
Total genes	61659	70337	50,264	54391	125,878	62,929
GC content	39.89	39.82	38.96	39.26	39.21	38.32
N50 length	2101	2027	2,455	2036	2,317	2,492
Average length	1049.33	1011.11	1,244.16	1048.26	1,027.03	1,212.45
Complete BUSCOs (insecta_odb10)	93.71%	94.37%	95.17%	94%	99.71%	98.91%
BUSCOs fragmented %	4.24	3.58	3.22	2.37	0.15	0.58%
% mapped to genome*	88.56%	88.30%	87.97%	88.63%		

* preliminary genome unpublished

Odorant receptors in *I. duplicatus* and *I. acuminatus*

ORs represent one of the most important olfactory proteins in insects. Our manual annotations of *I. duplicatus* transcriptomes using separate and combined assemblies revealed 69 ORs, including the co-receptor, ORCo. The typical 7 transmembrane regions were predicted from 50 IdupORs and considered full-length, whereas 19 were partial sequences. Similar annotation in *I. acuminatus* revealed a total of 66 ORs. All ORs were named based on their orthology to ItypORs (Yuvaraj et al., 2021a). The ML phylogeny reconstructed using JTT+F+R9 based on the Bayesian information criterion (BIC) score revealed the seven reported coleopteran OR subfamilies (Gonzalez et al., 2021; Mitchell et al., 2020). The phylogeny was rooted with *IdupGR1*. The OR subfamily 2, had 12 members divided equally into subfamilies 2a and 2b (Figure 1). No bark beetle ORs were grouped into OR subfamily 3 was reported only in *M. caryae* (Mitchell et al., 2012). Seven IdupORs and five IacuORs were grouped into subfamily 5. Interestingly, we found bark beetle-specific OR expansions in this subfamily with 1:1 orthology except for DponORs. 17 IdupORs and 11 IacuORs were grouped as OR subfamily 1 and (Figure 1.1). 31 IdupORs and 27 IacuORs were included in the subfamily-7, the largest in coleopteran OR subfamilies (Figure 1.1). We also found three members in this subfamily with variants, but not isoforms, named with the suffix 'a' and 'b' for identification (Figure 1.1). A conserved orthology was identified between ItypORs, IacuORs and IdupORs throughout the phylogeny, including a 1:1 orthology in a well-characterized *Ips* spp. specific OR clade in subfamily-7. However, *A. glabripennis* specific expansions are found in all identified coleopteran subfamilies (1-7) as observed in bark beetle specific expansion. Within the bark beetle specific OR expansions, *D. ponderosae* showed more divergence. The characterized ORs from *I. typographus* were found to be the most divergent clade of ORs (Hou et al., 2021; Yuvaraj et al., 2021).

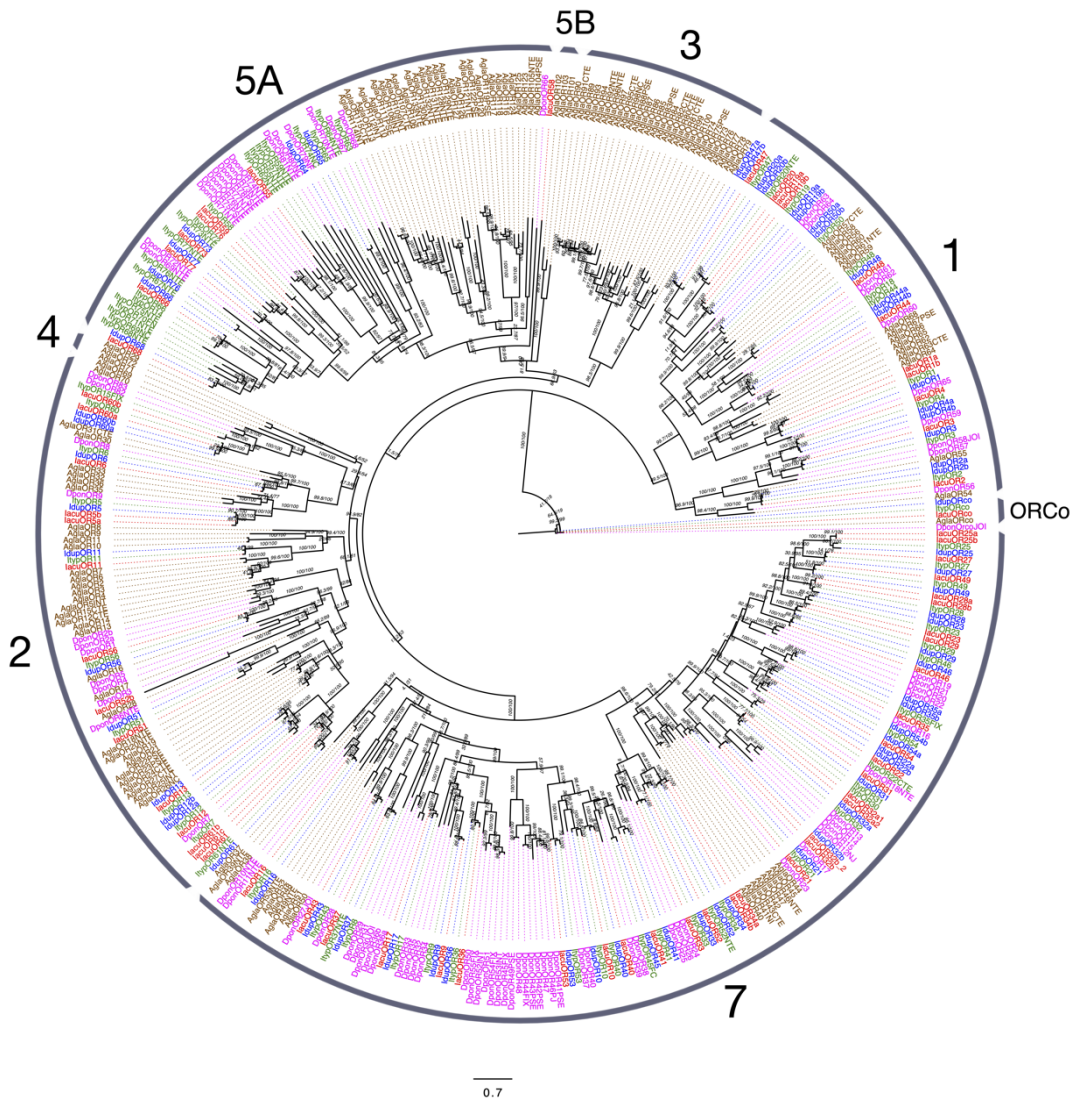

Figure 1.1. Maximum likelihood phylogeny of bark beetle ORs. The tree was reconstructed using predicted OR sequences from *I. duplicatus* (blue), *I. acuminatus* (red), *I. typographus* (green) and selected coleopteran species. The tree was rooted with IdupGR with ORCo at the basal node. Each Coleopteran OR subfamily is marked with respective numbers. ORs from the other species were coloured as: *D. ponderosae* (magenta), and *A. glabripennis* (brown). The branch labels indicate SH-like approximate likelihood ratio test (SH-aLRT) value/bootstrap value. The scale represents amino acid substitutions per site.

Ionotropic receptors and iGulR family receptors in bark beetles

Manual annotations based on insect ionotropic glutamate receptor family proteins (iGluRs) led to the identification of 69 and 59 transcripts in *I. duplicatus*

and *I. acuminatus* antennae respectively as iGluRs. As iGluRs are further classified based on sequence homology, a maximum likelihood phylogeny was reconstructed using all well-reported classes of iGluRs (Benton et al., 2009; Croset et al., 2010). The ML phylogeny rooted with non-NMDA iGluRs from *D. melanogaster* revealed six groups of iGluRs as non-NMDA iGluRs, IR8a, IR25a, NMDA-receptors, antennal IRs, and divergent IRs. 11 and 16 iGluRs were found to be non-NMDA in both *I. duplicatus* and *I. acuminatus*, including kainite receptors (Figure 2). Representative IRs for each IR co-expressing receptors IR8a and IR25a (Figure 2) were found in both annotations. Six in IdupiGluRs and four IacuiGluRs were found to be NMDA-receptors (Figure 1.2). Among the remaining iGluRs, 28 IdupIRs were identified as antennal IRs, and 22 were classified as divergent IRs based on the phylogeny (Figure 1.2). Whereas in *I. acuminatus* 19 antennal IRs and 10 divergent IRs were identified. The number of IRs identified was similar to the genome-based IR annotation reported from *D. ponderosae* (Andersson et al., 2019). The IR phylogeny revealed the divergence of antennal and divergent IRs in Coleoptera and Diptera. Interestingly, bark beetle-specific expansions were identified in divergent IRs, similar to the species-specific expansion found in *D. melanogaster* divergent IR clade.

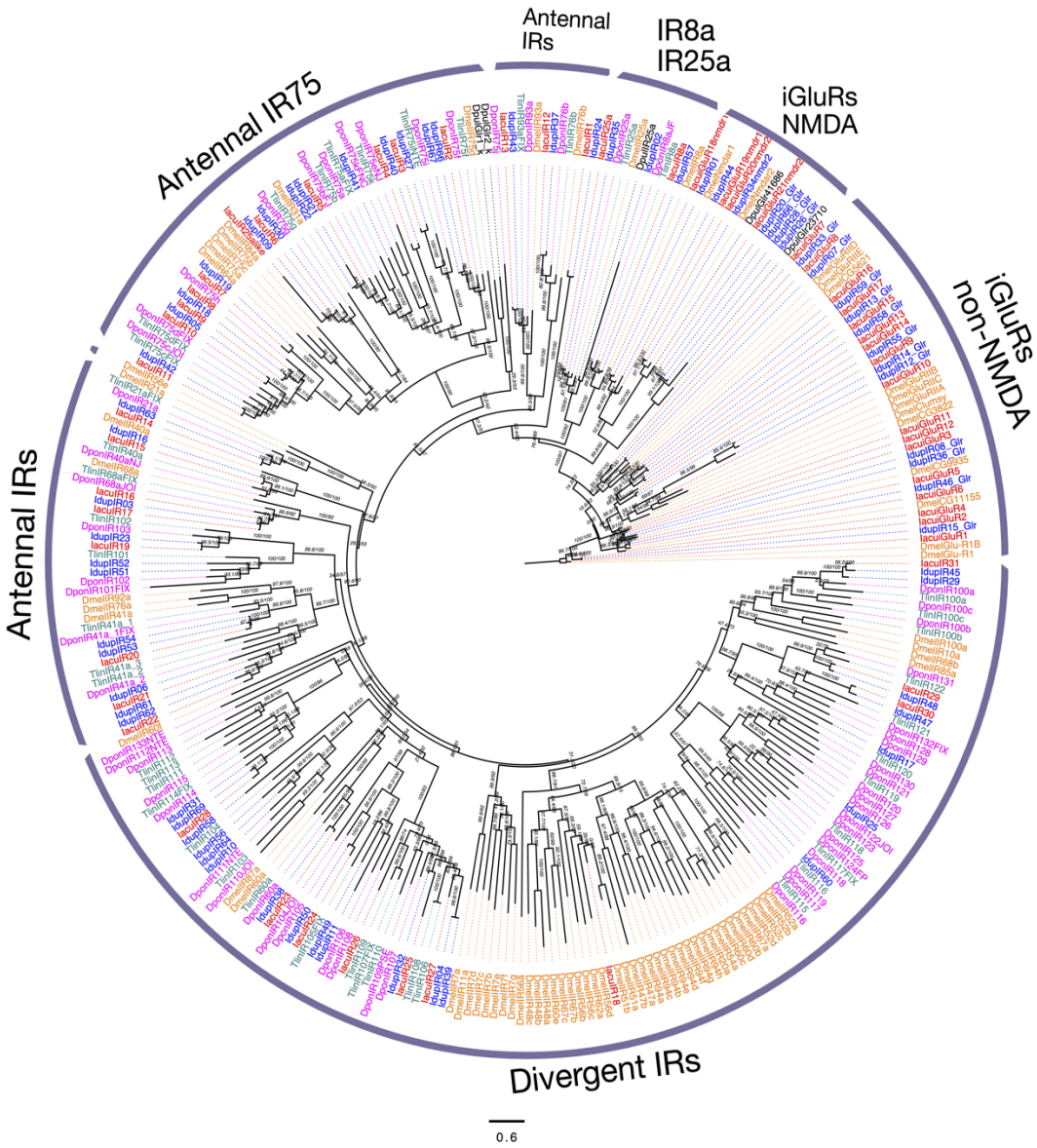

Figure 1.2. Maximum likelihood phylogeny of bark beetle IRs. The tree was reconstructed using predicted IR sequences from *I. typographus, I. duplicatus, I. acuminatus* and selected coleopteran species and *D. melanogaster*. The tree was rooted with a non-NMDA iGluR, *DmelGlu-R1.* All iGluR sub-families are marked at taxa labels. The IdupIRs and IacuIRs were colored in blue and red, respectively, and the other species were colored as *D. ponderosa* (magenta), *D. melanogaster* (orange), *Daphnia pulex* (black) and *T. lineatum* (green). The branch labels indicate SH-aLRT value/bootstrap value. The scale represents amino acid substitutions per site.

Gustatory receptors in bark beetles

GRs are essential for detecting tastants and nonvolatile pheromones in insects (Montell, 2009). Our antennal transcriptome analysis revealed a total of 25 and 26 GRs, respectively in *I. duplicatus* and *I. acuminatus*. The ML phylogeny revealed different classes of GRs based on their similarities with well-characterized GRs from *D. melanogaster* (Joseph and Carlson, 2015; Montell, 2009). The phylogenetic tree was constructed based on the LG+F+R6 amino acid substitution model identified based on the BIC score and was rooted with *DmelGR21a*. Both *DmelGR21a* and *DmelGR63a* are known to detect CO_2 in *D. melanogaster* (Montell, 2009). Three IdupGR candidates were found in the clade of GRs sensing CO_2 with 1:1 orthology to DponGRs (Figure 1.3). Two main GR classes identified were sugar and bitter-sensing receptors based on characterized DmelGRs (Chahda et al., 2019; Dahanukar et al., 2001; Delventhal and Carlson, 2016). Five candidate IdupGRs were identified within the clade of sugar-sensing receptors with orthology to DponGRs (Figure 1.3). Whereas in bitter-tasting receptor clades, species-specific expansions were detected (Figure 1.3). Similarly, coleopteran-specific GR expansions were found in the phylogeny with 15 IdupGRs and a similar number of DpondGRs (Figure 1.3). A large clade of those GRs was found between CO_2 and sugar-sensing receptors but not classified as bitter sensing due to the lack of well-characterized GRs within the clade. An ortholog for two *DmelGR68a* and *DmelGR32*, involved in *D. melanogaster* courtship behavior (Andrews et al., 2014; Bray and Amrein, 2003; Montell, 2009) was also identified. Interestingly, conserved orthology was identified between IdupGRs and DponGR but not with AplaGRs, indicating bark beetle-specific GR expansions.

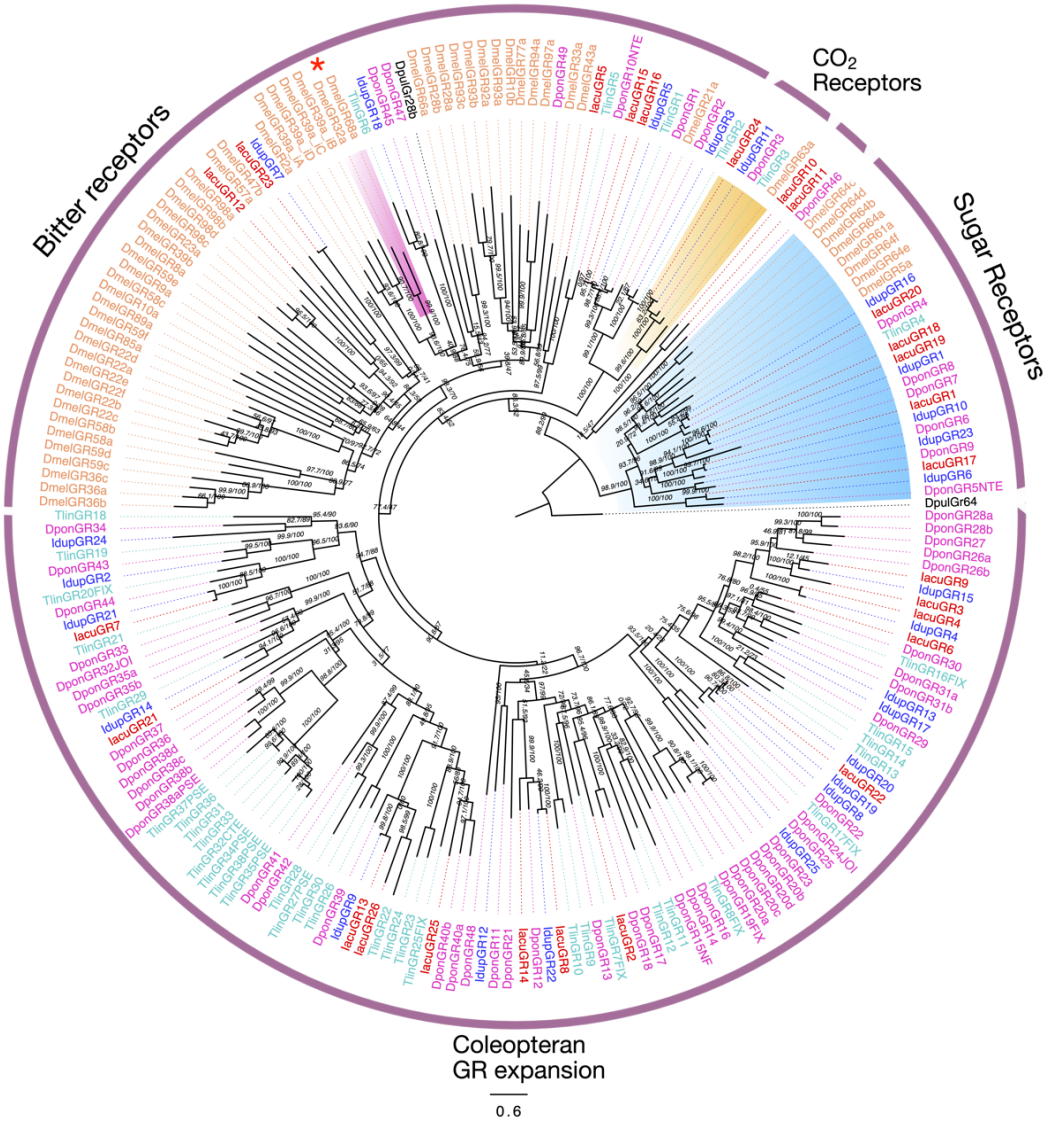

Figure 1.3. Maximum likelihood phylogeny of bark beetle GRs. The tree was reconstructed using predicted GR sequences from *I. duplicatus* and *I. acuminatus* along with selected coleopteran IR sequences and *D. melanogaster* IRs. The tree was rooted with a DpulGR64. The IdupGRs and IacuGRs are red blue, respectively, and the other species were colored *D. ponderosa* (magenta), and *T. lineatum* (light green). (*) indicates the DmelGRs involved in courtship behavior. The branch labels indicate SH-aLRT value/bootstrap value. The scale represents amino acid substitutions per site.

Odorant binding proteins in bark beetles

OBPs are important non-receptor proteins involved in peripheral olfactory detection, transporting odorants to the receptors through the olfactory sensilla lymph. We identified 27 and 36 OBPs, respectively, from *I. duplicatus* and *I. acuminatus* antennae. OBPs are generally classified based on function, antennal expression, and structural features (Hekmat-Scafe et al., 2002). As bark beetle OBPs were uncharacterized, we used sequence characteristics to classify them as Classic OBPs, Minus-C OBPs, Plus-C OBPs, and atypical OBPs (Venthur et al., 2014). 11 Classic-OBPs (six conserved Cysteine residues) and five Minus-C OBPs identified in *I. duplicatus* whereas 16 minus-C OBPs were found in *I. acuminatus*. We found four IdupOBPs with one additional cysteine at the C-terminal region and classified them as atypical OBPs.

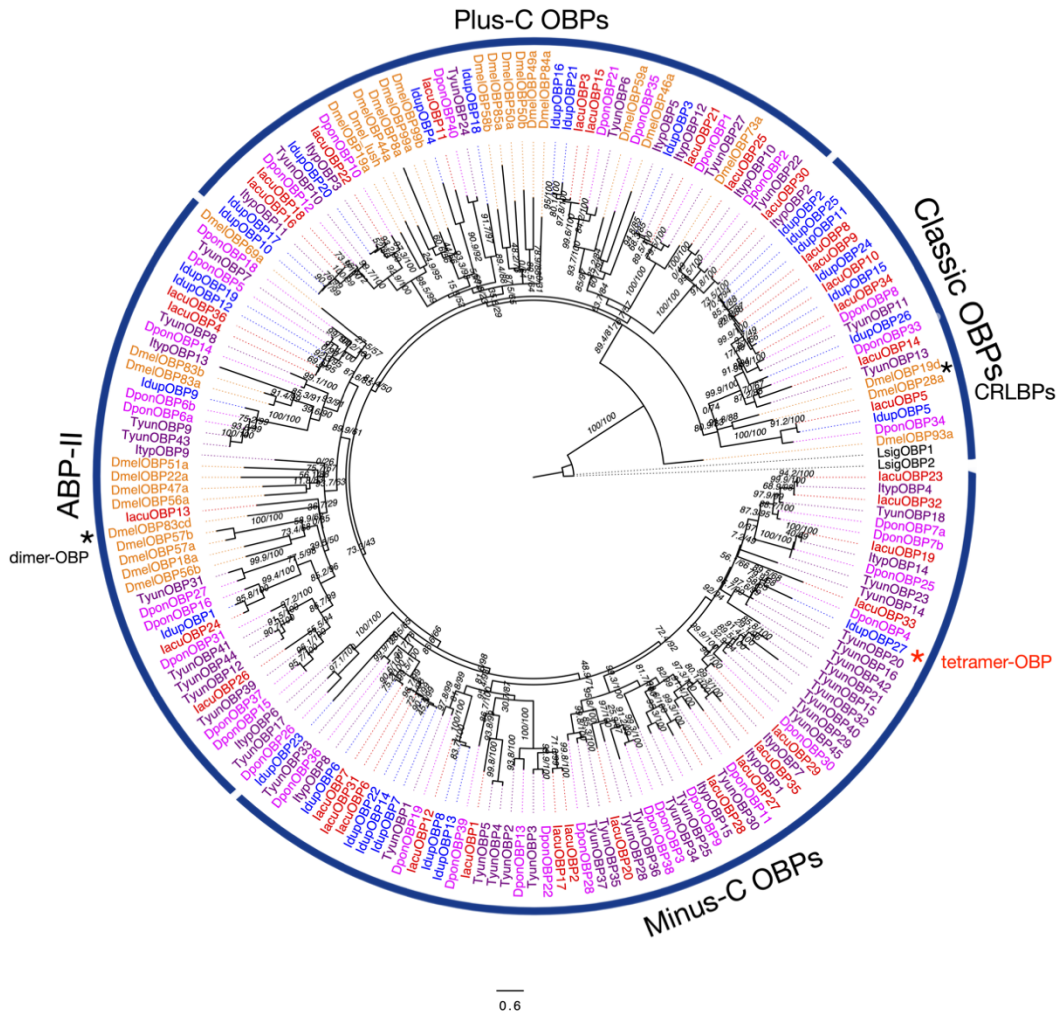

Figure 1.4. Maximum likelihood phylogeny of bark beetle OBPs. The tree was reconstructed using predicted OBP sequences from bark beetles. The IacuOBPs were colored in red and other OBPs were colored as: *I. typographus* (maroon), *D. ponderosae* (magenta), *D. melanogaster* (orange), *T. yunnanensis* (violet), and *L. y-signata* OBPs were used as outgroup. OBP subfamilies are marked at taxa labels. CRLBPs and dimer-OBP from *D. melanogaster* are marked with (*), and tetramer-OBP from *I. duplicatus* is marked with (red text and *). The branch labels indicate SH-aLRT value/bootstrap value. The scale represents amino acid substitutions per site. OBP subfamilies are marked at taxa labels based on sequence analysis; however, sequence homology is less between the groups.

Based on the BIC score, the maximum likelihood IdupOBP phylogeny was reconstructed using LG+R4 as an amino acid substitution model. The phylogeny provided more resolution to the classification, as OBPs show less sequence similarity across insect orders (Venthur and Zhou, 2018). Based on these functions, OBPs are classified into general OBPs and antennal binding proteins (Venthur et al., 2014) which potentially include pheromone binding proteins (PBPs). PBPs are key OBPs specifically involved in the binding and transport of pheromones to the receptor and are well characterized in many insect orders (Antony et al., 2018; Große-Wilde et al., 2006). The antennal OBPs are highlighted in orange in the phylogeny, in which a clade of PBPs is highlighted in yellow (Figure 1.4) based on the sequences from characterized PBPs (Wojtasek et al., 1998). The Minus-C OBP clade is highlighted in blue, whereas the remaining OBPs are considered general OBPs. The chemical-sense-related lipophilic-ligand-binding protein (CRLBP) (Hekmat-Scafe et al., 2002) is marked with (*); however, no orthologs were found in bark beetles.

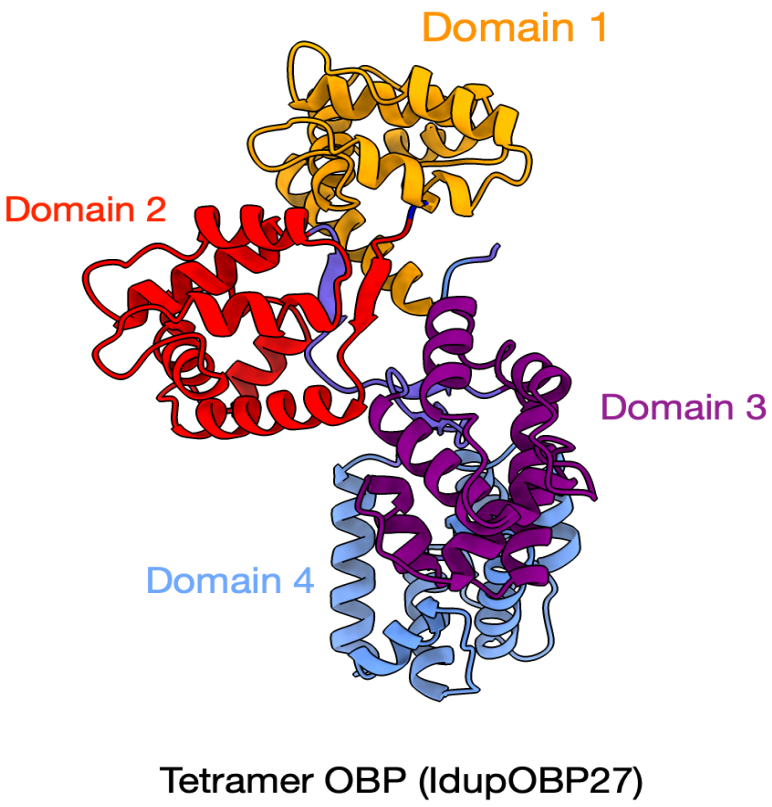

Figure 1.5. Predicted 3D structure of the tetramer-OBP, *IdupOBP27*. The structure was predicted using Alphafold3, excluding the signal peptide predicted using SignalPv6.

Interestingly, IdupOBP27 was found to have 12 cysteine residues in the C-terminal region; however, it showed no orthology to the dimer OBP DmelOBP83cd, reported from *D. melanogaster* (Hekmat-Scafe et al., 2002; Sánchez-Gracia and Rozas, 2008) (Figure 1.4). Further analysis revealed its sequence similarity to OBPs from *Dendroctonus adjunctus* (ACN: QKV34985.1) and *D. ponderosae* (ACN: AGI05167.1), and the presence of four structural domains concluded *IdupOBP27* as a tetramer-OBP (Figure 1.5). The predicted structure of the protein is provided in Figure 1.5. However, no ortholog was found from both *I. acuminatus* and *I. typographus* (Figure 1.4), but DponOBP4 was found to be the most similar bark beetle sequence in phylogeny.

Chemosensory proteins (CSPs) in bark beetles

Chemosensory proteins are characterized by the four conserved cysteine residues that bind to odorants and pheromones in insects (Pelosi et al., 2006). We identified nine chemosensory proteins each *in I. duplicatus* and *I. acuminatus* in our antennal transcriptome screening. Eight of them were found to have four conserved cysteine residues, except IdupCSP6. Interestingly, IdupCSP7 was found to have a long C-terminal chain. The maximum likelihood phylogeny of CSPs reconstructed based on the LG+R4 amino acid substitution model (based on the BIC score) allowed further comparisons between species (Figure 1.6). Only six CSPs were reported from *I. typographus* (Andersson et al., 2013), and we found orthologs of five ItypCSPs except for *ItypCSP6*. However, 1:1 orothology was found in *D. ponderosae* CSPs, as 11 CSPs were reported from that species (Andersson et al., 2013). Unlike other protein families studied, bark beetle-specific expansions were not found in CSPs (Figure 1.6). However, species-specific expansions were observed only in *D. melanogaster* CSPs (Figure 1.6).

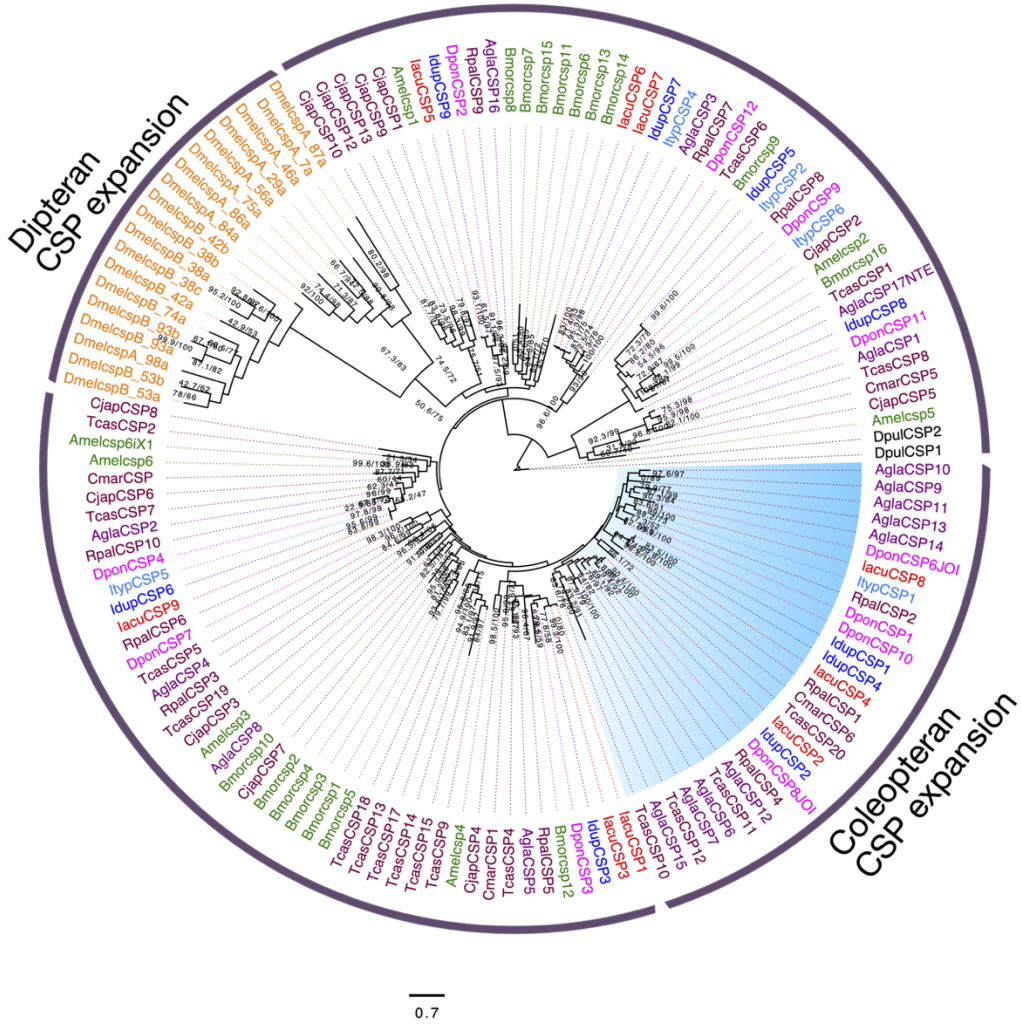

Figure 1.6. Maximum likelihood phylogeny of bark beetles CSPs. The tree was reconstructed using predicted CSP sequences from *I. duplicatus*, *I. acuminatus* and selected CSPs from other insect orders. The IdupCSPs and IacuCSPs were colored in blue and red respectively. The other coleopterans were colored in (brown) and *A. glabripennis* in magenta. *Bombyx mori* and *Apis mellifera* were represented in green. *D. pulex* (black) CSPs were used as an outgroup. The Diperan and Coleopteran-specific CSP expansions are marked at the taxon labels with later highlighted in blue. The branch labels indicate SH-aLRT value/bootstrap value. The scale represents amino acid substitutions per site.

Sensory neuron membrane proteins in bark beetles

A total of six SNMPs in *I. duplicatus* and five in *I. acuminatus* were initially annotated as SNMPs. Using a maximum likelihood phylogeny, three IdupSNMPs were grouped as SNMP classes 1 (Figure 1.7) and 2. Two SNMP candidates, *IdupSNMP1a* and *IdupSNMP1b,* were identified as SNMP1a and SNMP1b classes of proteins, respectively (Figure 1.7). Only one SNMP2 class of protein identified in *I. duplicatus* belonged to the 2b group and was named *IdupSNMP2b* (Figure 1.7). However, no IdupSNMP1s were detected as Group 3, and none in the IdupSNMP2 class belonged to Group 4. Whereas in *I. acuminatus* one each was identified from all four SNMP classes Ia, 1b, 2a, 2b and none were found from group 3 and group 4. Three candidates: *IdupSNMPc6*, *IdupSNMPc10* and *IdupSNMPc12* were identified as SNMPs with low blast identity scores respectively to *Anthonomus grandis* (ACN: AWF93834.1), *Drosophila navojoa* (ACN: XP_017969087.1) and *Meteorus pulchricornis* (ACN: QCS38482.1). These three SNMPs were highly divergent in the phylogeny (Figure 7), but were not related to CD36 croquemort protein used as outgroup but shared sequence similarity to scavenger receptor class B proteins. Ungrouped SNMPs were named with the suffix 'c' followed by a contig number. SNMP1 candidates shared orthology to IdupSNMPs and ItypSNMPs, whereas SNMP2b protein was an ortholog of *DponSNMP2b* as the only one SNMP2 protein was reported in *I. typographus* (Andersson et al., 2013) was grouped into SNMP2a sharing orthology to *DponSNMP2a*.

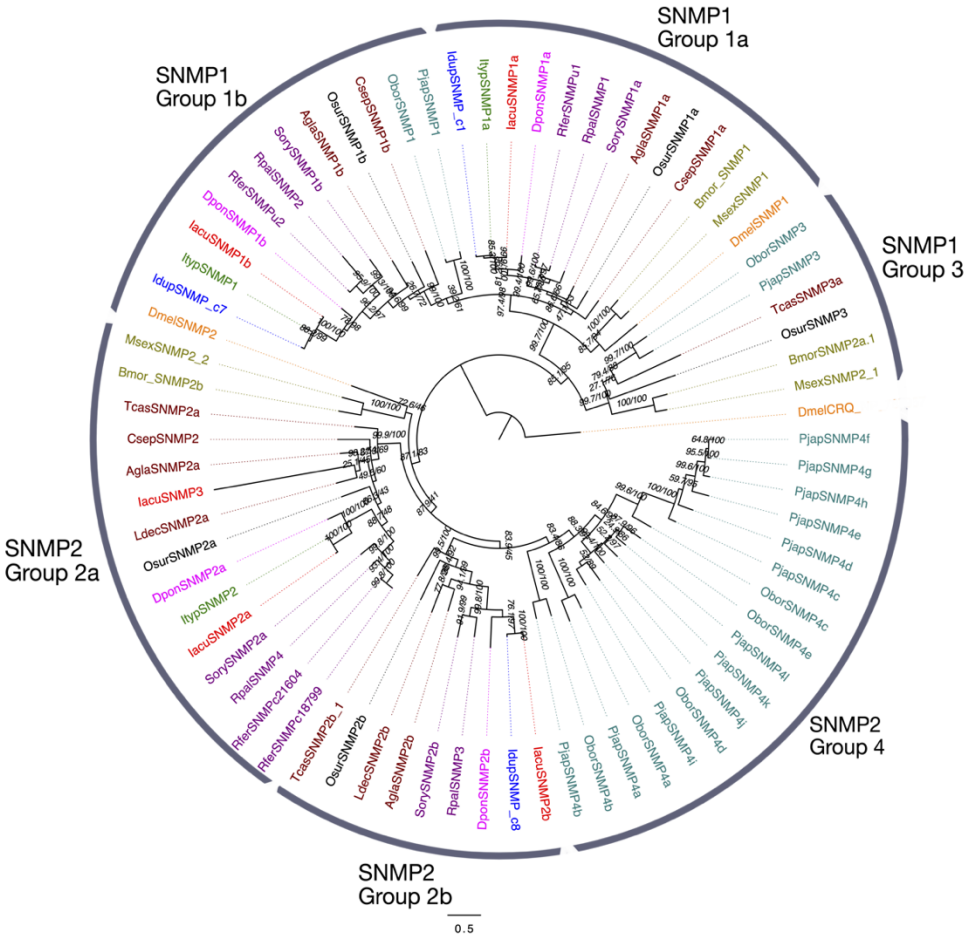

Figure 1.7. Maximum likelihood phylogeny of bark beetle SNMPs. The tree was reconstructed using predicted SNMP sequences from *I. duplicatus*, *I. acuminatus* and selected CSPs from other insect orders. The SNMP1 and SNMP2 proteins were highlighted in orange and blue, respectively. The IacuSNMPs and IdupSNMPs were coloured in red and blue, respectively; and others were colored as *I. typographus* (green), *D. ponderosae* (magenta), *R. palmarum, R. ferrugineus and S. oryzae* in violet, *A. glabripennis* (brown), *D. melanogaster* (orange), *T. castaneum* (dark blue), *Manduca sexta and B. mori* in olive; *Popilio japonica* and Scarabaeidae-specific SNMPs were used for SNMP2 Group 4 classification in light green. All SNMP subgroups are marked outside taxon labels. A non-SNMP protein, croquemort (crq) from *D. melanogaster* was used as an outgroup. The branch labels indicate SH-aLRT value/bootstrap value. The scale represents amino acid substitutions per site.

Results

Chapter 2: Conserved orthology in termite chemosensory gene families

Partially based on the manuscript:

> **Johny, J.**, Diallo, S., Lukšan, O., Shewale, M., Kalinová, B., Hanus, R., Große-Wilde, E., 2023. Conserved orthology in termite chemosensory gene families. *Front. Ecol. Evol.* 10. doi:10.3389/fevo.2022.1065947

Results

De novo antennal transcriptome sequencing and assembly

The antennal transcriptome data for *N. cubanus, P. simplex* and *I. inquilinus* were generated using Illumina-generated paired-end sequencing. This yielded 48.0 million read pairs from *N. cubanus* libraries, resulting in 247,031 transcripts. Trinity *de novo* assembly, with a total of 53,949 predicted ORFs. The same approach generated 46.3 million read pairs, yielding 180,250 transcripts with 58,126 predicted ORFs in *P. simplex*, and 30.6 million read pairs assembled into 203,568 transcripts that included 52,980 predicted ORFs in *I. inquilinus*. Next, we performed BUSCO 5.3.2 analysis as a measure for completeness of the transcriptomes, using the insecta10 dataset as a reference. This analysis showed 97.4 %, 97.3% and 97.2% completeness for *N. cubanus, P. simplex* and *I. inquilinus*, respectively. An overview of the sequencing and assembly statistics is provided in Table 2.1.

Table 2.1: *De novo* transcriptome assembly statistics of the three species of termites, *N. cubanus*, *P. simplex* and *I. inquilinus*

	Neotermes cubanus	*Prorhinotermes simplex*	*Inquilinitermes inquilinus*
Total number of raw reads	48,033,206	46,252,432	30,635,256
Number of transcripts	247,031	180,250	203,568
Full length ORFs	53,949	58,126	52,980
N50 length	2,273	3,482	2,356
GC content	39.57	39.55	40.19
Complete BUSCOs (insectaodb_10, %)	97.4	97.3	97.2
Fragmented BUSCOs (insectaodb_10, %)	1.0	1.0	0.6

Termite odorant receptors

A total of 30, 54 and 28 ORs from the antennal transcriptomes of *N. cubanus, P. simplex* and *I. inquilinus,* respectively were annotated. Of these, 24, 48 and 27 predicted proteins, respectively, presented an OR-typical transmembrane profile in TMHMM analysis (Krogh et al., 2001), and a length of >350 aa, which we considered to be full-length ORs. Next, we reconstructed a maximum likelihood phylogeny using the predicted amino acid sequences of our candidate ORs from the three studied species, as well as other termite-ORs that were previously reported from *Z. nevadensis* (Terrapon et al., 2014)*, C. secundus* (Harrison et al., 2018) and *R. speratus* (Mitaka et al., 2016). We also added OR coding sequences of *B. germanica* (Robertson et al., 2018), as well as ORs from other major insect orders, to stabilize the phylogenetic analysis and assist in the examination of our newly identified termite ORs. To add more resolution to the phylogeny, we also added a set of recently reported 'primitive ORs' from the silverfish *Lepisma saccharina* (Thoma et al., 2019). Finally, we included gustatory receptors from *D. pulex* that had previously been shown to be an outgroup for all insect ORs (Pẽalva-Arana et al., 2009).

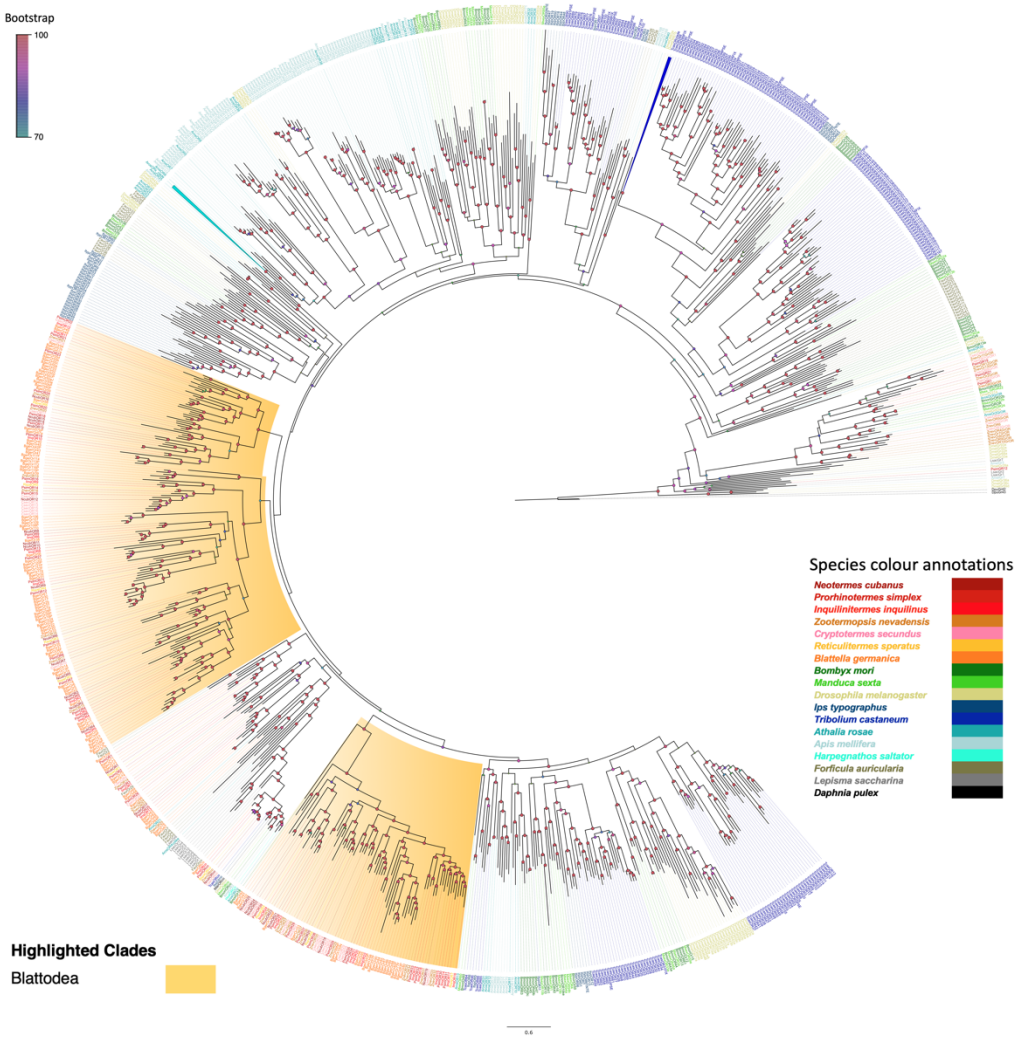

Figure 2.1. Maximum likelihood phylogeny of termite ORs. The tree was reconstructed using predicted OR sequences from termites and other insect orders and rooted using GRs as an outgroup. ORs from other species were named and colored as follows: Blattodea: *R. speratus* (Rspe, orchid blue), Csec: *C. secundus* (Magenta), Ncub: *N. cubanus* (Green), Psim: *P. simplex* (Blue), Iinq: *I. inquilinus* (Pink), Znev: *Z. nevadensis* (Purple), and Bger: *B. germanica* (Orange); Lepidoptera (Green): *B. mori* and *M. sexta*; Diptera (yellow): *D. melanogaster*; Coleoptera (blue): *I. typographus* and *T. castaneum*, Dermaptera (purple): Faur: *F. auricularia* and Hymenoptera (teal-green): *A. rosae* and *A. mellifera*; Outgroup: *D. pulex* and primitive ORs (Maroon): *L. saccharina*. Node colour indicates the bootstrap support value based on 1000

replicates. The scale bar indicates the estimated amino acid substitutions per site.

The phylogeny, rooted using the *D. pulex* GR outgroup, revealed the monophyly of OR and GR gene families with high bootstrap support. Between these two major clades was a group of 'GR and OR-like' sequences representing mainly termites and Lepidoptera. Adding a larger number of non-Isoptera sequences, including ORs of the basal insect *L. saccharina* helped stabilising the phylogeny of this clade of GR and OR-like sequences, with Isoptera sequences sharing more sequence similarity with GRs than with the highly expanded OR families across other insect orders. We found representatives from *Z. nevadensis, C. secundus* and *P. simplex* within this clade, but not from other termite species or *B. germanica*. The ORs of Zygentoma formed an ancestral clade with high bootstrap support, and within this, ORCos appeared as highly derived sequences, fitting reports of the evolutionary origin and ancestral nature of ORCo sequences (Brand et al., 2018; Missbach et al., 2014; Thoma et al., 2019). The termite ORCos formed a subset with primitive ORCo from *L. saccharina* as an ancestral sequence. The three Isoptera-specific expansions in ORs are in accordance with the other insect orders and indicate an evolutionary pattern, i.e. an ancestral set of ORs that share orthologous sequences between most insect orders, and a rapidly evolving set with multiple species-specific expansions, as mainly observed in Coleoptera and Hymenoptera (Andersson et al., 2019). We found 13 isopteran ORs in the ancestral clade, sharing orthologs to different insect orders and the remaining ORs formed two independent Isoptera-specific expansions of 50 ORs and 37 ORs. Within these two expansions, the most recently evolved one (37 ORs) shares sequence similarity with Hymenopteran ORs whereas the other one (50 ORs) was similar to the ancestral isopteran clade (Fig. 2.1).

Termite gustatory receptors

The manual annotation revealed 20, 25 and 26 GRs respectively from the antennal transcriptomes of *N. cubanus*, *P. simplex* and *I. inquilinus*. Several of these sequences already were identified in the search for Ors, and have been

labelled as GRs in figure 1. Among these candidate genes, 8, 7 and 6 receptors respectively from *N. cubanus, P. simplex* and *I. inquilinus* belong to the clade containing the *D. melanogaster* CO2 receptor clade. Similarly, 6, 3 and 4 candidates respectively from *N. cubanus, P. simplex* and *I. inquilinus* belong to the sugar receptor clade, and 4, 15 and 16 candidates respectively to the bitter taste receptor clade (Fig. 2.2). Isoptera- specific expansions were observed in all three subclades. However, we found no clear 1:1 orthologous for the *D. melanogaster* pheromone sensitive or CO2 GRs in any of the Isopteran GRs compared. Putative orthologs for the *D. melanogaster* fructose receptor Gr43a were present in *Z. nevadensis, C. secundus* and *T. castaneum.*

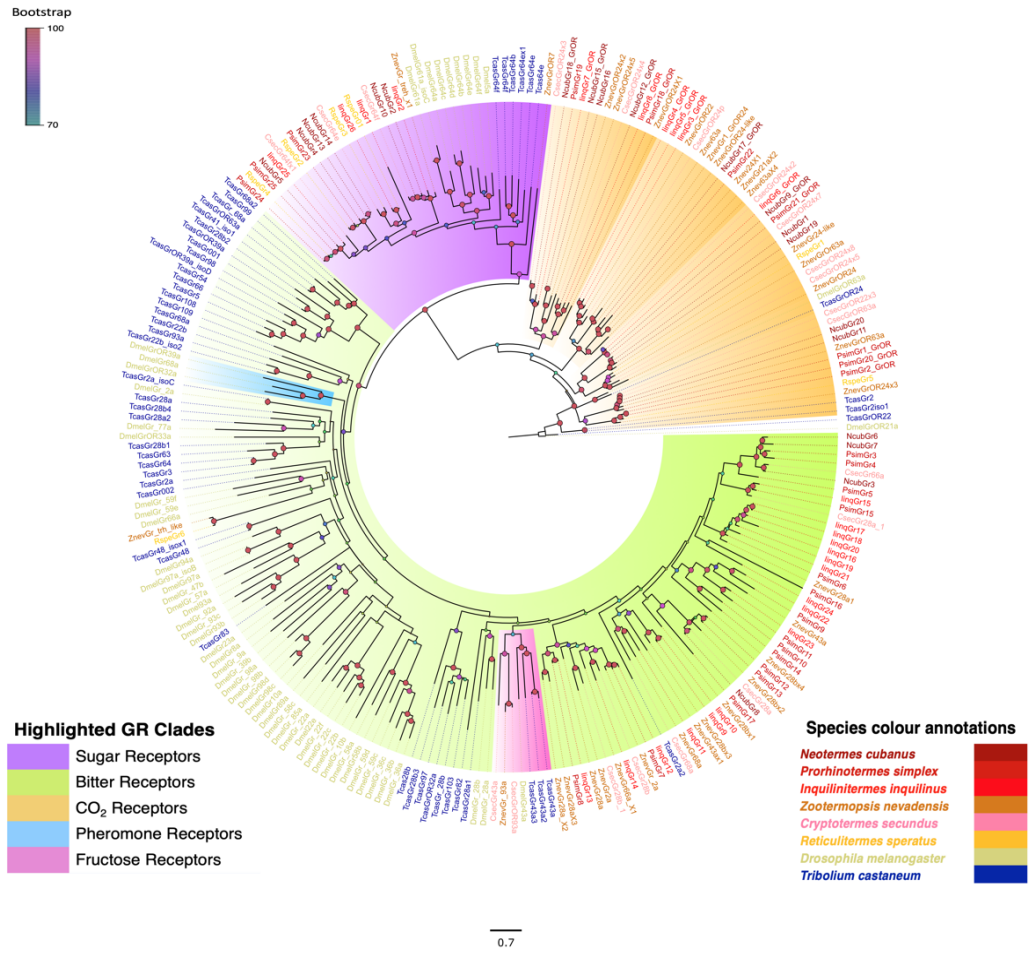

Figure 2.2. Maximum likelihood phylogeny of termite GRs highlighting known taste and CO_2 receptors. The highlighting details are provided in-figure legend. The tree was constructed using JTT+F+G4 as best-fit amino acid substitution model and rooted with CO_2 and sugar receptors

as their basal location reported earlier in analyses with GRLs of other animals (Robertson, 2015; Robertson et al., 2018). Species included in the phylogeny are *N. cubanus* (Ncub), *P. simplex* (Psim), *I. inquilinus* (Iinq), *Z. nevadensis* (Znev) *R. speratus* (Rspe), *C. secundus* (Csec), *T. castaneum* (Tcas) and *D. melanogaster* (Dmel). Sequence names are colored according to species, and color codes are provided in the in-figure legend. Node color indicates the bootstrap support value based on 1000 replicates. The scale bar indicates the estimated amino acid substitutions per site.

Isoptera-specific expansions in termite antennal ionotropic glutamate receptors

BLASTx searches were performed using well-annotated IR and iGluR sequences from different insect orders, Coleoptera, Lepidoptera, Hymenoptera and Diptera (Croset et al., 2010) recovered, 98, 95 and 77 transcripts from the antennal transcriptomes of *N. cubanus, P. simplex* and *I. inquilinus,* respectively. Based on length of the predicted protein, as well as presence of all IR-typical domains, we considered 33, 53 and 29 transcripts from *N. cubanus, P. simplex* and *I. inquilinus*, respectively, as complete. In multiple sequence alignment, we confirmed iGluRs family members by the presence of a characteristic conserved arginine (R) residue in the S1 domain involved in binding the glutamate α-carboxyl group (Benton et al., 2009; Croset et al., 2010). We further classified these receptors into the three distinct iGluR subfamilies (AMPA, NMDA, kainate) based on homology.

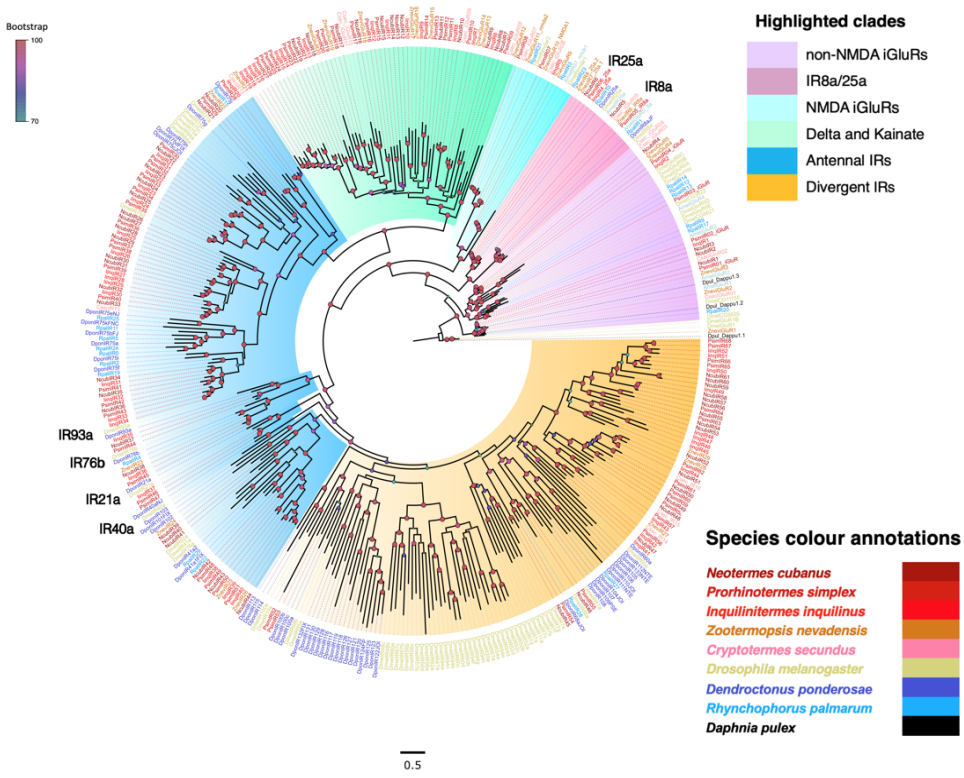

Figure 2.3: Phylogeny of termite IRs showcasing major iGluR subfamilies. The tree was reconstructed using the maximum likelihood method using LG+F+R as the best-fit amino acid substitution model and was rooted with non-NMDA iGluRs as an outgroup. Major iGluR subfamilies were highlighted as non-NMDA iGluRs (Purple), IR8a/25a (pink), NMDA (light blue), delta and kainate IRs (light green), antennal IRs (blue), Divergent IRs (orange). Known conserved IR subgroups are marked in the tree. Each species represented were named as *C. secundus* (Csec), *N. cubanus* (Ncub), *P. simplex* (Psim), *I. inquilinus* (Iinq), *Z. nevadensis* (Znev), *D. melanogaster* (Dmel), *D. ponderosae* (Dpon), *R. palmarum* (Rpal) and *D. pulex* (Dpul). Sequence names are coloured according to species and colour codes are provided in the in-figure legend. The node colours indicate the bootstrap support (1000 replicates) and the scale represents the estimated amino acid substitutions per site.

Finally, IR subfamily members were identified based on the absence of conserved aspartate (D) or glutamate (E) in the second half of the S2 domain that interacts with the α-amino group of the glutamate ligand. Partial sequences that were too short to include these protein domains were excluded from the analysis, but classified based on homology alone. For the phylogenetic analysis, we used our newly identified iGluR sequences, as well as other termite iGluR and IR sequences that were previously reported from *Z. nevadensis* (Terrapon et al., 2014) and *C. secundus* (Harrison et al., 2018). We also added iGluR coding sequences from other major insect orders to stabilize the analysis and assist in the annotation of newly identified termite IRs and iGluRs. Finally, we used non-NMDA iGluRs from the *D. pulex* as an outgroup as these receptors are considered ancestral to both NMDA iGluRs and IRs (Croset et al., 2010).

After rooting, the dendrogram revealed clear monophyletic clades for each major iGluR subfamily with maximum bootstrap support (Fig. 2.3). The non-NMDA iGluR subfamilies appeared basal in the phylogeny, with the IR8, IR25a, and the NMDA clades highly derived. We found representative sequences from all three clades in all three termite transcriptomes with the exception of IR8a, which was not found in *I. inquilinus.* The remaining iGluRs formed three separate clades; the most ancestral one was an Isoptera-specific clade with sequences similar to 'kainate' and 'delta' iGluR subfamilies. Based on the classification scheme used in *Drosophila* IRs the other two were antennal IRs and divergent IRs (Benton et al., 2009). In total, we found 13, 19 and 14 transcripts from *N. cubanus, P. simplex* and *I. inquilinus,* respectively, in the Isoptera-specific clade of ancestral IRs. The three species shared a nearly equal number of antennal IRs (*N. cubanus*: 23, *P. simplex*:18, *I. inquilinus*: 20), *i.e.* 18-25% of total IRs identified. These numbers were on par or slightly higher than previously reported for other termite species for example, 12 IRs reported from *R. speratus* (Mitaka et al., 2016). The clade of divergent IRs showed weak bootstrap support. However, the Isoptera-specific expansion of both antennal and divergent IRs was well supported (Fig. 2.3).

Sensory Neuron Membrane Proteins (SNMPs) in termite antennal transcriptome

The next family of chemosensory proteins investigated were sensory neuron membrane proteins (SNMPs). BLASTx query using well-annotated sequences of SNMP1 and SNMP2 recovered six transcripts each from *I. inquilinus* and *P. simplex* and five from *N. cubanus* as SNMPs. Previously reported SNMPs from *Z. nevadensis* (Terrapon et al., 2014), *C. secundus* (Harrison et al., 2018) and SNMPs from other insect orders were added to our data for phylogenetic analysis using maximum likelihood algorithms. Additionally, we used a non-SNMP CD36 family protein, croquemort (crq) from *D. melanogaster* as an outgroup (Fig. 2.4). Based on the phylogeny, we identified 5 out of 6 transcripts each from *P. simplex* and *I. inquilinus,* and 4 out of 5 from *N. cubanus* as SNMP1. We found four Isoptera-specific SNMP1 subclades with high bootstrap support and thus, further classification in subtypes 'a' and 'b' as in other orders was not attempted. We identified one SNMP2 protein each from all the three-termite species analysed, which also formed an Isoptera-specific clade.

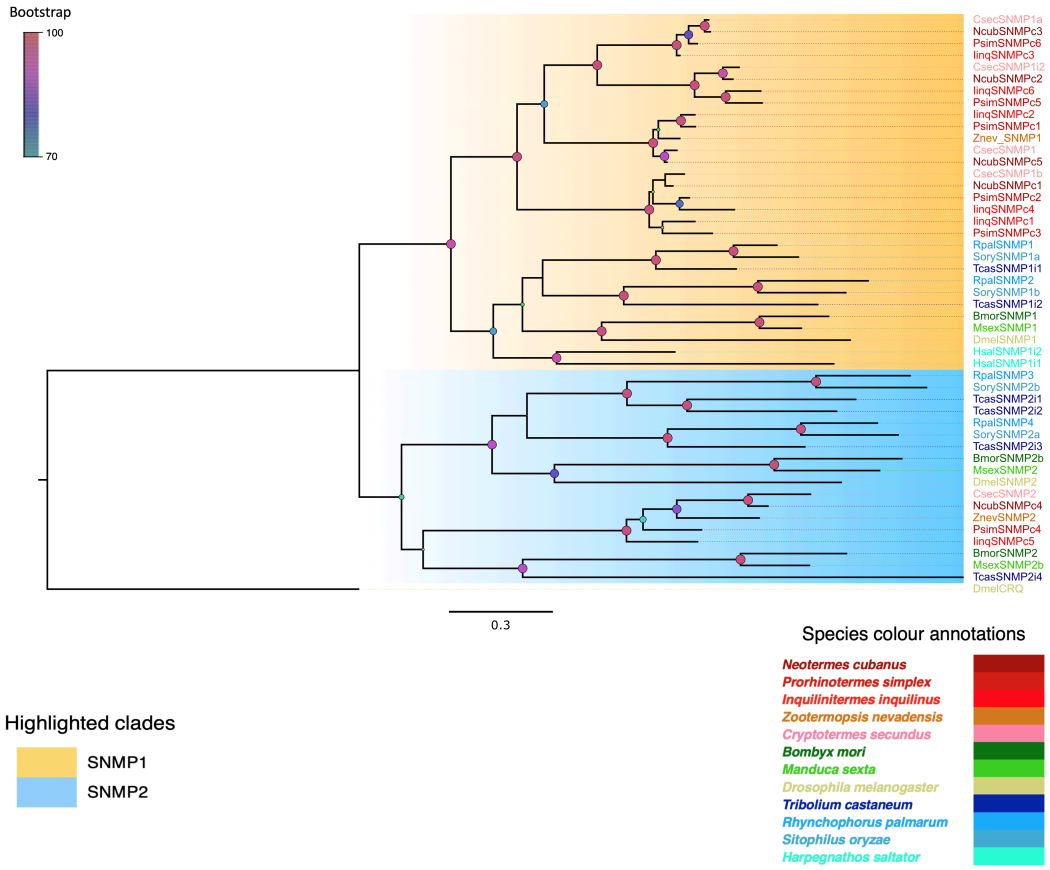

Figure 2.4. Maximum likelihood phylogenetic tree indicating two SNMP types in termites. The tree was constructed using LG+R as the best-fit amino acid substitution model under Bayesian information criterion. The two SNMP types are highlighted as SNMP1 (orange) and SNMP2 (blue). Species abbreviations and colours used are Bmor: *B. mori* (Green-Fern), Msex: *M. sexta* (Maroon), Dmel: *D. melanogaster* (Red), Csec: *C. secundus* (Magenta), Ncub: *N. cubanus* (Green), Psim: *P. simplex* (Blue), Iinq: *I. inquilinus* (Pink), Znev: *Z. nevadensis* (purple), Hs: *H. saltator* (Aqua Blue), Sory: *S. oryzae* (black), Tc: *T. castaneum* (Grey) and Rpal: *R. palmarum* (Teal). The node values indicate the bootstrap support (1000 replicates). The scale bar represents the estimated amino acid substitutions per site.

Soluble proteins (OBPs and CSPs) involved in termite chemoreception

Starting with well-annotated sequences from other insect species we also screened our transcriptomes for sequences encoding candidate OBPs. Using this approach, we recovered 29, 34 and 25 candidates from *N. cubanus, P. simplex* and *I. inquilinus,* respectively, with a predicted average amino acid length of 150 aa. Within the candidate OBPs, using the SignalP v6.0 (Teufel et al., 2022) signal peptides have been identified in numbers: 22 out of 29 from *N. cubanus,* 29 out of 34 from *P. simplex,* and 17 out of 25 from *I. inquilinus.* Based on sequence analysis we identified, 3, 2 and 2 transcripts each respectively from *N. cubanus, P. simplex* and *I. inquilinus* as Plus-C OBPs and 4, 3, 3 respectively from the same as Minus-C OBPs. Adding OBP sequences from other insect orders we constructed a maximum likelihood phylogeny to classify the new candidates. Besides the newly identified sequences, we included OBP protein sequences from two other termite species, i.e. *Z. nevadensis* (Terrapon et al., 2014) and *C. secundus* (Harrison et al., 2018), as well as OBPs from representative species of Diptera, Coleoptera, Lepidoptera and Hymenoptera (Brand et al., 2018; Große-Wilde et al., 2006; Vogt et al., 2015). OBPs of the basal hexapod *L. y-signata* were used as an outgroup (Missbach et al., 2015). The analysis allowed us to associate our candidates with the four major OBP sub-groups: classic, Minus-C, Plus-C and ABP-II types (Fig. 2.5). Additionally, we identified six Isoptera-specific expansions in the phylogeny. The 'Plus-C' subgroup contained two to three OBPs from each of our termite species at a basal position; classic and Minus-C OBP-subgroups each formed multiple clades in the phylogeny. Compared to other subgroups the most recently evolved Minus-C OBPs formed order-specific expansions (Blattodea, Coleoptera and Lepidoptera). Termite OBPs also possessed orthologs in multiple Isoptera-specific expansions.

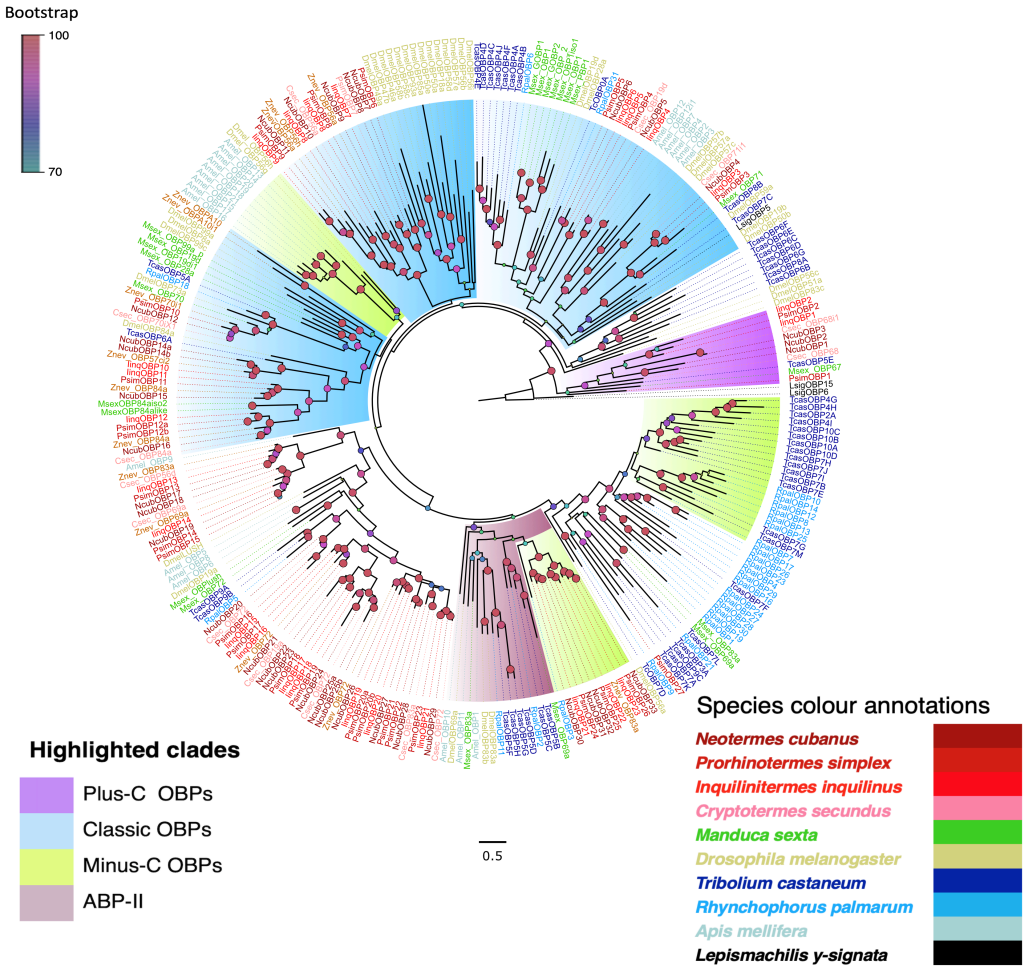

Figure 2.5. Maximum likelihood phylogeny of termite OBPs. The tree was constructed using LG+R as the best-fit amino acid substitution model and using *L. y-signata* OBPs as outgroup (cayenne). The major OBP groups are highlighted as Classic OBPs (blue), Minus-C (green), Plus-C (violet) and ABP-II (maroon). The isopteran-specific clade is highlighted in Orange. Sequences names are colored according to species. The species included are Msex: *M. sexta* (Maroon), Dmel: *D. melanogaster* (Red), Csec: *C. secundus* (Magenta), Ncub: *N. cubanus* (Green), Psim: *P. simplex* (Blue), Iinq: *I. inquilinus* (Pink), Znev: *Z. nevadensis* (purple), *T. castaneum* (asparagus) and Rpal: *R. palmarum* (Teal). The node values indicate bootstrap support and the scale bar represents estimated amino acid substitutions per site.

Next, we screened the transcriptomes for chemosensory proteins (CSPs), identifying 10, 6 and 9 CSPs from *N. cubanus*, *P. simplex* and *I. inquilinus,* respectively. We further examined these proteins by reconstructing a maximum likelihood phylogeny, using CSPs reported for *R. speratus* (Mitaka et al., 2016), and reference CSPs from species in other insect orders, while using *D. pulex* CSPs as an outgroup (Fig. 2.6). The phylogeny revealed species-specific CSP expansions in *D. melanogaster, T. castaneum* and *B. mori,* but not in termites. There were two evolutionary patterns observed in CSPs, one a highly divergent clade of CSPs from a large number of species and a second one with mostly single orthologs from each species.

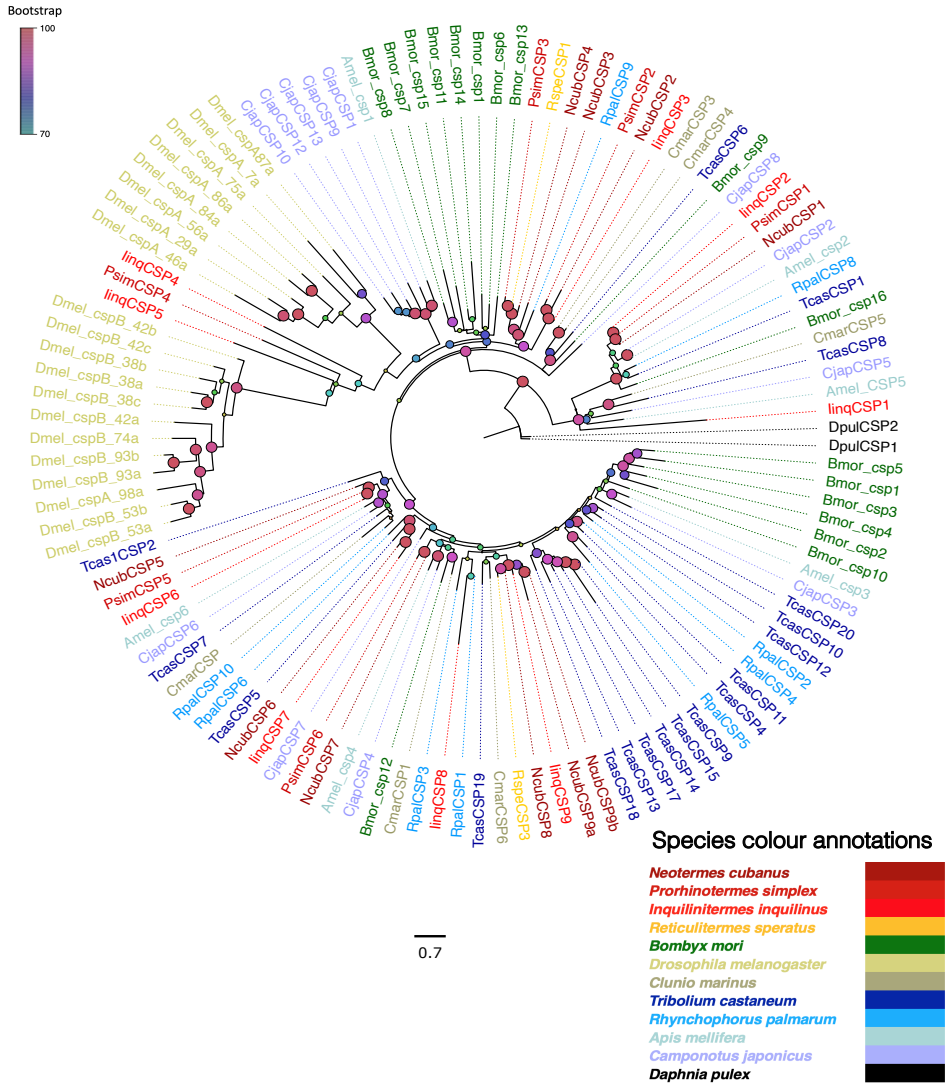

Figure 2.6. Maximum likelihood phylogeny of termite CSPs. The tree was constructed using LG+R as amino acid substitution model and rooted with *D. pulex* CSP sequences (black) as outgroup. Names of the newly identified termites CSPs were coloured as *N. cubanus* (Ncub, green), Psim: *P. simplex* (Psim, blue), *I. inquilinus* (Iinq, pink). The other species included were also colored *R. speratus* (Rspe, orchid blue), *T. castaneum* (Tc, grey), *R. palmarum* (Rpal, teal), *B. mori* (Bmor, aqua blue), *A. mellifera* (Amel, skyblue), *D. melanogaster* (Deml, red), *C. marinus* (Cmar, plum) and *C. japonicus* (Cjap, orange). The node labels represent bootstrap values (1000 replicates) and the scale bar represents estimated amino acid substitutions per site.

Results

Chapter 3

Population-level functional polymorphisms in *Ips typographus* pheromone receptor, *ItypOR33*

Partially based on the manuscript:

> **Johny, J.,** Diallo, S., Nadachowska-Brzyska K., Moliterno, A.A.C., Roy, A., Kalinová, B., Große-Wilde, E., Schlyter, F. 2024. Olfactory adaptations in bark beetle populations: Functional polymorphisms in Ips typographus pheromone receptor, ItypOR33. *Mol. Ecol.* (submitted)

Population genomics data credits: Dr. Krystyna Nadachowska-Brzyska

Identification and confirmation of natural polymorphism in ItypOR33

ItypOR33 belongs to subfamily-7 of coleopteran ORs (Figure S2) and was reported as a receptor with 403 amino acid residues (Andersson et al., 2013). The PCR amplifications recovered ItypOR33 with two mutations: F53Y and E266G (Figure 1A). Interestingly, we also found a population-level variant, *ItypOR33a,* with mutations K10E, L228I, K249Q, and K391R (Figure 1B). To confirm the existence of the variant, we repeated the PCR with proofreading polymerases and screened our antennal transcriptomes (n = 6) (Johny et al., unpublished). The screening confirmed mutations L228I and K249Q in *ItypOR33a*, but not the other two. Additionally, we cross-checked the gene (Figure 3.1F) with the published genome of *I. typographus* (Powell et al., 2021), but retrieved only *ItypOR33,* as the genome was from Scandinavia. Our analysis confirms that both versions of *ItypOR33* exist in the *I. typographus* populations, with key variations being L228I and K249Q in ItypOR33a (Figure 1B). We further analyzed the relative transcript abundance of *ItypOR33* compared to odorant receptor co-receptor (*Orco*) in the available antennal transcriptomes. The relative TPM (Transcripts Per Million) value of *ItypOR33* from the Scandinavian population has been estimated as 6.01 (Yuvaraj et al., 2021). However, in the Czech population (ItypOR33a), the relative TPM values were 5.54 and 4.77 in male and female antennal transcriptomes (n = 3 each) (Johny et al., unpublished). Further, we tested the selection pressure occurring at the clade of *ItypOR33* within the phylogeny of ItypORs and found purifying selection (ω = 0.088) occurring at the *ItypOR* sub-family 7, as expected.

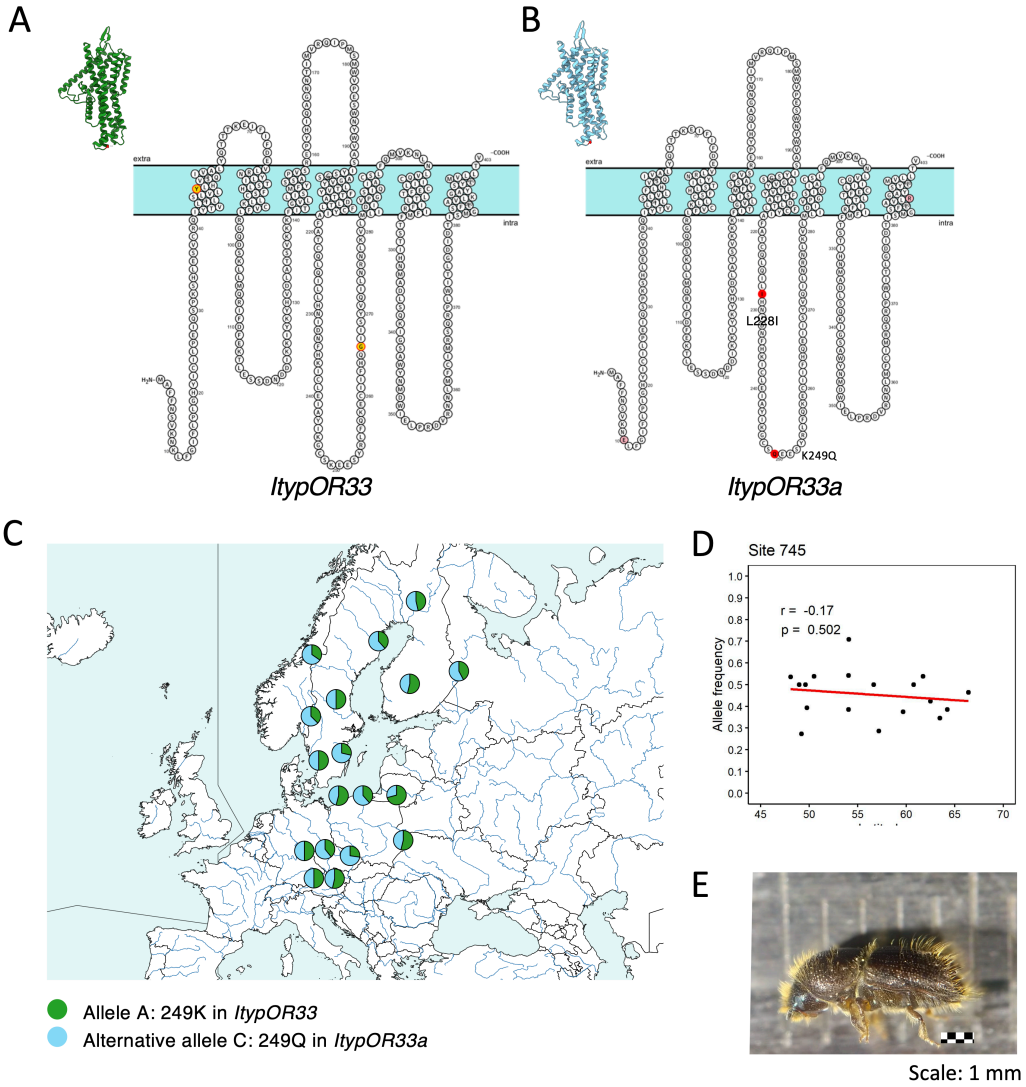

Figure 3.1. Polymorphic variants of *ItypOR33*. A and **B,** The membrane topology of *ItypOR33 and ItypOR33a*, marked with mutations (red) to the version published in (Andersson et al. 2013). The transmembrane regions were predicted by DeepTMHMM (Hallgren et al., 2022) and plotted using Protter v1.0 (Omasits et al., 2014). **C,** Geographic distribution of alleles present in nonsynonymous site 745 across European populations. The pie charts give frequencies of alleles in the reference genome (green) and an alternative allele (pale blue) identified in population genomic data. **D,** Correlation between allelic frequency

distribution of SNP site 745 (K249Q) and the latitude across 18 European populations **E,** Image of bark beetle *Ips typographus* indicating size **F,** Exon-intron map of *ItypOR33*. The dark boxes indicate exons, and connected lines indicate introns.

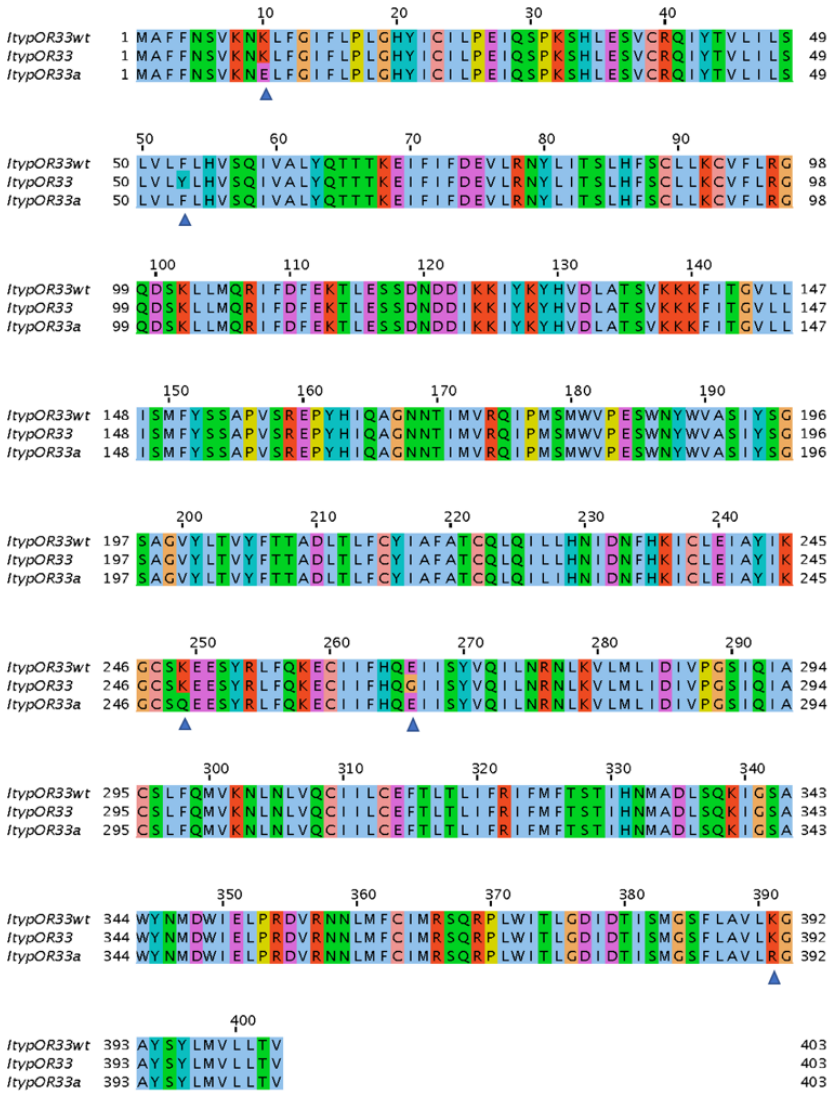

Figure 3.2: Multiple sequence alignment showing the variations in protein sequences of ItypOR33 and ItypOR33a against the published version of ItypOR33wt (Yuvaraj et al., 2021). The change in amino acids is marked below alignment.

Population genomics screening and analysis of *ItypOR33* variants

As the cloning confirmed the existence of variant *ItypOR33a* in our samples, we looked for this variant in bark beetle populations across Europe using

recent population genomics data, as *ItypOR33* has been recently mapped into one of the chromosomal inversions (Mykhailenko et al., 2023). However, we could confirm only one polymorphism (K249Q) within the analyzed 18 European populations (Table 3.2). Interestingly, the original version from Sweden and the new variant were present in similar frequencies among European populations (Figure 3.1 C and D). We found no correlation between the frequency of the K249Q variant and latitude (r = −0.17; p = 0.50) (Figure 3.1C) and no association between particular alleles at the site and inversion haplotype in European spruce bark beetle populations. The allele C for K249Q was present at both inversion haplotypes. Additionally, we found two nonsynonymous mutations that were polymorphic in the population dataset but not in samples used in the experiment. Similar to K249Q, both the additional sites were polymorphic across species range (Figure S3 A and C), and there was no correlation between longitude (r = 0.18 and r = −0.08; p = 0.47 and p = 0.75) nor association with inversion haplotype. Tajima's D value of −1.83 indicates more low-frequency alleles in *ItypOR33*, possibly due to population expansions. No specific sites were under positive selection as per MEME analysis.

Deorphanization of ItypORs using DNS

Effectiveness of the DNS in bark beetle OR deorphanization

We used ItypOR46, a receptor previously characterized using *in vitro* expression systems, to standardize the DNS deorphanization protocol, as it was not previously attempted in *I. typographus*. *ItypOR46* was cloned and expressed in *Drosophila* ab3 sensilla and screened with an odor panel that includes its previously reported ligand (*S*)-(−)-phenol. ItypOR46 responded strongly to a racemic mixture of ipsenol and ipsdienol with a strong neuronal response to ipsenol compared to ipsdienol as reported in HEK293 cell studies (Yuvaraj et al., 2021). Other compounds also elicited only weak secondary responses with average spikes/s less than 20 (Figure 3.3 A). We analyzed the dose response of the enantiomers for both ipsenol and ipsdienol and found that (*S*)-(−)-ipsenol elicited higher responses compared to the weak response

found only at the higher concentrations of (*R*)-ipsenol. Whereas (*R*)-ipsdienol, and (*S*)-ipsdienol did not show a strong response at any of the tested concentrations (Figure 3.3 B).

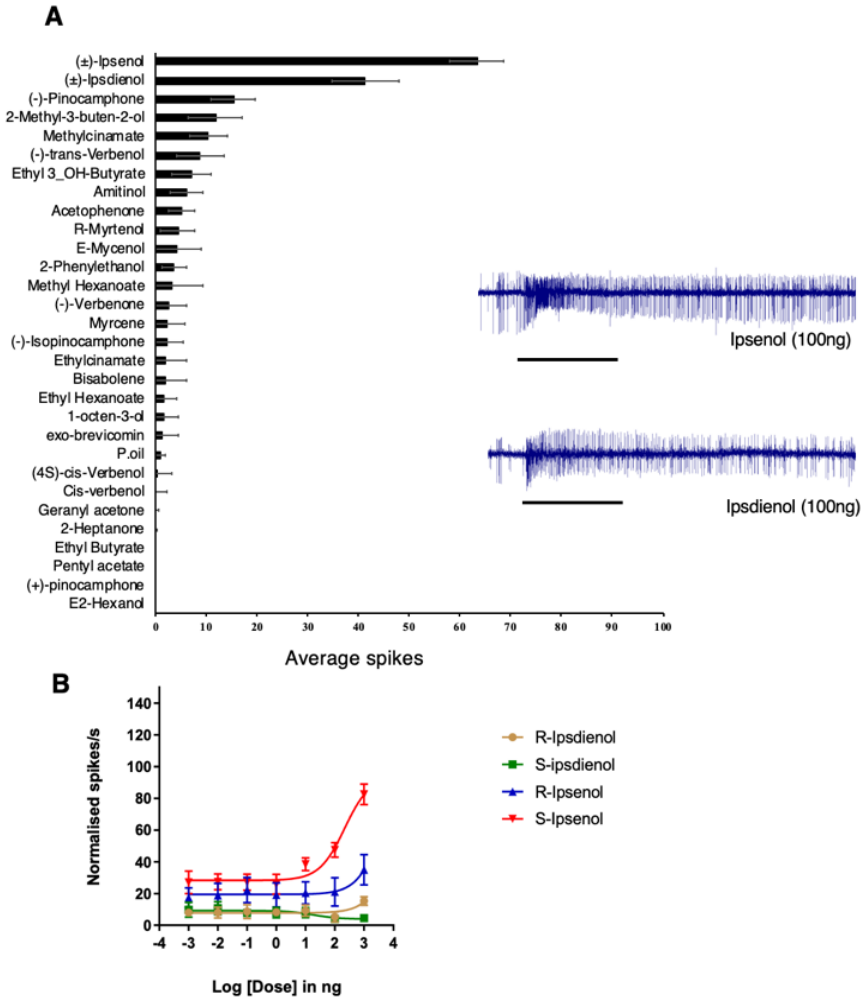

Figure 3.3. Tuning of ItypOR46 towards selected ligands. A, Mean response of ItypOR46 to a range of odorant doses and indicative trace for its response pattern. Error bars indicate SEM (n = 6-14) Traces of ab3A expressing *ItypOR46* responding to ipsenol and ipsdienol at 100ng provided in the insight. **B,** Dose-response curves of *ItypOR46* with n = 9 for (*R*)-(−)-ipsenol and (*S*)-(−)-ipsenol, n = 5 for (*S*)-(−)-ipsdienol and (*R*)-(−)-ipsdienol. Error bars indicate SEM.

ItypOR33 was tuned to *Ips* spp. pheromone component amitinol

After deorphanizing ItypOR46, we proceed to ItypOR33 following the same protocol. Using a panel of 88 compounds, including *Ips* pheromone, host, and non-host volatiles, we investigated the tuning pattern of *ItypOR33* in ab3 sensilla. We found that only four compounds (amitinol, ipsdienol, myrtenol, and ipsenol) elicited considerable spiking (>20 spikes/s) in SSR. Amitinol showed the strongest response, followed by *R*-myrtenol and ipsdienol (Figure 3.4). After finding the four ligands with variable response patterns, we checked the dose-response pattern to identify the key ligand of this receptor. We found that *ItypOR33* strongly responded to amitinol (<40 spikes/s) at 1 ng with a neuronal adaptation at a dose higher than 100 ng (Figure 2C). *R*-myrtenol and *S*-ipsdienol also elicited a moderate response (~40 spikes/s) at doses starting from 1ng (Figure 3.4 C). *R*-ipsdienol and *S*-ipsenol showed a secondary response (~30 spikes/s) at doses starting from 1 ng. *R*-ipsenol did not elicit any significant physiological responses. These results confirm amitinol as the primary ligand for ItypOR33.

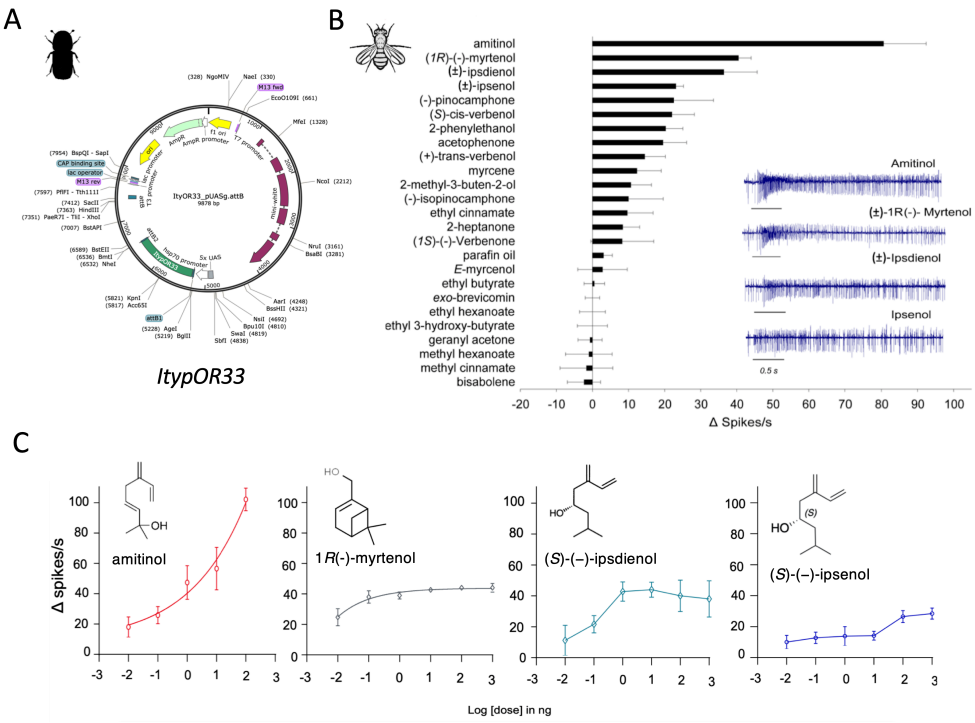

Figure 3.4. Functional characterization of *ItypOR33.* A, Expression vector constructed with ItypOR33 and pUASg.attB for the fly embryo injection. **B,** Mean response of ItypOR33 to a range of odorants and indicative traces for its response pattern. Error bars represent SEM (n > 4). **C,** Dose–response curves of ItypOR33 for amitinol, myrtenol, (*S*)-(−)-ipsdienol and (*S*)-(−)-ipsenol. Error bars represent SEM.

The variant *ItypOR33a* was tuned to (*S*)-(−)-ipsenol

After verifying the mutations and the existence of both variants *ItypOR33* and *ItypOR33a* in *I. typographus* populations, we cloned and expressed *ItypOR33a* in the *Drosophila* ab3 sensilla. To compare the response pattern of two receptor variants, we used the same odor set (Table 3.4). The receptor *ItypOR33a* responded to the racemic mixture of ipsenol with secondary responses to a few other compounds (Figure 3B). We also noted that several tested compounds generated a higher response after the stimulation with ipsenol and ipsdienol. A dose-response test evaluated the ligand specificity towards the two ipsenol enantiomers. *R*-ipsenol showed a weaker response in all the concentrations, while (*S*)-(−)-ipsenol showed dose-dependent responses. The ipsdienol enantiomers showed no consistent results at the tested doses (Figure 3.5 C). We also tested myrcene, (−)-pinocamphone, and ethyl cinnamate at various concentrations but found no strong responses. Our results confirm that ItypOR33a is tuned to (*S*)-(−)-ipsenol. As we found the new variant of ItypOR33a tuned to (*S*)-(−)-ipsenol, we compared its response pattern with ItypOR46, a receptor already deorphanized with the same ligand. We found a slightly lower response in ItypOR33a than ItypOR46 (Figure 3.5 D).

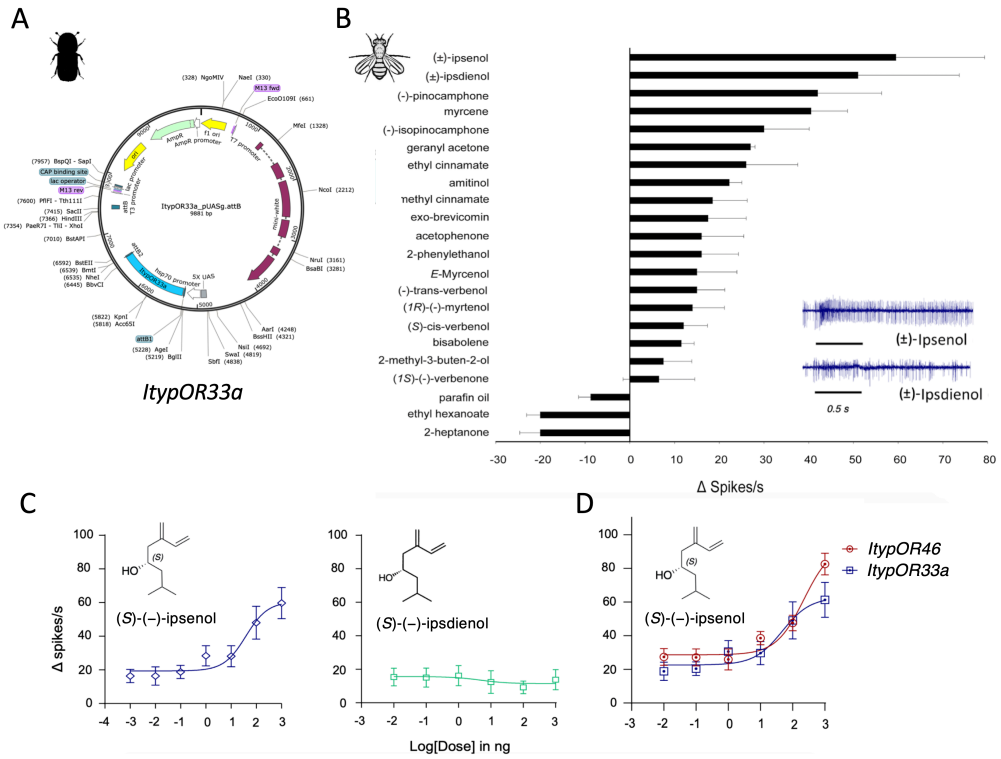

Figure 3.5. Functional characterization of *ItypOR33a*. A, Expression vector constructed with ItypOR33a and pUASg.attB for the fly embryo injection. **B,** Mean response of ItypOR33 to a range of odorants and indicative traces for its response pattern. Error bars represent SEM (n > 4). **C,** Dose–response curves of ItypOR33 for amitinol, myrtenol, (*S*)-(−)-ipsdienol and (*S*)-(−)-ipsenol. Error bars represent SEM.

Protein structural predictions and molecular docking

The Alpha fold 2, and AlphaFold3-based protein structural predictions showed high pLDDT values (predicted local-distance difference test) for monomeric and the predicted template modeling (pTM) score for the multimeric structures. AlphaFold3 predictions had pTM scores of 0.9 and 0.8, respectively, for the monomeric and heteromeric structure predictions. For each ItypOR33 monomeric structure, two potential binding sites were predicted (Figure 3.6 A-C). The binding site 2 (deep cavity) was selected for the docking based on the draggability score predicted by the Caver Analyst 2.0 (Jurcik et al., 2018).

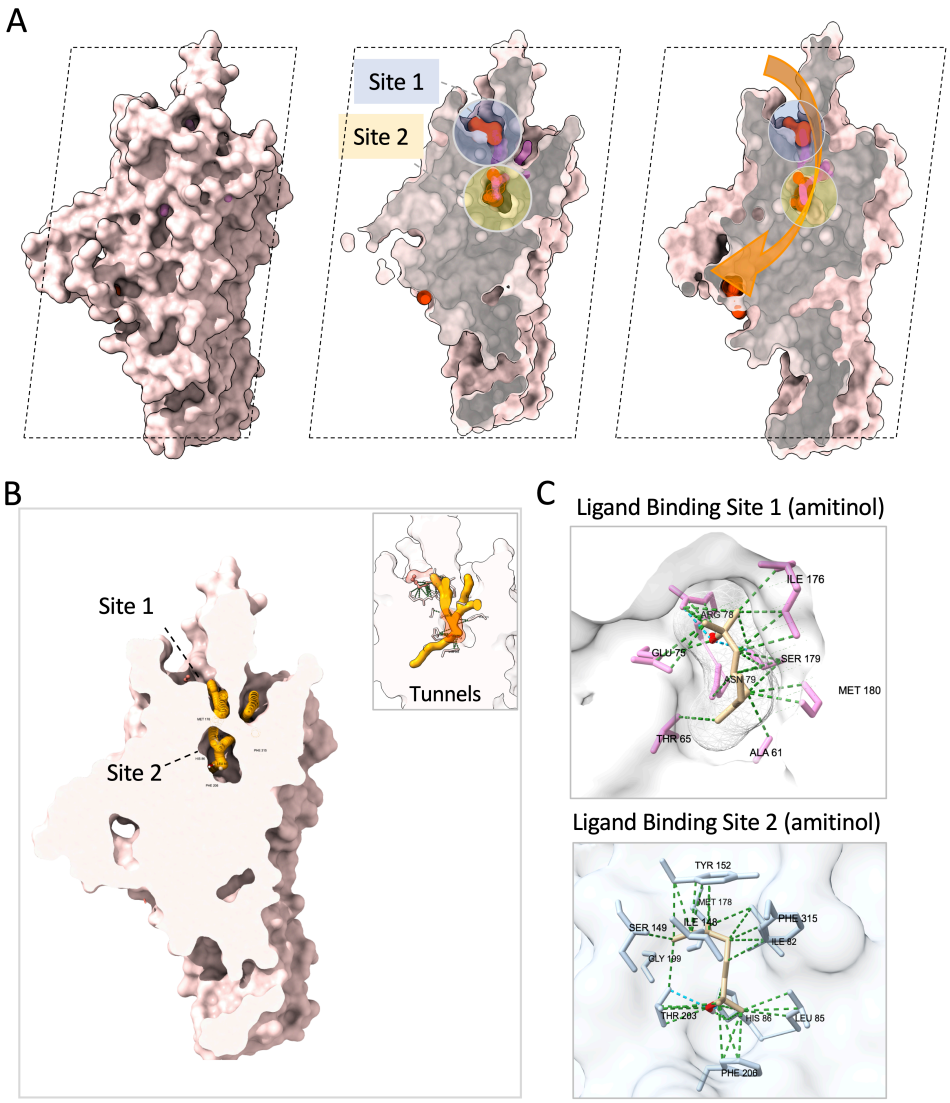

Figure 3.6. Ligand binding sites identified in *ItypOR33*. A, Surface model of *ItypOR33* with cross sections i) indicating the two ligand binding sites, ii) proposed ligand movement during ligand binding and activation. **B,** Cross section of the surface model showing predicted tunnels that connect ligand binding sites 1 and 2 (detailed in insight). **C,** *ItypOR33* Interactions with its ligand amitinol at ligand binding site 1(top) and site 2 (bottom). Hydrogen bonds are indicated in dashed blue lines and other atomic interactions in green. All interacting residues are labelled as three letter code and position.

For ItypOR33, the druggability scores were 0.35 and 0.75 for Sites 1 and 2, respectively. For ItypOR33a, the scores were 0.08 and 0.79, respectively, for Sites 1 and 2. The docking results at binding site 2 showed similar binding affinities for ItypOR33^{WT} and ItypOR33^{53Y,226G} as, −6.6 kcal/mol and −6.55 kcal/mol correspondingly for amitinol; and −6.2 kcal/mol, −6.3 kcal/mol respectively for ipsenol. For the polymorphic variant ItypOR33a^{10E,288I,249Q,319R}, the binding affinity towards amitinol was reduced to -6.1 kcal/mol, whereas affinity towards ipsenol increased to −6.5 kcal/mol. These results were in coherence with our SSR data. Analysis of ligand binding residues in *ItypOR33* revealed H-bond formations involving residues, 86H (Histidine) and 203T (Threonine) respectively, from second and fourth transmembrane (TM) helices (Figure 3.7 B) in its interaction with amitinol. Similarly, ItypOR33a's interaction with ipsenol also involved the same amino acids, indicating the role of additional contacts or interactions in ligand binding (Figure 3.8A and B). For *ItypOR33* key residues involved in additional bonds were Ile82, Leu85, Ile148, Ser149, Tyr152, Met178, Phe206 and Phe315 (Figure 5B). For *ItypOR33a,* the same residues, along with Val145, were found to be interacting with ipsenol, confirming the key residues of ligand binding site 2 (Figure 3.8 B). Although the predicted draggability was less, we also tested the binding affinities of both receptors with the same ligands at site 1. The analysis confirmed Site 1's lesser binding affinities than Site 2 with the results: ItypOR33 Site 1: amitinol (-4.88 kcl/mol) and ipsenol (-4.87 kcl/mol) and ItypOR33a Site 1: amitinol (-5.98 kcl/mol) and ipsenol (-4.98 kcl/mol). The analysis of tunnels allowed us to predict the possible ligand movement between binding sites 1 and 2. From *ItypOR33,* six tunnels have been identified, whereas, from ItypOR33a, 13 tunnels have been identified spanning site 2 (Figures 3.7A and 3.8A). The AlphaFold3-based multimeric predictions with ItypOR33:ItypOrco in a 1:1 ratio showed higher ipTM and pTM scores as 0.79 and 0.8, respectively, compared to 1:3 ratio (ipTM = 0.76, pTM = 0.79) indicating that 1:1 heteromeric structures are more likely to be true (Figures 3.7 and 3.8).

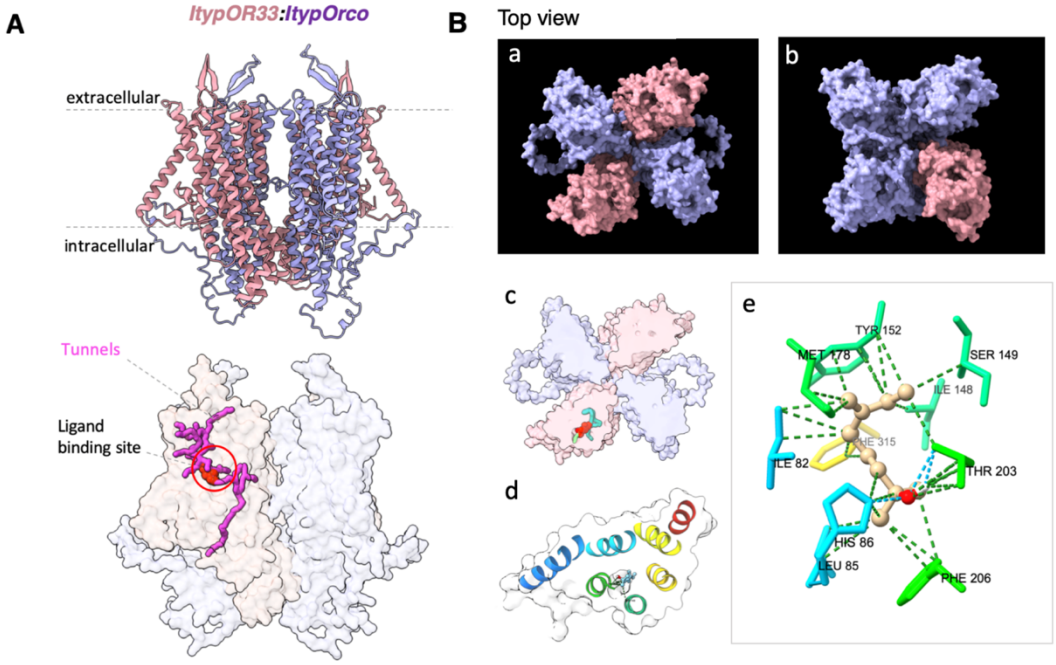

Figure 3.7. Predicted structural basis of ligand binding in *ItypOR33.* **A** (top), Predicted Heterotetrameric (closed) structure of ItypOR:ItypORco (1:1) using AlphaFold3. Monomers are colored as in the figure title. Extracellular and intracellular regions are marked, showing the loops. **A** (bottom), The surface model of ItypOR33:ItypOrco complex with marked ligand amitinol, (red) at binding site 2. The tunnels identified by Caver Dock are colored magenta. **B,** The surface top view of ItypOR33: ItypOrco complex in 1:1 ratio (a) and 1:3 ratio (b). A cross-section of the ligand binding site 2 with tunnels is shown in (c). The seven TM helices (colored) in the ItypOR33 monomeric model are shown in (d), and interactions with amitinol at the ligand binding site 2 are shown in (e). Hydrogen bonds are shown in dashed blue lines, and other interactions are in green. All interacting residues are labeled as three-letter codes and positions. Residues' colors (e) correspond to the seven TM helices in (d).

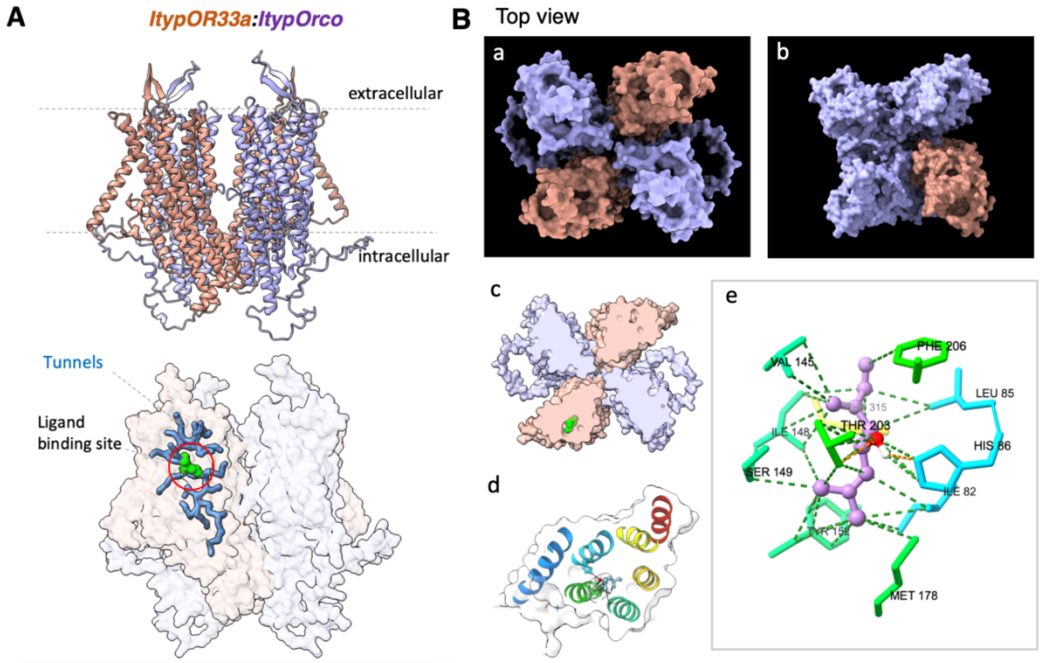

Figure 3.8. Predicted structural basis of ligand binding in *ItypOR33a.* **A** (top), Predicted heterotetrameric structure of ItypOR:ItypORco (1:1) based on AlphaFold3. Monomers are colored as in the figure title. Extracellular and intracellular regions are marked, showing the loops. **A** (bottom), The surface model of ItypOR33a: ItypOrco complex with marked ligand ipsenol (green) at binding site 2. The tunnels identified are colored in magenta. **B,** The surface top view of ItypOR33: ItypOrco complex in 1:1 ratio (a) and 1:3 ratio (b). The cross-section of ligand binding site 2 is shown in (c). The seven TM helices (colored) in the ItypOR33a monomeric model are shown in (d) and the interactions with ipsenol at ligand binding site 2 are shown in (e). Hydrogen bonds are shown in dashed blue lines and all other interactions are in green. All interacting residues are labeled as three-letter codes and positions. Residue colors (e) indicate the respective TM helices colors shown in (d).

Relative quantification of *ItypOR33* expression

The qRT-PCR results showed no significant changes in ItypOR33 expression in males and females with a mean fold change of 1.03 ± 0.28 and 1.18 ± 0.32

respectively for males and females (Mean ± SEM) and *T* (0.77) statistics $p = 0.48$ (Figure 7A).

Behavioral assays

As amitinol has been known as a heterospecific pheromone component in *Ips* spp., we evaluated its hitherto unstudied behavioral role in *I. typographus* using dual-choice bioassays. The bioassays were performed as described in the methods section. The bark beetles' preference index towards the test zone is provided in Figure 7B as a violin plot. The count data represented as a percentage of responses are also provided in Figure 3.10. In assay 1, known pheromone blend [2-methyl-3-buten-2-ol (MB) + *cis*-verbenol (cV)] against the solvent hexane was tested and found a significant attraction in the beetles to pheromone (χ^2 15.0, $p = 0.0001$) as expected, confirming the assay efficiency (Figure 7B). In assay 2, we tested amitinol against the solvent but found no significant attraction ($p = 0.19$) as expected, as amitinol is known to elicit responses in combination with pheromones in other *Ips* species (Kohnle et al., 1988). The amitinol and pheromone blend (MB+cV) with varying doses of amitinol (1 to 100 µg/mL) were tested in assays 3 to 5 and confirmed the highest attraction in the medium dose (10 µg/mL) plus MB+cV against pheromone alone (MB10:cV1) (χ^2 3.83, $p = 0.05$) (Figure 3.9B). We tested the sex-specific differences in amitinol responses using males and females (Schlyter and Cederholm, 1981), respectively in assays 6 and 7. We found significant attraction towards (MB10: cV1) + amitinol (10) *vs* control (MB10: cV1) in males (χ^2 4.33, $p = 0.04$), whereas female beetles' preferences to the same pheromone combinations were not significantly different from their preference to controls (χ^2 0.24, $p = 0.62$) (Figure 7B). Finally, we compared the preference indexes towards the amitinol plus pheromone blend in males and females using the Shapiro-Wilk test, followed by one-way ANOVA with Tukey's multiple comparisons, which confirmed that no sex-specific differences at $p < 0.05$ between assay 6 (males) and 7 (females), $p = 0.28$. The direct correlations of these responses to *ItypOR33* require gene knockout experiments, which are not currently established in *I. typographus* and are

beyond the scope of this research. However, we confirm these pheromone responses by electrophysiological methods.

Electroantennogram recordings

We quantified and confirmed the bark beetles' responses to amitinol using EAG. Moderate responses were observed to all the three stimuli (pheromone, amitinol, and a blend of pheromone + amitinol) in beetles tested (n = 10) with an amplitude range of 2 to 4.6 mV, respectively. However, we found no significant difference between males and females in any of the three stimuli with p values 0.40, 0.22, and 0.70, respectively, for pheromone, amitinol, and pheromone + amitinol (Shapiro-Wilk test for normality followed by one-way ANOVA and Turkey's multiple comparisons) (Figure 7C). We performed the dose-response analysis (n = 7) using different doses of amitinol ranging from 1ng/mL to 100 µg/mL. A dose-dependent increase in responses was observed in the higher doses (1 µg/mL, 10µg/mL, and 100µg/mL) with p <0.001 in mixed-effects model analysis. However, no significant differences were observed between males and females in any of the doses tested (Shapiro-Wilk test followed by t-tests) (Figure 3.9D).

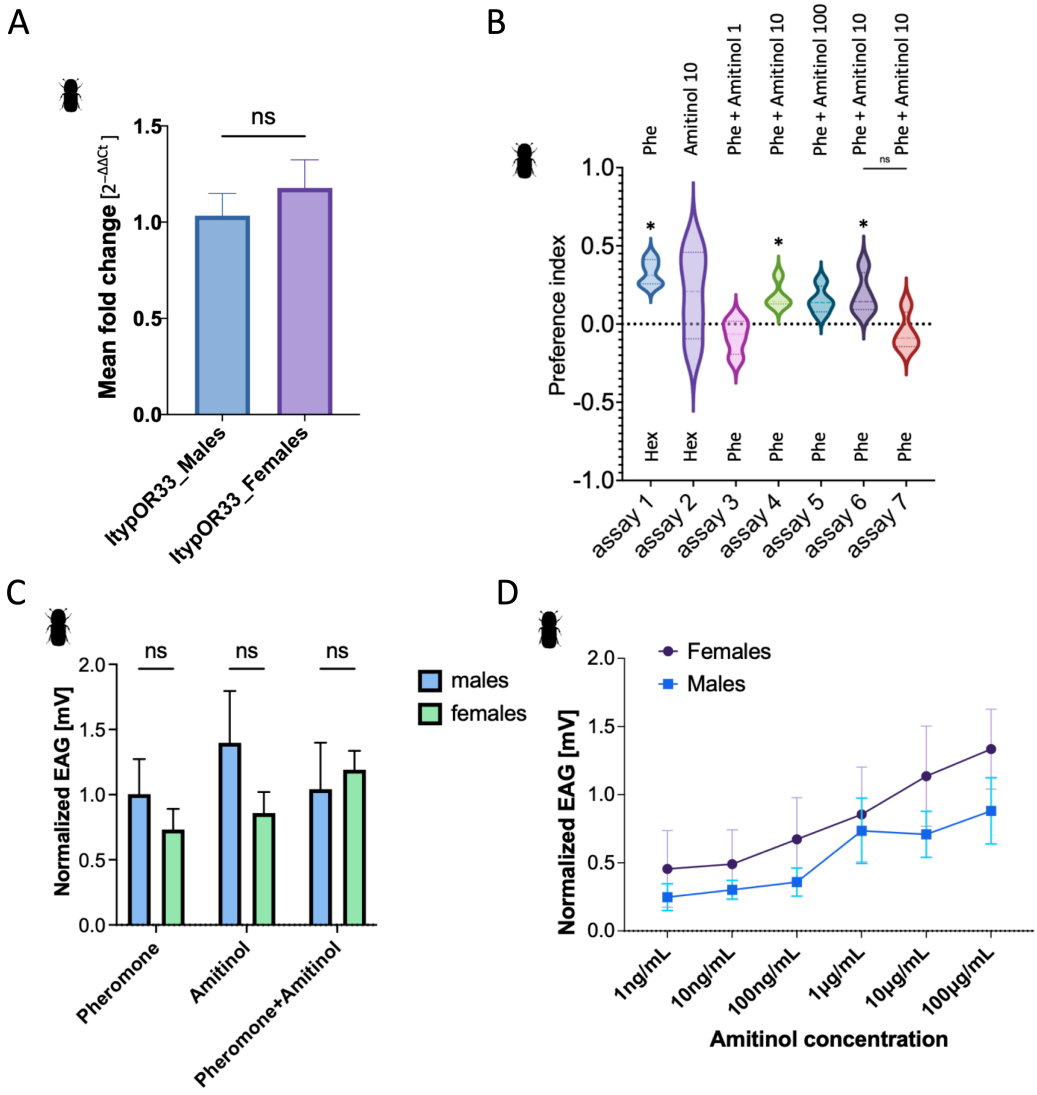

Figure 3.9. Comparison of *ItypOR33* expression and responses to its key ligand amitinol between *I. typographus* males and females. A, Relative quantification of *ItypOR33* expression. RT-qPCR-based mean fold change in *ItypOR33* expression compared to *ItypOrco* estimated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Expression in males (ItypOR33_Males) and in females (ItypOR33_Females) are represented as a bar graph. The significant difference in expression between the males (blue) and females (violet) was tested using a *t*-test at alpha level 0.05. 'ns' indicates non-significance and error bars represent SEM. **B,** Two-choice bioassays indicating *I. typographus* response to amitinol. The bark beetle preferences towards test *vs* control were represented as

a preference index ranging from 1 to -1. Seven bioassays were performed as assay1: pheromone (Phe) *vs* Hexane (Hex), assay2: amitinol vs solvent (Hex); assay 3: (pheromone + amitinol 1) *vs* pheromone alone; assay 4: (pheromone + amitinol 10) *vs* pheromone alone; assay 5: (pheromone + amitinol 100) *vs* pheromone alone; assay 6 (males): (pheromone + amitinol 10) *vs* pheromone alone and assay 7 (females) (pheromone + amitinol 10) *vs* pheromone alone. The pheromone refers to the blend (2-methyl-3-buten-2-ol and *cis*-verbenol, MBcV 10:1). Chi-square test based significant preferences of beetles towards the test zone compared to control zone are indicated with (*) at α level 0.05. No significant difference was observed between the preference indexes of males and females (ns). **C,** Electroantennogram responses of *I. typographus* males and females towards amitinol. EAG responses normalized with solvent were plotted for each set of stimuli as 1) pheromone (a blend of 2-methyl-3-buten-2-ol and *cis*-verbenol,10:1). 2) amitinol and 3) Pheromone+amitinol. The significant difference between males and females in three sets was calculated using one-way ANOVA at alpha level 0.05. 'ns' indicates non-significance, and error bars represent SEM. n = 10 for each gender. **D,** EAG dose responses in *I. typographus* males and females towards amitinol. Normalized EAG responses from *I. typographus* males and females plotted against six doses of amitinol stimuli (1 ng/mL to 100 µg/mL). n = *7* for each gender, and error bars represent SEM.

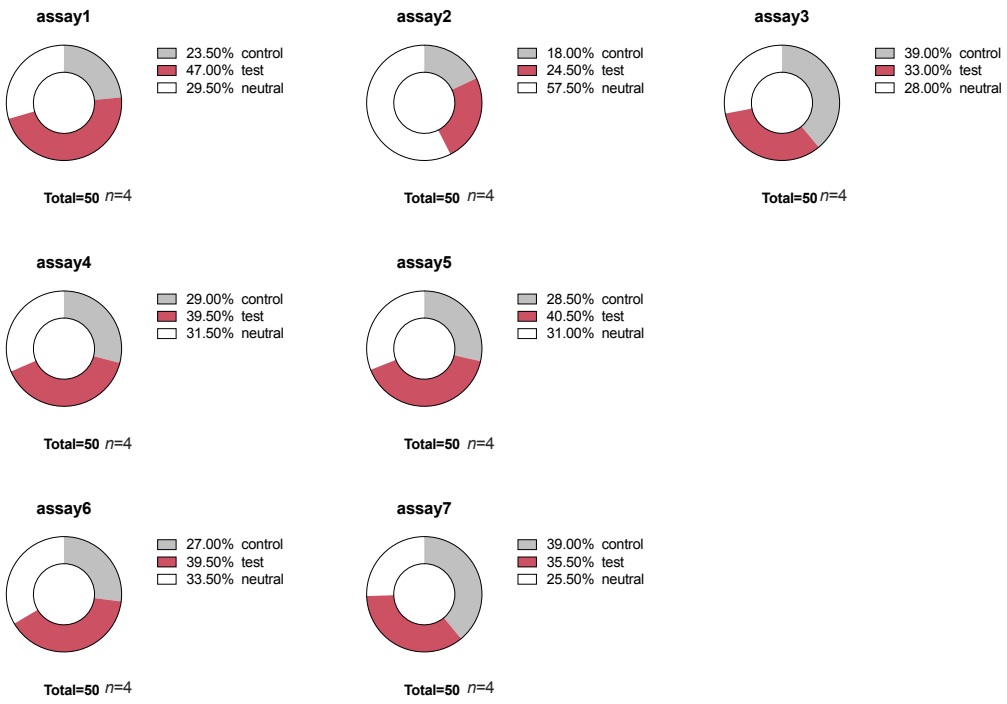

Figure 3.10 Two-choice bioassays. Raw data results of two-choice bioassay with adult *I. typographus* provided percentages of choices between three zones (test, control, and neutral). Assay 1: hexane (control) *vs* pheromone (test); Assay 2: hexane (control) *vs* amitinol (test)Assay 3: pheromone (control) *vs* amitinol 1 + pheromone (test); Assay 4: pheromone (control) *vs* amitinol 10 + pheromone (test); Assay 5: pheromone (control) *vs* amitinol 100 + pheromone (test); Assay 6 (males): pheromone (control) *vs* amitinol 10 + pheromone (test); Assay 7 (females): pheromone (control) *vs* amitinol 10 + pheromone (test) ;Pheromone = 10:1 [2-methyl-3-buten-2-ol (MB): (4*S*)-*cis*-verbenol) (cV)]

Table 3.1: List of Primers used in the experiments.

Primer	Primer sequence in 5' to 3' direction	Use
ItypOR33F	ATGGCGTTTTTCAATTCAGTAAAGAAC	Cloning
ItypOR33R	TCAAACTGTTAACAACACCATCAAATAGG	Cloning
ItypOR46_F	ATGAATGCTTTCCCAGATTCCG	Cloning
ItypOR46_R	TTAATTGTTACTTGTAAACACAGTTATGT	Cloning
Ityp_control_F	CCCACGACGGAAGAACTATT	cDNA check
Ityp_control_R	CCCGGATGTCTTTCTCTGTT	cDNA check
GW1	GTTGCAACAAATTGATGAGCAATGC	Colony PCR, Sanger Sequencing
GW2	GTTGCAACAAATTGATGAGCAATTA	Colony PCR, Sanger Sequencing
UAS1	TAGCGAGCGCCGGAGTATAAATAG	Sanger Sequencing
UAS2	ACTGATTTCGACGGTTACCC	Sanger Sequencing
DmOr22a_F	TCTCCAGCATCGCCGAGTGT	Single-Wing PCR
DmOR22a_R	CGGCAGAGGTCCAGTCCGAT	Single-Wing PCR
ItypOR33a_Fa	CAGAAATCCAGAGTCCCAAGAG	Single-Wing PCR
ItypOR33a_Ra	GAAACTGGAGCCGAACTGTAA	Single-Wing PCR
ItypOR33b_Ra	AGCATTGAAAATTATCAATATTATGTAACA GTA	Single-Wing PCR
ItypOR33_qRT_F	CCACTTGCCAACTTCAGATAC	RT-qPCR
ItypOR33_qRT_ R	ACTTTGAGATTGCGGTTCAATA	RT-qPCR
ItypOrco_qRT_F	CAATGTCTGGGATGACCTACTA	RT-qPCR
ItypOrco_qRT_R	GTCCAAAGAGGCTGATAATTCC	RT-qPCR
β-Tubulin_F	TGATGACGAGTACGAAGCGG	RT-qPCR
β-Tubulin_R	CAAAGCAAGGCA CTCTTGGTC	RT-qPCR

Table 3.2. List of populations used to screen *ItypOR33* polymorphism/variants in the population genomics analysis. The table was adapted from (Mykhailenko et al., 2023). 'N' represents the number of individuals collected per site.

Population ID	**Population name**	**Latitude**	**Longitude**	**Country**	**N**
AAS	Ås	59,667	10,793	Norway	13
ASA	Asa	57,165	14,783	Sweden	14
BAW	Bavarian Forest	48,960	13,395	Germany	11
BIL	Bílkovice	49,761	14,848	Czechia	14
BOR	Borki	54,090	21,912	Poland	12
EFI	Eastern Finland	62,492	30,010	Finland	13
GOS	Gościnno	54,047	15,657	Poland	14
LAN	Länsi	61,723	23,633	Finland	13
LIN,(BAD, LAM, FRE)	Linz	48,092	13,874	Austria	14
LUB	Lubaszki	54,057	17,556	Poland	14
MEL	Mellakoski	66,399	24,440	Finland	14
ROZ, (BRO, BUK, KUK, DEB)	Roztocze	50,508	22,786	Poland	14
SIL	Siljanfors	60,757	14,066	Sweden	14
STE	Steigerwald	49,622	10,263	Germany	14
STJ	Stjørdal	63,469	10,918	Norway	13
SVA	Svartberget	64,236	19,570	Sweden	13
TON	Tönnersjö	56,643	13,070	Sweden	13
TRE	Třebíč	49,212	15,879	Czechia	13

Table 3.3. European populations carrying *ItypOR33* Allele A (249K) and Allele C (249Q).

249K Populations	**Country**	**249K Populations**	**Country**
AAS2	Norway	SIL2-6	Sweden
AAS22	Norway	SIL4-3	Sweden
ASA125	Sweden	SIL4-6	Sweden
ASA13	Sweden	SIL5-2	Sweden
ASA20	Sweden	STE2	Germany
BAD3	Austria	STE22	Germany
BAW160	Germany	STE31	Germany
BAW164	Germany	STE33	Germany
BAW165	Germany	STJ14	Norway
BIL11	Czechia	SVA11	Sweden
BIL40	Czechia	SVA3	Sweden
BIL7	Czechia	TON10	Sweden
BOR14	Poland	TON5	Sweden
BOR15	Poland		
BOR18	Poland		
BOR23-Q	Poland		
BOR24	Poland		
BOR5	Poland		
BRO3	Poland		
BUK7	Poland		
DEB2	Poland		
DEB9	Poland		
EFI1-4	Finland		
EFI1-8	Finland		
EFI2-4	Finland		
GOS10	Poland		
GOS15	Poland		
GOS17	Poland		
LAM7	Austria		
LAN11	Finland		
LAN16	Finland		
LAN3	Finland		
LUB17	Poland		
LUB21	Poland		
MEL22	Finland		
MEL25	Finland		
MEL32	Finland		
SIL1-6	Sweden		

249Q Populations	**Country**	**249Q Populations**	**Country**
AAS21	Norway	LUB2	Poland
AAS25	Norway	LUB6	Poland
AAS28	Norway	LUB7	Poland
AAS29	Norway	LUB8	Poland
AAS30	Norway	MEL17	Finland
ASA10	Sweden	MEL19	Finland
ASA11	Sweden	MEL20	Finland
ASA12	Sweden	MEL28	Finland
ASA126	Sweden	SIL1-5	Sweden
ASA14	Sweden	SIL3-4	Sweden
ASA2	Sweden	SIL3-5	Sweden
ASA21	Sweden	SIL3-6	Sweden
ASA47	Sweden	SIL5-7	Sweden
BAW156	Germany	STE20	Germany
BAW162	Germany	STE25	Germany
BAW178	Germany	STE4	Germany
BIL1-DUP	Czechia	STE5	Germany
BIL10	Czechia	STJ1	Norway
BIL20	Czechia	STJ12	Norway
BIL34	Czechia	STJ4	Norway
BIL5	Czechia	STJ6	Norway
BIL9	Czechia	STJ9	Norway
BOR2	Poland	SVA1	Sweden
BRO9	Poland	SVA10	Sweden
DEB1	Poland	SVA4	Sweden
EFI1-9	Finland	SVA7	Sweden
EFI2-12	Finland	SVA8	Sweden
EFI2-6	Finland	TON7	Sweden
EFI2-8	Finland	TON9	Sweden
EFI2-9	Finland	TRE1	Czechia
GOS16	Poland	TRE4	Czechia
GOS3	Poland	TRE6	Czechia
KUK9	Poland	TRE8	Czechia
LAM10	Austria	TRE9	Czechia
LAN19	Finland		
LAN27	Finland		
LAN9	Finland		
LUB19	Poland		

Table 3.4: List of volatile organic compounds (VOCs) used in SSR

Compound's name	**Manufacturer/Provider**	**Purity**
(S)-cis-Verbenol	Acros organics	97%
(+)-trans-Verbenol	Lab Stock	GC-MS
2-Methyl-3-buten-2-ol	Acros organics	97%
(1S)-(−)-Verbenone	Acros organics	94%
Acetophenone	Sigma Aldrich	99%
2-Phenylethanol	Acros organics	99%
Ipsenol	B.K	GC-MS
Ipsdienol	Chem Cruz and B.K	GC-MS
S-Ipsenol	B.K	GC-MS
R-Ipsenol	B.K	GC-MS
S-Ipsdienol	B.K	GC-MS
R-Ipsdienol	B.K	GC-MS
(−)-Pinocamphone	R.U via A.J	GC-MS
(−)-Pinocamphone	R.U via A.J	GC-MS
(−)-Isopinocamphone	R.U via A.J	GC-MS
(−)-Isopinocamphone	R.U via A.J	GC-MS
Myrcene	Sigma Aldrich	Technical grade, GC-MS
Aminitol	A.J	GC-MS
Ethylcinnamate	Sigma Aldrich	99%
Methylcinnamate	Sigma Aldrich	99%
Bisabolene	Thermo-Fisher	
E-Mycenol	Fyto-Farm	95%
(1*R*)-(−)-Myrtenol	Sigma Aldrich	95%
exo-brevicomin	Lab Stock	
Geranyl acetone	Sigma Aldrich	≥97%
(±)-1-Octen-3-ol	Thermo-Fisher	98%
4-Vinyl anisole	Sigma Aldrich	97%
4-Ethylguaiacol	Sigma Aldrich	≥98%
(5S,7S)-trans-Conophthorin	A.J	
1-Hexanol	Sigma Aldrich	99.5%
3-Octanol	Sigma Aldrich	≥97%
Styrene	Thermo-Fisher	99%
2,3-Dihydrobenzofuran	Thermo-Fisher	99%
Geranyl acetate	Thermo-Fisher	98%
Beta-Farnescence	Sigma Aldrich	Technical grade
Butyl Acetate	Sigma Aldrich	

Benzyl acetate	Sigma Aldrich	≥99.7%
3,4 dimethoxytoluene	Thermo-Fisher	98%
2-Methyl-1-butanol	J&K Scientific Ltd.	98%
3-Methyl-1-butanol	VWR Life Science	GC-MS
(±)-2-Methylbutyl acetate	Sigma Aldrich	99%
Citral	Sigma Aldrich	≥96%
trans-pino-carveol	Synergy Ltd	
4-methyl anisole	Sigma Aldrich	97%
(−)-Terpinene-4-ol	Sigma Aldrich	≥95%
1,8-Cineole	Acros organics	99%
3-Carene	Acros organics	90%
Estragole	Sigma Aldrich	98%
R-(+)-limonene	Thermo-Fisher	97%
4-Thujanol	Sigma Aldrich	≥97%
(±)-Camphor	Sigma Aldrich	≥95%
(+)-a-Pinene	Sigma Aldrich	98%
p-Cymene	Sigma Aldrich	99%
γ-Terpinene	Sigma Aldrich	97%
(*E*)-(−)-caryophyllene	Sigma Aldrich	98%
Eugenol methyl ether	Sigma Aldrich	≥98%
Isoamyl acetate	Sigma Aldrich	≥99%
2-Phenylethyl acetate	J&K Scientific Ltd.	≥98%
α-longifolene	Phyto Lab	
Terpinolene	Sigma Aldrich	≥85%
Toluene		
farnesyl acetate	Sigma Aldrich	≥95%
2-campholene	Synergy Ltd	
Trans-anethole	Sigma Aldrich	99%
trans-a-ocimene	TRC Canada	
Chalcogran	Thermo-Fisher	96%
1-Nonanol	Thermo-Fisher	95%
1-Octanol	Honey well	99%
Benzyl alcohol	VWR Chemicals	1005%
E2-hexenol	Thermo-Fisher	97%
E2-hexenal	Acros organics	100%
Z2-hexenol	Thermo-Fisher	97%
Z3-hexenol	Thermo-Fisher	98%
(±)-Sabinene	Angene	75%
Pentyl acetate	Sigma Aldrich	≥99%
Ethyl acetate	VWR Chemicals	99%
linalool	Thermo-Fisher	97%

gamma nonalactone	Sigma Aldrich	98%
Methyl Eugenol	Sigma Aldrich	98%
P-cresol	Sigma Aldrich	≥99%
m-cresol	Sigma Aldrich	≥99%
Citronellol	Sigma Aldrich	≥95%
1-Heptenal (Heptanal)	Thermo-Fisher	97%
Limonene oxide	Sigma Aldrich	97%
a-Terpineol	Sigma Aldrich	90%
Methyl jasmonate	Sigma Aldrich	≥98%
Isophorone	Sigma Aldrich	
Hexyl acetate	Sigma Aldrich	≥98%
Ethyl hexanoate	Sigma Aldrich	≥98%
Methyl hexanoate	Sigma Aldrich	≥99%
Ethyl butyrate	Sigma Aldrich	≥98%
2-Heptanoe	Sigma Aldrich	≥98%
Ethyl-3-hydroxybutyrate	Sigma Aldrich	≥97%

Abbreviations used for the names of scientists kindly provided the compounds: B.K.- Blanka Kalinová, R. U. - Rikard Unelius, A.J.- Anna Jirošová

Results

Chapter 4

Functional evolution of odorant receptors in termites

Partially based on the manuscripts:

1. **Johny, J.**, Diallo, S., Lukšan, O., Shewale, M., Kalinová, B., Hanus, R., Große-Wilde, E., 2023. Conserved orthology in termite chemosensory gene families. *Front. Ecol. Evol.* 10. doi:10.3389/fevo.2022.1065947
2. Diallo, S., Kašparová, K., Šulc, J., **Johny, J.,** Křivánek, J., Nebesářová, J., Sillam-Dussès, D., Kyjaková, P., Vondrášek, J., Machara, A., Lukšan, O., Grosse-Wilde, E., Hanus, R., 2024. Identification of the trail-following pheromone receptor in termites. bioRxiv 2024.07.24.605012. doi:10.1101/2024.07.24.605012 (submitted)

The trail-following pheromone receptor in termites

The functional characterization studies of the four *P. simplex* (Rhinotermitidae) ORs, using the Drosophila empty-neuron system, revealed one of tested recepotr, PsimOR14, tuned to the TFP compound monocyclic diterpene neocembrene, with moderate responses to another compound geranylgeraniol. No hypothetical assumptions on the functional evolution of these genes or the specificity of ligands have been attempted in this thesis, as further investigations are in progress. The transcriptome-based gene expression analysis revealed moderate expression of PsimOR14 compared to other PsimORs. The identified TFP receptor belongs to a lineage of isopteran specific OR expnaisons but not from the most derived one. The deorphanization of candidates from the most deroved lineage is in progress.

Discussion

Chapter 1: Conserved orthology in bark beetle chemosensory gene families

The northern bark beetle, *I. duplicatus,* is one of the emerging economic pests of forests in Central Europe, with reported seasonal local outbreaks (Holusa et al., 2010; Holuša et al., 2013; Jeger et al., 2017; Wermelinger et al., 2020). Like most insects, olfaction plays a central role in the survival and host selection of these beetles in the forest (Byers et al., 1990; Schlyter et al., 1992; Zhang et al., 2007), and pheromone traps are widely used for monitoring purposes (Duduman, 2014; Holuša et al., 2012; Schiebe et al., 2011). However, the current management strategies remain inefficient as they attack upper parts of shaded trees and overwinter (Davídková et al., 2023), and co-habitants like *I. typographus*, further complicate management strategies.

The pine bark beetle *I. acuminatus* is an equally important pest in Eurasia, especially in pine forests. The recent outbreaks reported highlight the importance of developing more sustainable pest control strategies against these beetles (Papek et al., 2024; Seybold et al., 2006). These beetles show remarkable diversity in pheromone composition and carry a phloeomycetophagous lifestyle and pseudogamy (Papek et al., 2024). With the identification of recent ItypORs tuned to fungal volatiles, the ORs from *I. acuminatus* offer attractive candidates for OR characterization studies.

The presented research explored the olfactory gene familes in *I. duplicatus* (Johny et al., 2024a) and *I. acuminatus*. The number of ORs identified from both *I. duplicatus* and *I. acuminatus* was similar to that reported from the genomes of other bark beetles *I. typographus* (Andersson et al., 2013; Yuvaraj et al., 2021) and *D. ponderosae* (Andersson et al., 2019). A similar number of ORs were reported in the genome and transcriptome-based annotations from other Coleopterans (Antony et al., 2016; Gonzalez et al., 2021). Our phylogenetic analysis revealed a conserved orthology in most bark beetle chemosensory

gene families, except in OBPs. Such conserved orthology has been recently reported in other insect orders, like Blattodea (Johny et al., 2023).

Interestingly, bark beetle-specific OR expansions were observed in Coleopteran OR subfamilies 7 and 5. In which the subfamily-7 OR expansions are of special interest as they include *ItypOR46* and *ItypOR49*, the pheromone receptors detecting (*S*)-(–)-Ipsenol and (*R*)-(–)-Ipsdienol (Yuvaraj et al., 2021), respectively. Interestingly, the five-member clade was found to be detecting monoterpenoids with different ecological origins, and we report orthologs of these proteins. Importantly, *IdupOR23* and *IdupOR29* were found to be orthologs of ItypORs detecting fungal volatiles, and *I. duplicatus* are known to carry specific plant pathogenic fungi (Zimová et al., 2019). As the deorphanization of ItypORs is of great interest, reporting the orthologs from a sister species provides valuable insights into the functional evolution of odorant receptors in *Ips* spp. Similarly, Coleopteran ORs have been extensively characterized in recent years, to mention a few species: *M. caryae* (Mitchell et al., 2012), *I. typographus* (Hou et al., 2021; Roberts et al., 2022; Yuvaraj et al., 2021, 2024), *Holotrichia parallela* (Wang et al., 2020), *R. ferrugineus* (Antony et al., 2024, 2021; Ji et al., 2021), *R. palmarum* (Brajon et al., 2024a), *D. ponderosae* (Roberts et al., 2022), and *Hylobius abietis L.* (Roberts et al., 2022). The ItypOR orthologs reported in this research showed at least two bark beetle-specific OR expansions within Coleopteran ORs. Characterizing these receptors and orthologs will be of special interest as ORs, specifically the pheromone receptors, hold true potential for developing pest control strategies (Anderson and Newcomb, 2021; Venthur and Zhou, 2018). Additionally, conserved responses to ecologically relevant odors have been reported recently from ORs orthologs in conifer-feeding beetles (Roberts et al., 2022). OR-based biosensors have huge potential in food sensing (Bohbot and Vernick, 2020) and pest monitoring (Anderson and Newcomb, 2021; Hoddle et al., 2024).

Interestingly, the number of IRs from both species was similar to that reported from the genome of *D. ponderosae* (57 IRs) (Andersson et al., 2019) and higher than IRs reported from *I. typographus* transcriptomes. While the antennal IRs

generally share only a fraction of total IRs in Diptera, (Croset et al., 2010) the Coleoptern IRs share a nearly equal number of antennal and divergent IRs. Unlike in Diptera, no species-specific expansions are found in Coleopteran divergent IRs. The bark beetle-specific expansion in divergent IRs indicates the orthologous nature of these proteins and the commonalities in environmental stimuli they shared. GRs were classified into sugar, bitter, and CO_2 sensing receptors (Chahda et al., 2019; Dahanukar et al., 2001; Delventhal and Carlson, 2016). The number of GRs reported was similar to that reported from the *A. planipennis* genome but lower than from *D. ponderosae* (Andersson et al., 2019). However, GRs from the antennal transcriptome are essential for chemosensory detection. The phylogeny rooted with CO_2 sensing receptors showed two distinct clades that sense sugar and bitter sensing receptors with orthology between bark beetle GRs. This indicates their shared gustatory preferences as wood-boring insects and bark beetle-specific GR divergence (Andersson et al., 2019). The 1:1 orthology observed within CO_2 receptors signifies their responses to the common environmental stimuli that apply to Dipteran GRs from *D. melanogaster.* The large uncategorized clade with no orthologs of *D. melanogaster* shows the probable bitter-tasting receptor expansion in Coleoptera, which includes bark beetle-specific GR expansions. Such expansions are important for insects as bitter sensing receptors are known to detect versatile chemical stimuli, including metals, fatty acids, and bacterial components (Arntsen et al., 2024).

OBPs are known to increase the sensitivity of odorant receptors to odorants (Große-Wilde et al., 2006). These are some of the well-studied chemosensory proteins in insects. We identified a similar number of OBPs reported from other Coleopteran species (Andersson et al., 2019; Gonzalez et al., 2021; Liu et al., 2018). The OBPs were classified based on their six conserved Cysteine residues (Venthur and Zhou, 2018), and we found Classic, Minus-C, and atypical OBPs but not Plus-C OBPs (Zhou et al., 2004). Interestingly, we found a tetramer-OBP in *I. duplicatus,* while only dimer OBPs have been reported and described in the literature (Rihani et al., 2021; Zhou et al., 2004). While no functional studies have been reported in bark beetle PBPs, our data provides

interesting candidates, as OBPs expressed in the antenna are more likely to be involved in pheromone detection (Antony et al., 2018). Due to the diverse tasks performed, the function of OBPs still remains unclear (Rihani et al., 2021). SNMPs are another class of membrane protein that are involved in insect chemoreception. They belong to a large family of CD36 proteins that perform various functions and thus often require a clear phylogeny to differentiate the SNMP sub-groups (German et al., 2013; Gomez-Diaz et al., 2016; Nichols and Vogt, 2008; Vogt, 2003a). Although we identified six SNMPs in *I. duplicatus* based on a blastx homology search, only three were classified; two of them as SNMP1 class 1a and 1b, and the third was SNMP2 Group-2a. The structural and functional analysis of SNMP1 proteins has proposed their role in pheromone detection as a tunneling protein for transferring odorants from OBPs to ORs (Benton et al., 2007; Gomez-Diaz et al., 2016; Johny et al., 2024b; Rogers et al., 2001). However, the functional distinction between the 1a and 1b groups remains unclear (Vogt et al., 2009). The SNMP2b protein *IdupSNMP2b* could be involved in the pheromone-clearing process, according to the proposed functions of SNMP2 proteins in insects. However, unlike SNMP1 proteins, no orthology was observed in SNMP2 proteins between I. *typographus* and *I. duplicatus*.

The study provides a comprehensive coverage of candidate chemosensory proteins in bark beetles. Multiple antennal transcriptomes were generated, and a high-quality assembly of *I. duplicatus and I. acuminatus* was performed using a traditional assembly and mapping approach. The identified gene repertoire includes multigene family proteins, ORs, IRs, GRs, OBPs, CSPs, and SNMPs, with numbers comparable to that reported from the genomes of other bark beetles. The phylogenetic analysis revealed the divergence in each chemosensory protein and the conserved orthology in bark beetle chemosensory genes. Finding the orthologs of *I. typographus*, one of the actively studied coleopteran species, provides valuable functional insights and is a resource for future research.

Discussion

Chapter 2: Conserved orthology in termite chemosensory gene families

The number of ORs in the transcriptomes of *N. cubanus* (30), *P. simplex* (54) and *I. inquilinus* (28) was roughly similar to that reported from the genomes of *Z. nevadensis* (69) and *C. secundus* (42), and higher than *R. speratus* (22) (Harrison et al., 2018; Mitaka et al., 2016; Terrapon et al., 2014). However, the highest number of ORs among Blattodea was found in *B. germanica* (134 ORs), which may be partially explained by its large genome size, chromosomal translocations, and a higher rate of gene family expansions (Harrison et al., 2018). Ants, belonging among eusocial Hymenoptera, also possess massive OR expansions, leading to ~350 ORs in *H. saltator* and *Camponotus floridanus* (Zhou et al., 2012). It has been hypothesised that these expansions are connected to their eusocial behaviour (Zhou et al., 2012). However, high number of ORs has been reported also in non-eusocial Hymenoptera, such as *Nasonia vitripennis* (301, Robertson et al. 2010), suggesting that the expansion of ORs is an ancestral trait shared by Hymenoptera, which might potentially had facilitated the multiple independent evolutions of eusociality in hymenopteran insects. By contrast, the high OR repertoire reported recently in basal solitary apoid wasps phylogenetically positioned between ants and bees indicates that the OR repertoire in fact, reduced during the evolution of eusocial apoids (Obiero et al., 2021).

Termites, despite being eusocial insects, exhibit numbers of OR genes comparable to non-eusocial insects (Mitaka and Akino, 2021). If an expansion of ORs preceded the emergence of eusociality in Hymenoptera, the same is not true for Isoptera. The phylogenetic analysis revealed the highly conserved ORCo lineage and multiple Isoptera-specific OR expansions, which were analogous to the recent report in *C. secundus* based on the gene tree analysis (Harrison et al., 2018). Within these Isoptera-specific expansions, we found 1:1 orthologous relationship between the ORs of distinct termite species. This is

somewhat unusual for the highly divergent OR family, indicating a high degree of OR conservation across termites.

The transcriptome screening performed in the three species of termites yielded 20, 25 and 26 GRs, respectively from *N. cubanus, P. simplex* and *I. inquilinus*. The phylogeny (Fig. 2.2) also reveals the isopteran specific expansion of GRs in all three major subclades: sugar, bitter and CO_2 receptors. The dendrogram clearly separates different GR sub-classes as taste and CO_2 and pheromone receptors. The basal clade includes *D. melanogaster* GR5a and Gr64a, which are tuned towards trehalose and sucrose respectively and who exhibit complementary functional profiles in *D. melanogaster* (Jiao et al., 2008). We have identified 6, 3 and 4 putative sugar receptors each respectively from *N. cubanus*, *P. simplex* and *I. inquilinus*. The CO_2 receptor-containing clades were also largely expanded in termites. There were no orthologs found for Drosophila pheromone receptors Gr32a and Gr68a. The fructose receptor (Gr43a) subclade was located within the large bitter receptor clade, as reported in the *B. germanica* GR expansions (Robertson et al., 2018), indicating a conserved phylogenetic pattern across insect orders.

IRs, a subfamily of iGluRs, were found to be involved in detecting environmental as well as intracellular chemical signals (Ai et al., 2013; Benton et al., 2009; Croset et al., 2010). They were first identified in *D. melanogaster* and are well described in terms of their functional and evolutionary origins (Croset et al., 2010; Rytz et al., 2013). In contrast to other insect orders, IRs are numerous in Isoptera; in fact, the IR expansion in termites is considered to be analogous to OR expansions in Hymenoptera, signifying the importance of this protein family (Harrison et al., 2018; Robertson et al., 2018). A recent genome-based annotation in the cockroach *B. germanica* recovered 455 IRs, the highest number reported in insects. Nevertheless, nearly half of them were pseudogenes (Harrison et al., 2018; Robertson et al., 2018). While our findings of 98, 95 and 77 IRs from *N. cubanus, P. simplex* and *I. inquilinus*, respectively, exceed the numbers identified in most other insect species, they fall in the range of the numbers reported from other termites (*Z. nevadensis*: 141; *C.*

secundus: 135) (Terrapon et al., 2014, Harrison et al., 2018). Although the olfactory perception of social signals in ants (Slone et al., 2017; Trible et al., 2017) and TFPs in termites (Gao et al., 2020) have been demonstrated as OR/ORCo dependent, it was also proposed that a parallel ionotropic receptor gene family expansion has favored the evolution of colony communication in termites (Harrison et al., 2018). The total count of IR coding genes will likely be higher within the full genomes, but not substantially so. In contrast, only 12 IRs reported from *R. speratus* could be explained by the limited coverage of chemosensory genes in the whole-body transcriptome (Mitaka et al., 2016). It should be noted that genes with expression limited to one or a few tissues, like antennal IRs, will be underrepresented in a whole-body RNA pool, which is why we used antennal transcriptomes in our study. The expansion and positive selection in IRs have been reported recently in *Z. nevadensis* and *B. germanica* (Harrison et al., 2018). Rapid expansions in chemosensory receptor gene families provide functional divergence, crucial for adaption to different niches (Arguello et al., 2016). The caste and sex-biased expression of IRs reported in *Z. nevadensis* and *C. secundus* indicates the possible role of these genes in the pheromone communication (Harrison et al., 2018). The different subsets of iGluRs including IRs were added to the phylogenetic analysis. iGluRs exist across kingdoms, including plants, animals and prokaryotes (Croset et al., 2010; Rytz et al., 2013). We found orthologs of all major iGluR subfamilies in all three transcriptomes; our analysis revealed both antennal and divergent IRs. Similar to ORs, Isoptera-specific expansions were previously observed in termite IRs (Harrison et al., 2018). The antennal IRs are considered to be involved in olfaction, divergent IRs in gustation (Abuin et al., 2011; Benton et al., 2009; Croset et al., 2010; Prieto-Godino et al., 2017). As per the functional studies in *Drosophila*, the IR20a clade includes both taste and pheromone receptors (Koh et al., 2014). In our analysis this clade grouped with the divergent IRs. We identified five candidates in this clade, two each from *N. cubanus*, *P. simplex*, and one from *I. inquilinus*, and further research is required to confirm the role of these receptors.

SNMPs are broadly conserved CD36 (cluster of differentiation 36) family of transmembrane proteins in animals and are reported to be involved in the detection of lipid-derived pheromones in insects (Benton et al., 2007; Pregitzer et al., 2014). Among the two SNMP types reported in insects, SNMP1 was found to be expressed in both sensory neurons and supporting cells of insect pheromone-sensitive sensilla, whereas SNMP2 was found only in the sensory supporting cells as reported in the moths *Heliothis virescens* and *Antheraea polyphemus* (Forstner et al., 2008). Recent structural studies indicate that SNMP1 might function as a co-receptor or act as a tunnel to pass the signal molecules to the pheromone receptor (Gomez-Diaz et al., 2016; Johny et al., 2024b). The number of SNMP1 proteins identified from our antennal transcriptomes was similar to the number reported from *C. secundus* (5) and higher than the one reported from *Z. nevadensis* (Harrison et al., 2018; Terrapon et al., 2014). Like termite ORs and IRs, SNMP1 showed 1:1 orthologous pattern among the five-termite species compared. The higher number of SNMP1 proteins in termites could be correlated with the pheromone diversity in termites (Mitaka and Akino, 2021). In SNMP2 proteins, we found a single orthologous transcript in all five-termite species compared. SNMP2, proteins are mainly found in the sensory neuron supporting cells and are proposed to be involved in pheromone clearance processes (Forstner et al., 2008).

OBPs and CSPs expressed in antennae and pheromone glands, respectively, are involved in both the reception and broadcast of the chemical message (Pelosi et al., 2018). OBPs are highly abundant in the insect sensillar lymph and thus found abundantly in antennal transcriptomes (Venthur and Zhou, 2018). In Isoptera, OBPs and CSPs have been found to be differentially expressed among castes (Mitaka et al., 2016). The number of OBPs identified, i.e. 37, 35, 28 from *N. cubanus, P. simplex* and *I. inquilinus*, respectively, are higher than the OBPs reported from other termites (*Z. nevadensis*: 19; *C. secundus*: 19; *R. speratus*: 9) (Mitaka et al., 2016). Since OBPs are highly divergent in amino acid composition, using a basal hexapod *L. y-signata* (Missbach et al., 2014) as an outgroup helped in understanding OBP evolutionary pattern (Pelosi et al., 2005). All four major OBP sub-groups (classic, Minus-C, Plus-C and ABP-II

types) have been identified based on the earlier structure-based annotations (Venthur et al., 2014). Unlike in Lepidoptera and other insect orders, Isopteran OBPs are understudied. However, we found two transcripts each in our transcriptomes with similarity to the well-studied protein *BmorPBP* from the moth *B. mori* (Lautenschlager et al., 2007). The 1:1 orthologous pattern observed in the other termite chemosensory genes continued in the case of OBPs. The number of CSPs identified was also higher in our transcriptomes as these were not annotated from the genomes of the other two termite species *Z. nevadensis* and *C. secundus* (Harrison et al., 2018).

This research provides candidate genes of the major insect chemosensory gene families from three termite species belonging to three families of Isoptera of different phylogenetic positions, life histories and social complexities. We found comparatively large repertoires of chemosensory genes in all studied gene families as in other analysed termite species. The evolutionary analysis of termite chemosensory proteins revealed Isoptera-specific expansions with 1:1 orthologous pattern, indicating the existence of conserved olfactory functions. Our findings on basal eusocial insects will further enhance our understanding of the molecular underpinnings of eusociality.

Discussion

Chapter 3: Population level-functional polymorphisms in *Ips typographus* pheromone receptor, ItypOR33

Bark beetle outbreaks greatly impact the dynamics of forest ecosystems and global climate change. As olfaction plays a crucial role in their survival and adaptation, we provide molecular evidence for such olfactory adaptations within *I. typographus* populations by identifying functional variants of one of the highly expressed odorant receptors, *ItypOR33*. The varinats were successfully expressed in *D. melanogaster* using the DNS method, despite the challenges of expressing them in an evolutionary distant order Diptera (De Fouchier et al., 2017). Deorphanizing our target receptors, ItypOR33 and ItypOR33a, revealed the functional significance of these population-level variations. Further, the population genomics revealed both variants' spread and potential ecological relevance in different *I. typographus* populations. Additionally, we used *in silico* and *in vivo* methods to understand the structural aspects of ligand binding and behavioral aspects of the identified ligand in bark beetles. Key findings are discussed below.

ItypOR33: an amitinol receptor in *I. typographus*

ItypOR33 was found to be a pheromone receptor within the coleopteran OR subfamily 7 that detects amitinol, a pheromone component used by *Ips* spp. Other pheromone receptors identified within this subfamily were ItypOR28, ItypOR46, and ItypOR49, detecting bark beetle pheromone components *E*-myrcenol, (*S*)-(–)-ipsenol and (*R*)-(–)-ipsdienol respectively (Hou et al., 2021; Yuvaraj et al., 2021) (Figure D1). Amitinol is structurally similar to other ligands found in this clade, indicating the functional evolution of these receptors (Hou et al., 2021). At the cellular level, OSN classes that detect amitinol have been reported in *I. typographus,* however, with additional responses to ipsdienol and 2-methyl-3-buten-2-ol (Andersson et al., 2009).

Similarly, secondary responses were observed to ipsdienol and *R*-myrtenol, compounds produced by several *Ips* spp. (Symonds and Gitau-Clarke, 2016).

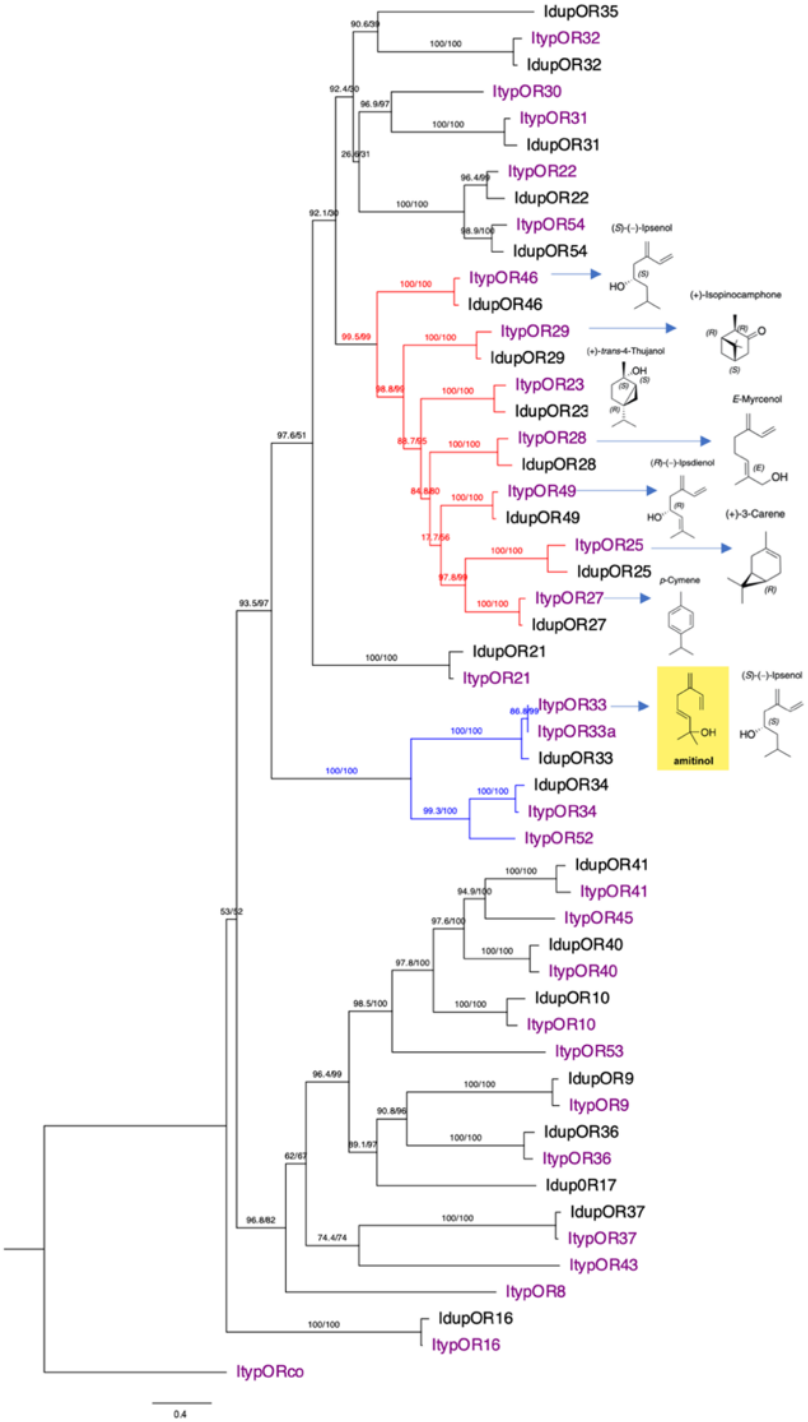

Figure D1. Functional evolution of ItypORs in coleopteran OR subfamily 7. Maximum-likelihood phylogeny of *Ips typographus* (Ityp) *and Ips duplicatus* (Idup) ORs from subfamily 7 rooted with *ItypORco*. The scale represents amino acid substitutions per site. Branch labels indicate SH-aLRT support (%) / bootstrap support (%). Ligand

structures from already known ItypORs clade (Hou et al., 2021; Yuvaraj et al., 2021)(red branches) are provided, along with our result - ItypOR33 ligand (highlighted) and its variant ItypOR33a's.

Amitinol has been reported as a major aggregation pheromone for *I. amitinus,* mainly produced by males (Kohnle et al., 1988). *I. amitinus* and *I. typographus* infest the same host, *Picea abies* at different areas of the tree (Gitau et al., 2013). Males from both species initiate galleries in weak or dying trees and produce the same major *Ips* pheromone components except amitinol (Gitau et al., 2013). Field assays have demonstrated that amitinol, in combination with aggregation pheromones, enhances trap catches of *I. cembrae* and *I. sexdentatus,* but reduces attraction in *I. acuminatus* (Kohnle et al., 1988). Our behavioral assays also report a better *I. typographus* preference for amitinol when combined with the pheromone blend. Trace amounts of amitinol were found in the hindguts of males *I. sexdentatus*, and *I. acuminatus* (Francke et al., 1986). Interestingly, the first conclusive field data demonstrating amitinol as an attractive pheromone component was in *I. duplicatus* (Zhang et al., 2007), a co-habitant of *I. typographus*. High expression of amitinol receptor *ItypOR33* in *I. typographus* could indicate the importance of this pheromone in inter-species communication, especially in early infestation stages. However, our relative quantification experiments did not find any sex-specific expression pattern.

The use of amitinol in *I. typographus* pheromone communication has not yet been explored in detail except for its weak antennal level detection (Andersson et al., 2009). The behavior assays showed similar results, indicating that *I. typographus* males are more attracted to MB+cV+amitinol than the *I. typographus* pheromone alone, suggesting the possible use of amitinol by males to detect the trees already attacked by other *Ips* spp. especially the co-habitant *I. duplicatus* (Zhang et al., 2007). However, our behavioral assays indicate that these detections are not sex-based, as males and females showed no significant difference in their preference for the amitinol + pheromone blend. Additionally, our EAG results confirm dose-dependent amitinol responses with a significant increase in higher doses. Although the behavioral assays,

supported by EAG recordings, showed that amitinol could enhance the attraction of *I. typographus* as a pheromone blend, the actual forest context may involve more complex interactions, as other *Ips* spp. like *I. duplicatus, I. acuminatus, I. cembrae,* and *I. sexdentatus* are known responders of this compound (Kohnle et al., 1988).

Natural polymorphisms alter the ligand selectivity of *ItypOR33*

Our study reports *ItypOR33* as a pheromone receptor in *I. typographus* that detects an *Ips* spp. pheromone component amitinol, and identifies a variant of this gene, *ItypOR33a,* within the same species from the Czech Republic. The finding of a variant for a highly expressing OR within a clade undergoing purifying selection led us further to explore its functional and ecological relevance. Such adaptive variations within insect populations are not well explored, as in mammals (Young et al., 2003). Currently, 63% of human ORs have SNP-derived variations that alter their olfactory responses (Mainland et al., 2014). However, the only known case of natural polymorphism in insect populations at the receptor level is the altered *OR59B* responses in *D. melanogaster* (Pellegrino et al., 2011). The large pool of antennae used for the RNA preparation technically supports the possible inclusion of population-level variants as the first version ItypOR33 was reported from Sweden (Yuvaraj et al., 2021) and the variant ItypOR33a was from the Czech Republic. We confirmed the existence and frequency of these variants in European populations by using population genomics.

The pheromone receptors and other ecologically relevant receptors usually undergo purifying selection as the deleterious mutations could affect species' survival. Here, amitinol as a pheromone component of other *Ips* spp. may induce a relaxed purifying selection on the amitinol receptor ItypOR33 to detect the conspecific pheromone component (*S*)-(−)-ipsenol. In turn, selection depends on the adaptability of this receptor and the extended benefit of the mutation. Interestingly, both ligands are structurally similar compounds, signifying minimum sequence divergence required to detect a new compound. Our *in silico* docking studies support this possibility. Moreover, amitinol is

formed by an allylic rearrangement of ipsdienol, another component of *I. typographus* pheromone. In *I. typographus,* ipsdienol has dedicated OSN classes with opposite enantiomeric specificity (*S* and *R*) (Andersson et al., 2009), but in our analysis, both *ItypOR33a* and *ItypOR46* were tuned to the same ipsenol enantiomer, (*S*)-(−)-ipsenol, eliminating them as for detecting enantiomers. Additionally, such variations could be explained in terms of ecological relevance, as both species attack the same host tree, calling for more refined inter-species communication.

In contrast to the narrowly tuned pheromone receptors in most insect orders, many coleopteran pheromone receptors are broadly tuned, with secondary responses to structurally similar compounds (Antony et al., 2021; Hou et al., 2021). For *ItypOR46*, we found primary responses to ipsenol and secondary responses to ipsdienol as reported in *in vitro* HEK cell expressions (Yuvaraj et al., 2021), but not to amitinol as reported in the oocytes expression system (Yuvaraj et al., 2021). Moreover, only one OSN class has been reported for (*S*)-(−)-ipsenol (Andersson et al., 2009). Among the two *I. typographus* receptors that respond to (*S*)-ipsenol, our dose-response analysis confirmed *ItypOR46* as the primary receptor with a slightly higher response than *ItypOR33a*. Similarly, the expression of *ItypOR46* was also higher than *ItypOR33* in Swedish samples (Yuvaraj et al., 2021). The geographical distribution of this receptor and its variant in European populations further supports its active role in bark beetle communication. Such geographical variations in OR expression have been recently reported in wasps (Krishnan et al., 2023). Additionally, *ItypOR33* variations may correlate with the ecology and distribution of *I. amitinus,* which is reported to expand its range in northern Europe (Økland et al., 2019).

Insights on the structural basis of ligand selectivity in ItypORs

Another interesting finding is the two single-point mutations that alter the ligand selectivity of *ItypOR33*. Single-point mutations that alter the ligand selectivity of receptors have been reported in moths (Cao et al., 2021; Liu et al., 2023; Yang et al., 2018). However, we report population-level variants from the same species. Similar observations are made in *Drosophila* populations, where

a mutation in *OR59B* changed odor inhibition activity by 1-octen-3-ol (Pellegrino et al., 2011). Our molecular docking analysis allowed us to differentiate the effect of natural variations on ligand selectivity. Our results confirm that two residues, 203T and 86H, add ligand-binding properties to *ItypOR33*, forming H-bonds, whereas ten other residues are involved in atomic-level interactions.

Additionally, deeper insights into ItypOR33 ligand binding revealed two ligand binding sites, as reported in ItypOR46 (Yuvaraj et al., 2021). As both sites can bind to the ligand with different affinities, we propose Site 1, located below the extracellular loop, as the initial binding site with lesser affinity. Site 1 is more accessible to the other interacting olfactory proteins like OBPs and SNMPs, which aid in transferring ligands to the receptor (Antony et al., 2018; Vogt, 2003b; Vosshall and Stensmyr, 2005). Site 2, as a deeper cavity located below Site 1, provides stronger affinity and potential to change the OR:Orco, the heteromeric complex structure, leading to channel opening. Having two binding sites and one of them (Site 2) located in deeper cavity could provide additional specificity. Identifying tunnels also supports this movement of ligands in and out within the receptor, which is essential for its rapid function. Our docking results indicate the prominent role of Site 2 in ligand binding. However, more tunnels identified in ItypOR33a at Site 2 could indicate that more ligands get access to this site and better movement of ligands. That explains the SSR results, which show ItypOR33a responds to more compounds than ItypOR33. Taken together, our results indicate that, natural polymorphism as *ItypOR33a* adds flexibility to ligand selection by making Site 2 more accessible to its potential ligands, or structurally similar compounds. Further research on the role of Site 2 on receptor specificity will be interesting but is beyond the scope of our research. We speculate that this flexibility based on population-level variations could favor the detection of its pheromone component (ipsenol) in *I. typographus* over a heterospecific pheromone component (amitinol) or *vice versa* as per the population/ecological demands.

The eco-evolutionary dynamics of *ItypOR33* variants

With global scenarios like climate change, ecological disturbances have been accelerated to a point where fast evolutionary dynamics are essential for the adaptation and survival of species (Johnson and Haynes, 2023). Such adaptive variations co-exist within the population to exploit the environment effectively (Cain and Sheppard, 1954). Our current research provides evidence for such eco-evolutionary dynamics occurring in bark beetles in forests. With the identification and functional characterization of *ItypOR33* and its variant in *I. typographus* populations, we report a possible olfactory adaptation in bark beetles towards rapid changes in their odor space. Climate change, forest disturbances, or bark beetle range expansions could drive such changes (McDowell et al., 2020; Økland et al., 2019). The high expression of *ItypOR33* signifies its olfactory importance, either in detecting amitinol for possible inter-species communication or detecting ipsenol possibly as an anti-attractant (Raffa et al., 2016). The recent range expansion in *I. amitinus* and increasing bark beetle outbreaks in Europe support the demand for rapid evolutionary adaptations in ORs involved in inter-species communication (Marini et al., 2017; Økland et al., 2019). Thus, a highly expressed ItypOR is more likely to possess an adaptive potential. Our population genomics analysis revealed that *ItypOR33* and its variant *ItypOR33a* are spread across the European populations with additional SNPs. Locating *ItypOR33* in a polymorphic chromosomal inversion (Mykhailenko et al., 2023) shows early signs of adaptive potential at the genome level, as inversions can be associated with local adaptations, particularly in species like *I. typographus* with high gene flow (Ellerstrand et al., 2022; Faria et al., 2019; Harringmeyer and Hoekstra, 2022; Müller et al., 2022). However, the selection of this variant in the population cannot be concluded from our analysis as it could be frequency-dependent (Nei, 1987). If selection favors, such adaptive variations are advantageous for migrating beetles, as *ItypOR33a* carrying populations could technically double the ipsenol detection. Such numerical increases in detectors have a multiplicative effect that leads to better signal transduction in insects (Hansson

and Stensmyr, 2011). However, we could not exclude the possibility of perceiving this odor differently or only as an inter-species signal marker based on neuronal coding in the brain. Interestingly, sensory augmentations at the cellular level (OSNs) have improved host or oviposition detection in *Drosophila* spp. and in mosquitoes (Dekker et al., 2006; Shaw et al., 2021; Syed and Leal, 2009). Nevertheless, most OSNs are considered to be functionally conserved (Hansson and Stensmyr, 2011).

At the receptor level, ORs originate through gene duplications and are primarily under purifying selection to detect ecologically relevant odors (Benton, 2015). Such duplications in ORs could result in paralogs that detect structurally similar compounds. However, we lack evidence for such duplication events at the genome level (Powell et al., 2021). Also, such events of two ORs detecting the same compounds are not rare in insects (de Fouchier et al., 2015). Conversely, the variations at the receptor level could also be a random event in the peripheral olfactory reception, which is less likely to alter the olfactory perception in the brain (Auer et al., 2022).

Nevertheless, these variations are critical to the evolution of species; as Darwin stated, without variation, natural selection cannot occur (Darwin, 1859). However, ecological relevance determines the fixing of such rapid variations during selection, as highly relevant adaptations are known to coevolve and get fixed within decades (Thompson, 1998). While polymorphic inversions in these migrating beetles could indicate signs of olfactory adaptation, our research provides direct evidence for possible adaptations within populations at the molecular level.

Discussion

Chapter 4: Functional evolution of termite odorant receptors

Chemosensory genes have been identified in a wide range of insects using genomic and transcriptomic approaches (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999) , which, to a large extent, enabled us to understand their evolutionary and behavioral adaptations in different biological contexts, including eusociality (Auer et al., 2020; De Fouchier et al., 2017; Engsontia et al., 2014; Keesey et al., 2022; Obiero et al., 2021; Pask et al., 2017; Robertson and Wanner, 2006; Terrapon et al., 2014; Zhou et al., 2015, 2012). Termites, despite being eusocial insects with well-studied chemical ecology and pheromone biology, have not been examined in detail in this regard until recently. With the known pheromone diversity and evolutionary pattern, functional evolutionary studies on termite receptors were attempted, identifying a trail-following pheromone receptor for the monocyclic diterpene neocembrene. Identifying pheromone receptors from *P. simplex* (Rhinotermitidae) provides an opportunity to find orthologs from other species currently being studied, like *N. cubanus* (Kalotermitidae), and evaluate the functional evolution of these receptors. However, these research works are currently in progress.

Conclusions

The research aimed to explore the rapid heritable changes in olfactory perception at the molecular level in response to the observed evolutionary patterns in the pheromone composition in bark beetles and termites. Both are critical parts of forest ecosystems, and the research aimed at understanding the olfactory adaptations is an essential step toward understanding the ecosystem. To achieve this goal, our current knowledge of chemosensory gene families in both inset groups was expanded by antennal transcriptome analysis. A direct comparison of olfactory gene repertoire in bark beetles and termites indicated a significantly high diversification of odorant receptors in bark beetles. Whereas ionotropic receptors were highly diversified in termites, indicating their usage in eusocial behavior and moist living habits. However, the numbers were comparatively lower than others in Balttodea. However, this signifies the evolution of both gene families in both insects, indicated by the specific habitat and environmental stimuli.

One of the hypotheses tested in studying gene families using phylogenies was the multi-step origin of chemosensory genes, especially in the odorant receptors. The conserved orthology observed in bark beetles and termite receptors indicates their common evolutionary pattern based on gene duplications. The gustatory and primitive ORs at the well-supported basal nodes provide insights into their evolutionary origin. With the different lineages of OR expansions observed in termite phylogeny, together with identified bark beetle OR expansions in coleopteran OR subfamilies, it can be concluded that the multi-step origin of these receptors is more likely true, proving the first hypothesis. However, this has to be further verified by functional characterization studies.

Functional analysis was performed on ORs from different clades; however, such works are in progress and are not included in the thesis. However, the tested pheromone receptors in *I. typogrpahus* provided unexpected evidence for standing genetic variations within populations with functional significance. The population genomics analysis supported the data

as the two allelic variants were equally distributed across populations. This allowed me to prove the second hypothesis on the molecular basis of olfactory adaptations: that the standing genetic variations could act as a source for olfactory adaptations at the molecular level. This also supports the recent adaptive potential reported regarding chromosomal inversions in *I. typographus*.

The pheromone receptor ItypOR33 and ItypOR33a the two tested variants also the first report of a functional polymorphism in insects other than Drosophila. The ligands identified for the receptor variants were structurally similar compounds amitinol and ipsenol, similar to those reported in the most diverged and well-studied sister clade in coleopteran OR phylogeny. Finding an amitinol receptor within a clade of ORs that detect structurally similar compounds supports the functional evolution of these receptors, supporting the third hypothesis.

With the successful implementation of the *Drosophila* empty-neuron system, we report *ItypOR33* as a pheromone receptor in *I. typographus* tuned to amitinol, a pheromone component used for interspecies communication, and the variant ItypOR33a tuned to (*S*)-(−)-ipsenol, own pheromone component, indicating population-level olfactory plasticity at the molecular level. As amitinol, is mainly used by other Ips spp. the behavioral aspects of this pheromone are being evaluated. These results confirm that single amino acid changes can contribute to the functional change in odorant receptors, proving the fourth hypothesis. However, the in silico structural predictions on ligand binding sites prove that mutations outside the binding sites can also alter the receptor's ligand selectivity. The identified ligand binding sites also provide much helpful structural information needed for designing olfaction-based pest control strategies. Understanding the eco-evolutionary dynamics and finding a receptor with high adaptive polymorphisms within species highlights the long-term complexities of managing bark beetles in forests. Finally, this research contributes to our understanding of evolutionary adaptations occurring in insect populations.

Incorporating the structural analysis into the OBP sequence analysis in *I. duplicatus* also revealed a unique tetramer OBP, reported as IdupOBP27. Additionally, the study generated high-quality transcriptomes with 97-99% BUSCO coverage for three termites and two bark beetle species for the scientific community interested in the antennal expressed genes in these species.

The successful implementation of the Drosophila empty-neuron system in termites, OR characterization revealed the first trail following pheromone receptors in termites, as PsimOR14 is tuned to neocembrane. Further receptor characterizations are required to validate the functional evolution of termite ORs. Further research is required and is in progress to understand the functional evolution of ORs and to identify the functional significance of identified ligands.

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List of other publications

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1. **Johny, J.,** Nihad, M., Alharbi, H. A., AlSaleh, M. A., & Antony, B. (2024). Silencing sensory neuron membrane protein *RferSNMPu1* impairs pheromone detection in the invasive Asian Palm Weevil. *Scientific Reports*, *14*(1), 16541.
2. Antony, B., **Johny, J.,** Montagné, N., Jacquin-Joly, E., Capoduro, R., Cali, K., Persaud, K., Al-Saleh, M. A., & Pain, A. (2021). Pheromone receptor of the globally invasive quarantine pest of the palm tree, the red palm weevil (*Rhynchophorus ferrugineus*). *Molecular Ecology*, *30*(9), 2025–2039.
3. Gonzalez, F.*, **Johny, J.*,** Walker, W. B.*, Guan, Q., Mfarrej, S., Jakše, J., Montagné, N., Jacquin-Joly, E., Alqarni, A. A., Al-Saleh, M. A., Pain, A., & Antony, B.* (2021). Antennal transcriptome sequencing and identification of candidate chemoreceptor proteins from an invasive pest, the American palm weevil, *Rhynchophorus palmarum. Scientific Reports*, *11*(1), 1–14. (*) contributed equally
4. Antony, B., **Johny, J.,** Abdelazim, M. M., Jakše, J., Al-Saleh, M. A., & Pain, A. (2019). Global transcriptome profiling and functional analysis reveal that tissue-specific constitutive overexpression of cytochrome P450s confers tolerance to imidacloprid in palm weevils in date palm fields. *BMC Genomics*, *20*(1), 440.
5. Antony, B.*, **Johny, J.*,** & Aldosari, S. A. (2018). Silencing the Odorant Binding Protein *RferOBP1768* Reduces the Strong Preference of Palm Weevil for the Major Aggregation Pheromone Compound Ferrugineol. *Frontiers in Physiology*, *9*(March), 1–17. (*) contributed equally
6. Antony, B.#, **Johny, J.#,** Aldosari, S. A., & Abdelazim, M. M. (2017). Identification and expression profiling of novel plant cell wall degrading enzymes from a destructive pest of palm trees, *Rhynchophorus ferrugineus*. *Insect Molecular Biology*, *26*(4), 469–484. (# co-first authors)

List of conferences during the PhD

Oral presentations:

1. **Johny, J.***, Diallo, S., Kalinová, B., Große-Wilde, E., Schlyter, F., *Functional evolution of odorant receptors in bark beetles.* Joint symposium on insect chemical ecology: FLD, CZU and Max Planck Institute of Chemical Ecology, September 2023.
2. **Johny, J.*,** Diallo, S., Lukšan, O., Kalinová, B., Hanus, R., Grosse-Wilde, E., *Functional Evolution of Odorant Receptors in Termites.* 3rd ISCE-APACE Joint Meeting, Kuala Lumpur, Malaysia, August 8-12, 2022
3. **Johny, J.,** Biograd Conclave: Season 2: Undergraduate and Postgraduate Symposium, Department of Life Sciecnes, Kristu Jaienci College, Bangalore, India, 17th June 2024. (*Keynote address***)**

Poster presentations:

1. **Johny, J.***, Diallo, S., Nadachowska-Brzyska, K., Roy, A., Kalinová, B., Große-Wilde, E., Schlyter, F., *Natural polymorphisms altering ligand selectivity in a bark beetle pheromone receptor.* ISCE2024, 39th Annual Meeting of the International Society of Chemical Ecology, Prague, Czechia, 14–18 July 2024
2. **Johny, J.***, AlSaleh, M.A., Antony. B., *Putative role of antenna-enriched sensory neuron membrane protein RferSNMPu1 in Asian Palm Weevil pheromone detection.* ISCE2024, 39th Annual Meeting of the International Society of Chemical Ecology, Prague, Czechia, 14–18 July 2024
3. Diallo, S., Kašparová, K., **Johny, J.,** Křivánek, J., Nebesářová, J., Šulc J., Sillam-Dussès, D., Kyjaková, P., Vondrášek J., Lukšan, O., Grosse-Wilde, E., Hanus, R., *Identification of the trail-following pheromone receptor in termites.* 39th Annual Meeting of the International Society of Chemical Ecology, Prague, Czechia, 14–18 July 2024
4. **Johny, J.***, Diallo, S., Kalinová, B., Große-Wilde, E., Schlyter, F., *Functional evolution of odorant receptors in bark beetles.* ISCE2023, 38th Annual Meeting of the International Society of Chemical Ecology, Bangalore, India, July 2023.

5. **Johny, J.***, Diallo, S., Kalinová, B., Große-Wilde, E., Schlyter, F., *Functional evolution of odorant receptors in bark beetles*. SMBE2023, Ferrara, Italy, 23-27 July 2023.
6. **Johny, J.***, Kalinová, B., Große-Wilde, E., Schlyter, F., *Functional evolution of odorant receptors in bark beetles*, 63rd Annual Drosophila Research Conference; April 6–10, 2022 San Diego, CA online; Genetics Society of America.

Notes: