Adaptive evolution of odorant receptors is associated with elaborations of social organization in ants

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Abstract

Cooperation in social insect colonies depends on a complex vocabulary of chemical signals, which requires a correspondingly complex array of chemosensory receptors. In the genomes of ants, the odorant receptor (OR) gene family is dramatically expanded compared to other insects. This is most notable in the "9-exon" OR subfamily, which was implicated in responding to cuticular hydrocarbons, a major class of signalling compounds. The compilation of a large, high-quality ant genome dataset by the Global Ant Genomics Alliance allows investigating ant OR evolution in unprecedented detail. By analysing 55,068 ORs of 163 representative ant species, we tested for association between the development of sociobiological traits and adaptive evolution of ORs. We provide a detailed analysis of gene family expansions and adaptive sequence evolution at the OR subfamily level, allowing focused analysis of distinct ant lineages. We identified strong enrichment of positive selection on 9-exon ORs in the ancestor of the formicoid clade, the largest clade comprising 90% of the extant ants. Formicoid species are characterized by greater colony size and reproductive division of labour. Thus, our results support a key role of adaptive diversification of chemical communication systems for the evolution of more complex elaboration of social organization in ants. We also observed enrichment of positive selection on 9-exon ORs associated with the more recent evolution of continuous worker polymorphism in multiple lineages. Surprisingly, the recent evolution of other sociobiological traits was associated with depletion of positive selection on ORs. These results suggest that the evolution of worker polymorphism involves more extensive adaptation of chemical communication compared to other aspects of ant sociobiology. Our analysis of an unprecedented ant OR dataset offers new insights into the specific context in which ORs played a major role in the evolution of elaborations in social traits across the diverse phylogeny of ants.

Introduction

Chemical communication is a vital function of insect societies, mediating many aspects of colony organisation. The perception of chemical signals relies on a large repertoire of odorant receptors (ORs), as well as other smaller chemosensory receptor families (Bargmann 2006; Bear et al. 2016; Robertson 2019). Colonies of social insects such as ants are characterised by elaborate cooperation among nestmates. Communication is central to the efficient coordination of necessary activities, including foraging for food, caring for the queen and brood, nest construction and defence. One of the fundamental functions of chemical signalling is nestmate recognition. Cuticular hydrocarbons (CHCs) are a diverse group of long-chain hydrocarbons thought to be a major component of the chemical cues used for recognition, including sexual communication and colony discrimination, which requires classifying individuals based on their CHC profile (van Zweden and d'Ettorre 2010; Blomquist et al. 2020).

ORs are critical for social communications in ants (Trible et al. 2017). In particular, the 9-exon ("9e") subfamily of ORs was suggested to harbour many of the receptors that respond specifically to the diverse array of CHCs displayed by ants (Engsontia et al. 2015; Zhou et al. 2015; McKenzie et al. 2016; Pask et al. 2017; Slone et al. 2017). This clade of the OR gene tree is dramatically expanded in ants, accounting for about a third of all ORs (C.D. Smith et al. 2011; C.R. Smith et al. 2011; Zhou et al. 2012; Zhou et al. 2015; McKenzie et al. 2016). Pask et al. (2017) characterized 22 ORs from the 9e clade and showed that they are narrowly tuned to respond to specific CHCs. Most of the 9e ORs display worker-biased expression, in line with a role in social coordination in the colony (Zhou et al. 2012). These ORs are also mainly expressed in the ventral half of the antennal club that comes in contact with other ants when they antennate each other for nestmate recognition, in line with these being receptors of non-volatile contact pheromones such as CHCs (McKenzie et al. 2016). In social insects, discrimination against non-colony members is especially important to avoid any form of colony exploitation. Therefore, strong selection on these discrimination abilities is expected.

The transition from solitary to social organisation in ants was accompanied by a dramatic expansion of the OR gene family (C.D. Smith et al. 2011; Zhou et al. 2012). This may be limited to the ants, as recent work found no evidence of a general relationship between the evolution of eusociality in Hymenoptera and the expansion of the OR repertoire (Gautam et al. 2024). Ant genomes harbour hundreds of ORs, and up to 503 ORs reported for the clonal raider ant *Ooceraea biroi* (McKenzie and Kronauer 2018). Studies have also shown that the reductive social behaviour in social parasite species (e.g. *Formica selysi*, *Acromyrmex ameliae*, *Lasius cf. spathepus*, *Tetramorium atratulum*) was linked to a loss of OR genes (Schrader et al. 2021; Jongepier et al. 2022; Vizueta et al. 2025). Many ant ORs show evidence of positive selection on their protein sequence (Engsontia et al. 2015; Zhou et al. 2015; Saad et al. 2018) making them attractive candidate genes for the study of adaptive evolution of chemical communication in ants. In total, Saad et al. (2018) reported positive selection in 7.2% of the tested OR gene tree branches (252 out of 3507). Recent species-specific OR gene duplications were followed by a higher rate of positive selection compared to ORs that were not recently duplicated (Engsontia et al. 2015; Saad et al. 2018), as expected for adaptive evolution in the birth-

and-death model of gene family evolution (Ohno 1970; Demuth and Hahn 2009; Innan and Kondrashov 2010; Mendivil Ramos and Ferrier 2012).

The study of ant ORs greatly benefits from the recent high-quality genomic dataset produced by the Global Ant Genomics Alliance (GAGA; Vizueta et al., 2025). GAGA sequenced genomes of 145 species of ants, which, in addition to 18 published high-quality genomes, represent 27 tribes in 12 subfamilies. Most of these genomes were produced using long-read sequencing that resulted in highly contiguous genome assemblies. This is especially important for accurate and complete assembly and annotation of the large tandem arrays of OR genes. Previous studies based on short-read genome assemblies revealed only a partial picture, typically underestimating the number of ORs. For example, the first genome assembly of *Camponotus floridanus* that was based on Illumina short-read sequencing allowed the annotation of 352 complete OR genes and 55 partial genes (Zhou et al. 2012), while the more recent PacBio long-read assembly (Shields et al. 2018) resulted in annotations of 466 complete ORs and 61 partial genes. The unprecedented genomic dataset produced by GAGA allows us to study the dynamic evolution of the OR gene family in ants with greater precision.

The genome-wide analysis of single-copy gene families in the GAGA genomes (Vizueta et al. 2025) revealed a high rate of positive selection on protein sequences in the branch leading to the ancestor of formicoid ants, a clade containing most extant ant species, which split from the poneroid ants ca. 125-145 Mya. The formicoid clade exhibits the most elaborated forms of reproductive division of labour, large colony size (Dornhaus et al. 2012), worker polymorphism (Oster and Wilson 1978), and extended queen longevity (Keller and Genoud 1997). This enrichment of positive selection in this branch is consistent with previous work published by Romiguier et al. (2022) which identified this branch as a hotspot for genes under positive selection. The GAGA analyses also revealed expansions in multi-gene families in the ancestor of ants. Many of these expanded gene families were implicated in chemoperception (22 families) and biosynthesis of CHCs (17 families). These results are in line with the hypothesis that the evolution of sociality in the ant ancestor involved the evolution of sophisticated chemical communication.

Here, we focus on the evolutionary dynamics of ant ORs and further explore whether positive selection on ORs is linked to the evolution of traits such as diet, colony size, worker polymorphism, and worker reproduction. We tested hypotheses using sets of representative species across the entire phylogeny, focusing on branches with inferred trait shifts. We identified enrichment of positive selection on the 9e ORs subfamily in the formicoid ancestor. Surprisingly, more recent branches with a transition to bigger colony sizes were depleted for positive selection, and that depletion was mostly in the non 9e OR subfamilies. We generally observed more depletion than enrichment in the recent branches we tested. However, positive selection on 9e ORs was enriched in branches of lineages that evolved continuous worker polymorphism. Our analyses leverage the unprecedented large-scale data from the GAGA project to reveal a complex pattern of adaptive evolution of ant ORs associated with some, but not all, elaborations of sociobiology in the ant phylogeny.

Materials and methods

Traits of interest

The GAGA project included the compilation of a trait table with information on social and ecological traits for each species from literature and experts, including for example their habitat, diet, foraging stratum, colony size, worker polymorphism, etc. We focused on traits that we hypothesised to be related to ORs evolution: colony size, diet, worker polymorphism, and worker reproductive functions. These traits require sensory function such as recognition and discrimination, whether of nestmates or of food sources, as well as complex social interactions that require complex signalling. Therefore, ORs can be expected to play a major role in the evolution and elaboration of these traits. Ancestral state reconstruction for each trait across the ant phylogeny was conducted using the R package *ape* (version 5.7-1; Paradis and Schliep, 2019) evaluating the best fitting model by using the Akaike information criterion (AIC). Further details can be found in Vizueta et al. (2025). Trait reconstructions are plotted on the phylogeny in Supplementary Figures S1 to S4. We used these reconstructions to identify branches with inferred trait changes, which we refer to as "transition branches" (marked on Figure 1). We hypothesized that there was positive selection on ORs related to the evolution of the transitioning traits in these branches. For example, we identified branches with a transition from an omnivorous to a herbivorous diet (e.g. in the ancestor of *Messor* harvester ants) and branches where colony size increased (e.g. in the ancestor of *Lasius* and *Myrmecocystus*).

The number of species analysed here coupled with the large number of ORs annotated in each of them resulted in a dataset of 55,068 OR genes. Tests for positive selection using dN/dS models are not feasible for such a large gene tree. Therefore, we focused the positive selection analyses on nine clades with small subsets of species (four to nine species in each; 75 species in total), selected to include the transition branches to be tested. We also tested the three most ancestral branches of the formicoid clade (rectangles in Figure 1) using a selection of ten representative species across the entire ant phylogeny. The first of these branches is the ancestor of all formicoid ants, the second is the branch after the split of the *Dorylinae* subfamily, and the third is the ancestor of *Formicinae* and *Myrmicinae* after the split of the *Myrmeciinae*, *Pseudomyrmicinae*, *Aneuretinae* and *Dolichoderinae* subfamilies.

Genome sequences

Most of the species in the GAGA dataset have been sequenced using PacBio long-read sequencing (125 out of 163 species), which is advantageous relative to Illumina short-read sequencing for the assembly and annotation of repetitive regions such as the typically large arrays of tandem-duplicated OR genes. Twenty species, including some rare species like *Leptanilla*, had limited sample biomass and could only be sequenced with stLFR (single-tube long fragment read), a sequencing technique that requires fewer DNA amount than PacBio. Scaffolding of contigs into whole chromosomes was achieved for 12 species using HiC, a technique that captures genome-wide chromatin interactions (Belton et al., 2012). The published genomes of 18 additional species were retrieved from NCBI, giving a total of 163 genomes. Among the 75 genomes used in our analyses, 65

were sequenced by PacBio and seven sequenced by stLFR. Three of the non-GAGA genomes were based on 454 sequencing, which is an obsolete short-read technology that is expected to result in more partial and missing ORs. Further details about the genome sequencing and assembly can be found in Vizueta et al. (2025).

Gene annotation

OR gene annotation was done using the *HAPpy-ABCENTH* pipeline (https://github.com/biorover/HAPpy-ABCENTH). The *ABCENTH* (Annotation Based on Conserved Exons Noticed Through Homology) program is an algorithm for building gene models that was designed for multigene families like ORs, with high sequence divergence but a conserved exon structure. *ABCENTH* builds HMM profiles separately for each exon based on known protein coding genes, which increases sensitivity and precision. *ABCENTH* then looks for splice sites of the candidate exons according to the expected exon length and phase. The genes used as queries were taken from manually curated ant ORs of the species: *Harpegnathos saltator*, *Stigmatomma sp*, *Ooceraea biroi*, *Camponotus floridanus* and *Solenopsis invicta*. Subsequently, novel OR annotations were evaluated by comparing them to OR sequences from *Drosophila melanogaster* and the five abovementioned ants. We identified the OR co-receptor (ORCO) in each species. Annotated ORs were classified in three categories: complete genes with the expected exon structure, pseudogenes with in-frame stop codons, and partial or fragmented annotations with incomplete exon structures, which can be caused by pseudogenization, fragmented assembly or assembly and annotation errors.

Phylogenetic analysis

The large number of complete OR sequences – 55,068 – made it impossible to analyse the full dataset jointly. Therefore, each of the 29 OR subfamilies defined by McKenzie et al. (2016) was analysed separately, and the results were pooled together for statistical analyses. ORs were assigned to a subfamily using *BLASTP* (version 2.11.0; Altschul et al., 1990), based on the top hit to sequences classified in the published subfamilies. 98% of the ORs (54,051 out of 55,068) had their top five hits within the same subfamily. The 9e subfamily comprises 19,528 sequences in our dataset, so it was further subdivided into six monophyletic clades that had 100% bootstrap support (we also observed issues in the alignment of these clades to each other, which would result in false positives in the dN/dS tests).

We constructed a multiple sequence alignment and tree for each OR subfamily and subset of species chosen for the positive selection analysis. Nucleotide coding sequences were used to build codon alignments using *Guidance2* (version 2.02; Sela et al. 2015) with the aligner *PRANK* (version 140110; Löytynoja and Goldman, 2005). Unreliably aligned codons were masked at a cutoff on the *Guidance* confidence scores that was chosen ad hoc for each alignment. We started by masking each alignment at a threshold of 0.8, and if more than 20% of the codons were masked we lowered the threshold by 0.1 and tested the percentage of masked codons again. We repeated the operation until we achieved a maximum of 20% masked codons, to avoid losing too much information for the inference of positive selection. This procedure was demonstrated as good practice for controlling the false positive rate of the branch-site test for positive selection (Privman

et al. 2012). The OR gene trees were built using *RAxML* (version 8.1.15; Stamatakis, 2014) with the translated sequence alignments as input, using the PROTCALG model, the LG rate matrix, and 100 bootstraps repeats.

Positive selection inference

Positive selection was inferred using the branch-site test for positive selection (Zhang 2005) as implemented in *Godon* (Davydov et al. 2019), using the M0 model to estimate branch length, four categories for codon rate variation, and testing all branches. A likelihood ratio test was conducted for each gene tree branch contrasting between the null model, not allowing positive selection, and the alternative model, allowing for positive selection in that branch. The likelihood ratio test p-values were then corrected for multiple testing using the Benjamini-Hochberg (1995) method.

Testing for association between trait evolution and OR sequence evolution

We developed a novel method for mapping positive selection results from genes trees of multi-copy gene families to the species tree, which is described in detail in our methodological paper (Pellen and Privman 2025). Briefly, we tested for enrichment of positive selection on ORs in species tree branches where we inferred a change in our traits of interest (the “transition” branches). The *Generax* (version 1.1.0; Morel et al., 2020) algorithm for reconciliation between gene trees and species trees was used to map branches of the gene trees onto branches of the species tree, with the UndatedDL reconciliation model. This step allows mapping of gene tree branches that were tested for positive selection to the species tree branch that we wish to test for enrichment. We then used a hypergeometric test to test for enrichment of positive selection (i.e. gene tree branches having *Godon* p-value < 0.05) in the species tree “transition” branches. Since we expect it to be more likely that positive selection occurred in a long branch than in a short branch, we normalized the number of gene tree branches by a factor defined as their branch length relative to the average gene tree branch length of the studied clade. This normalized number of branches was used as the size of the population that is tested for enrichment in the hypergeometric test. The ratio of observed/expected frequency of gene tree branches with positive selection were calculated to determine by what factor was the transition branch enriched or depleted for positive selection.

OR gene family expansions and contractions

To analyse the evolution of OR genes numbers, we applied the BDI stochastic model implemented in the *BadiRate* program (Librado et al. 2012). To avoid bias due to genome incompleteness or fragmentation, we excluded the 20 low quality genomes (short-read based with N50 < 500Kb), except for the *Leptanilla* outgroup, and added *Apis mellifera* as an outgroup. We then applied a global rates model (BDI-GR-CSP in *BadiRate*; Sankoff parsimony) to the numbers of complete OR genes. We note that the number of ORs reconstructed for the ant ancestor may be an underestimate due to *Leptanilla sp.*, the most basal ant species with only 151 complete ORs annotated. This genome has a lower quality compared to the rest of the dataset, as it is sequenced with the stLFR method only. It has an N50 size of 0.13 Mb, while the average for the dataset is 4.55 Mb. This low

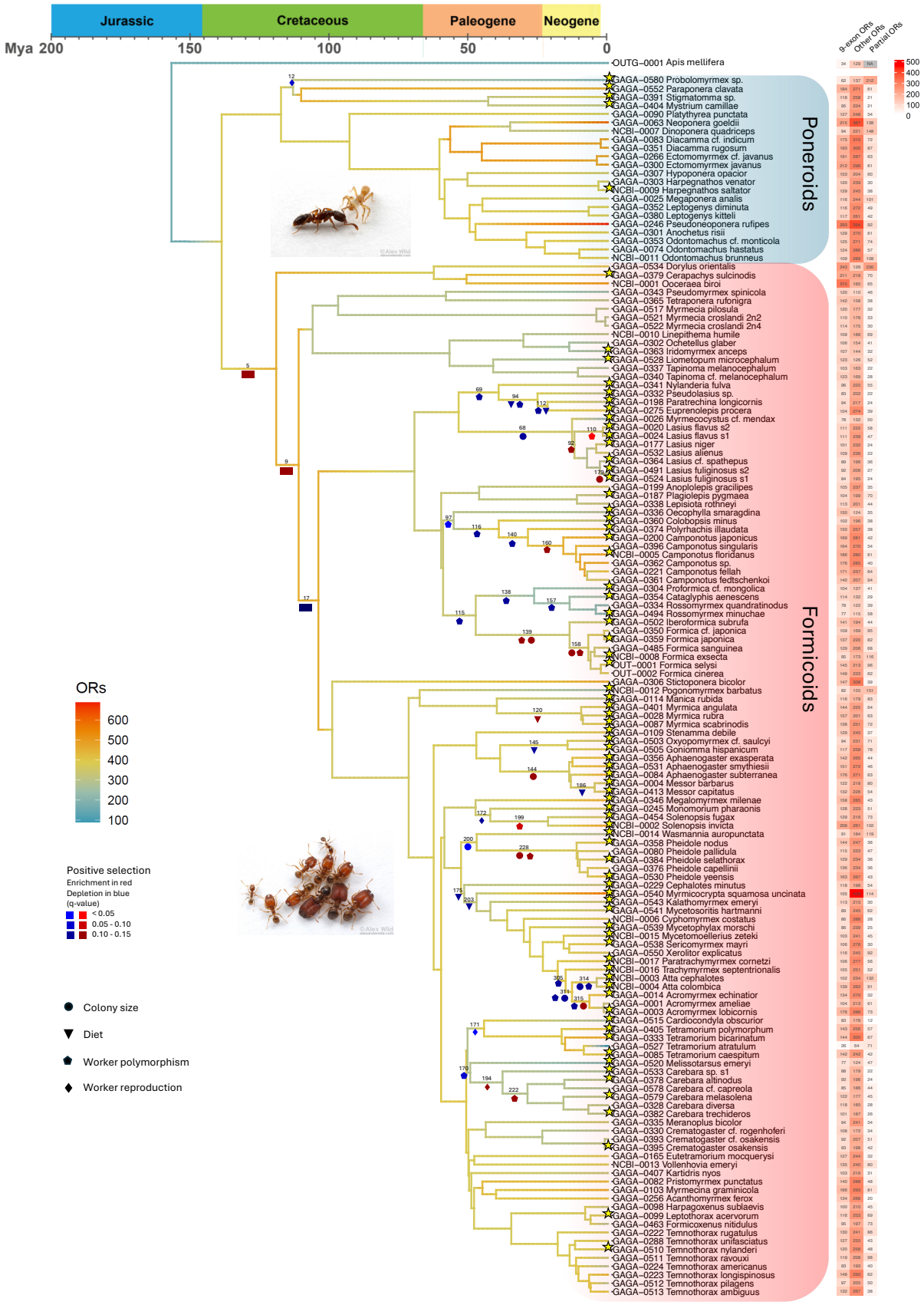
assembly contiguity may result in many ORs appearing to be partial genes which are not counted in the final dataset.

We also conducted a Phylogenetic Generalized Least Squares (PGLS) analysis using the *pgls* function in the R package *caper* (version 1.0.3, https://CRAN.R-project.org/package=caper) using the dated ant phylogeny. Again, we excluded the 20 species with lower quality genome assemblies by using *drop.tip* in the R package *treeio* to prune the species tree. We used PGLS to fit regression models between traits and ORs numbers (Supplementary Table S2). OR subfamilies with mostly single copy genes were removed from the final results to reduce spurious correlations.

Results

The odorant receptor repertoire of ants

The total number of ORs found through the annotation of 163 ant genomes was 66,169. This count includes partial ORs, which can be missing some terminal and/or internal parts of the full-length sequence. The percentage of partial ORs in each species varied from 4% to 56% (Supplementary Table S1) and was related to the contiguity of the genome assembly (scaffold N50 size), with fragmented assemblies having more partial ORs (Pearson correlation r = -0.28 between N50 scaffold size and the percentage of partial ORs, p-value = 0.00074). For example, *Gigantiops destructor* has the lowest N50 (8,895 bp) and the highest proportion of partial ORs (56%). Low quality genomes (N50 < 500Kb) were therefore excluded from analyses regarding gene numbers as they are expected to have biased under-estimates of OR numbers. We excluded partial ORs from further analyses, which left a total of 55,068 complete ORs across all 163 genomes, and 50,657 ORs without the low-quality genomes, ranging from 90 (*Tetramorium "Anergates" atratulus*) to 687 (*Pseudoneoponera rufipes*), with an average of 338 ORs per species (Figure 1). The ancestral reconstruction of OR gene numbers estimated 378 ORs for the ancestor of ants not including *Leptanilla*, which was excluded due to its low-quality genome (see inferred ancestral numbers in Supplementary Figure S5). The number of ORs expanded to 455 in the ancestor of the formicoid clade, while there was no further significant expansion in the poneroids ancestor (with 375 ORs).


Jurassic
Cretaceous
Paleogene
Neogene
Mya 200
150
100
50
0
9-exon ORs
Other ORs
Partial ORs
500
400
300
200
100
0
OUTG–0001 Apis mellifera
GAGA–0580 Probolomyrmex sp.
GAGA–0552 Paraponera clavata
GAGA–0391 Stigmatomma sp.
GAGA–0404 Mystrium camillae
GAGA–0090 Platythyrea punctata
GAGA–0063 Neoponera goeldii
NCBI–0007 Dinoponera quadriceps
GAGA–0083 Diacamma cf. indicum
GAGA–0351 Diacamma rugosum
GAGA–0266 Ectomomyrmex cf. javanus
GAGA–0300 Ectomomyrmex javanus
GAGA–0307 Hypoponera opacior
GAGA–0303 Harpegnathos venator
NCBI–0009 Harpegnathos saltator
GAGA–0025 Megaponera analis
GAGA–0352 Leptogenys diminuta
GAGA–0380 Leptogenys kitteli
GAGA–0246 Pseudoneoponera rufipes
GAGA–0301 Anochetus risii
GAGA–0353 Odontomachus cf. monticola
GAGA–0074 Odontomachus hastatus
NCBI–0011 Odontomachus brunneus
GAGA–0534 Dorylus orientalis
GAGA–0379 Cerapachys sulcinodis
NCBI–0001 Ooceraea biroi
GAGA–0343 Pseudomyrmex spinicola
GAGA–0365 Tetraponera rufonigra
GAGA–0517 Myrmecia pilosula
GAGA–0521 Myrmecia croslandi 2n2
GAGA–0522 Myrmecia croslandi 2n4
NCBI–0010 Linepithema humile
GAGA–0302 Ochetellus glaber
GAGA–0363 Iridomyrmex anceps
GAGA–0528 Liometopum microcephalum
GAGA–0337 Tapinoma melanocephalum
GAGA–0340 Tapinoma cf. melanocephalum
GAGA–0341 Nylanderia fulva
GAGA–0332 Pseudolasius sp.
GAGA–0198 Paratrechina longicornis
GAGA–0275 Euprenolepis procera
GAGA–0026 Myrmecocystus cf. mendax
GAGA–0020 Lasius flavus s2
GAGA–0024 Lasius flavus s1
GAGA–0177 Lasius niger
GAGA–0532 Lasius alienus
GAGA–0364 Lasius cf. spathepus
GAGA–0491 Lasius fuliginosus s2
GAGA–0524 Lasius fuliginosus s1
GAGA–0199 Anoplolepis gracilipes
GAGA–0187 Plagiolepis pygmaea
GAGA–0338 Lepisiota rothneyi
GAGA–0336 Oecophylla smaragdina
GAGA–0360 Colobopsis minus
GAGA–0374 Polyrhachis illaudata
GAGA–0200 Camponotus japonicus
GAGA–0396 Camponotus singularis
NCBI–0005 Camponotus floridanus
GAGA–0362 Camponotus sp.
GAGA–0221 Camponotus fellah
GAGA–0361 Camponotus fedtschenkoi
GAGA–0304 Proformica cf. mongolica
GAGA–0354 Cataglyphis aenescens
GAGA–0334 Rossomyrmex quandratinodus
GAGA–0494 Rossomyrmex minuchae
GAGA–0502 Iberoformica subrufa
GAGA–0350 Formica cf. japonica
GAGA–0359 Formica japonica
GAGA–0485 Formica sanguinea
NCBI–0008 Formica exsecta
OUT–0001 Formica selysi
OUT–0002 Formica cinerea
GAGA–0306 Stictoponera bicolor
NCBI–0012 Pogonomyrmex barbatus
GAGA–0114 Manica rubida
GAGA–0401 Myrmica angulata
GAGA–0028 Myrmica rubra
GAGA–0087 Myrmica scabrinodis
GAGA–0109 Stenamma debile
GAGA–0503 Oxyopomyrmex cf. saulcyi
GAGA–0505 Goniomma hispanicum
GAGA–0356 Aphaenogaster exasperata
GAGA–0531 Aphaenogaster smythiesii
GAGA–0084 Aphaenogaster subterranea
GAGA–0004 Messor barbarus
GAGA–0413 Messor capitatus
GAGA–0346 Megalomyrmex milenae
GAGA–0245 Monomorium pharaonis
GAGA–0454 Solenopsis fugax
NCBI–0002 Solenopsis invicta
NCBI–0014 Wasmannia auropunctata
GAGA–0358 Pheidole nodus
GAGA–0080 Pheidole pallidula
GAGA–0384 Pheidole selathorax
GAGA–0376 Pheidole capellinii
GAGA–0530 Pheidole yeensis
GAGA–0229 Cephalotes minutus
GAGA–0540 Myrmicocrypta squamosa uncinata
GAGA–0543 Kalathomyrmex emeryi
GAGA–0541 Mycetosoritis hartmanni
NCBI–0006 Cyphomyrmex costatus
GAGA–0539 Mycetophylax morschi
NCBI–0015 Mycetomoellerius zeteki
GAGA–0538 Sericomyrmex mayri
GAGA–0550 Xerolitor explicatus
NCBI–0017 Paratrachymyrmex cornetzi
NCBI–0016 Trachymyrmex septentrionalis
NCBI–0003 Atta cephalotes
NCBI–0004 Atta colombica
GAGA–0014 Acromyrmex echinatior
GAGA–0001 Acromyrmex ameliae
GAGA–0003 Acromyrmex lobicornis
GAGA–0515 Cardiocondyla obscurior
GAGA–0405 Tetramorium polymorphum
GAGA–0333 Tetramorium bicarinatum
GAGA–0527 Tetramorium atratulum
GAGA–0085 Tetramorium caespitum
GAGA–0520 Melissotarsus emeryi
GAGA–0533 Carebara sp. s1
GAGA–0378 Carebara altinodus
GAGA–0578 Carebara cf. capreola
GAGA–0579 Carebara melasolena
GAGA–0328 Carebara diversa
GAGA–0382 Carebara trechideros
GAGA–0335 Meranoplus bicolor
GAGA–0330 Crematogaster cf. rogenhoferi
GAGA–0393 Crematogaster cf. osakensis
GAGA–0395 Crematogaster osakensis
GAGA–0165 Eutetramorium mocquerysi
NCBI–0013 Vollenhovia emeryi
GAGA–0407 Kartidris nyos
GAGA–0082 Pristomyrmex punctatus
GAGA–0103 Myrmecina graminicola
GAGA–0256 Acanthomyrmex ferox
GAGA–0098 Harpagoxenus sublaevis
GAGA–0099 Leptothorax acervorum
GAGA–0463 Formicoxenus nitidulus
GAGA–0222 Temnothorax rugatulus
GAGA–0288 Temnothorax unifasciatus
GAGA–0510 Temnothorax nylanderi
GAGA–0511 Temnothorax ravouxi
GAGA–0224 Temnothorax americanus
GAGA–0223 Temnothorax longispinosus
GAGA–0512 Temnothorax pilagens
GAGA–0513 Temnothorax ambiguus
Poneroids
Formicoids
ORs
600
500
400
300
200
100
Positive selection
Enrichment in red
Depletion in blue
(q-value)
< 0.05
0.05 - 0.10
0.10 - 0.15
Colony size
Diet
Worker polymorphism
Worker reproduction

289

Figure 1: OR evolution along the ant phylogeny, including ancestral reconstruction of OR numbers and positive selection enrichment tests. Branch colour indicates the inferred ancestral number of ORs (see inferred ancestral numbers in Supplementary Figure S5). Coloured shapes mark branches with trait shifts that were tested for enrichment of positive selection on ORs. Shape colour indicates the statistical significance of enrichment or depletion based on the multiple testing corrected *p*-values (*q*-values). The species used in the positive selection analysis are marked with stars. The heatmap shows the number of complete ORs for each species partitioned to the 9-exon clade and the rest of the ORs, as well as partial genes.

We observed substantial variation in OR numbers along the phylogeny. Most species have fewer 9e ORs than non 9e ORs (Figure 1). The only exceptions being the small clade of army ants. *Ooceraea biroi* is the most extreme case with 315 9e ORs against 185 non 9e, as previously reported for this species (McKenzie et al. 2016; McKenzie and Kronauer 2018). Conversely, we note the *Myrmicocrypta* species as an extreme outlier for the number of non 9e ORs, with 458. Gene numbers of many OR subfamilies vary dramatically across ant species, most notably in the 9e subfamily and the other four largest subfamilies (E, L, U, V). Looking at the variation in the 9e ORs (Supplementary Figure S6), one species stands out, the workerless social parasite *Tetramorium atratulus*, which has the smallest number of 9e ORs. This species also shows drastic reductions in the other large OR subfamilies and is the only ant species that completely lost the medium-sized subfamilies P, T, M, and N. In subfamily P (Supplementary Figure S7), we noted a clade-specific expansion in the clade that includes *Camponotus*, *Polyrachis* and *Anoplolepis* (with smaller number in *Oecophylla*), and ponerine clade-specific expansions in *Neoponera* and in the clade of *Odontomachus* and *Leptogenys*. In subfamily H (Supplementary Figure S8), *Myrmicocrypta squomosa uncinata* stands out with 236 ORs, against an average of 12 over the rest of the ants. This seems to be due to extensive tandem duplications of subfamily H ORs that are restricted to this species (Supplementary Figure S9).

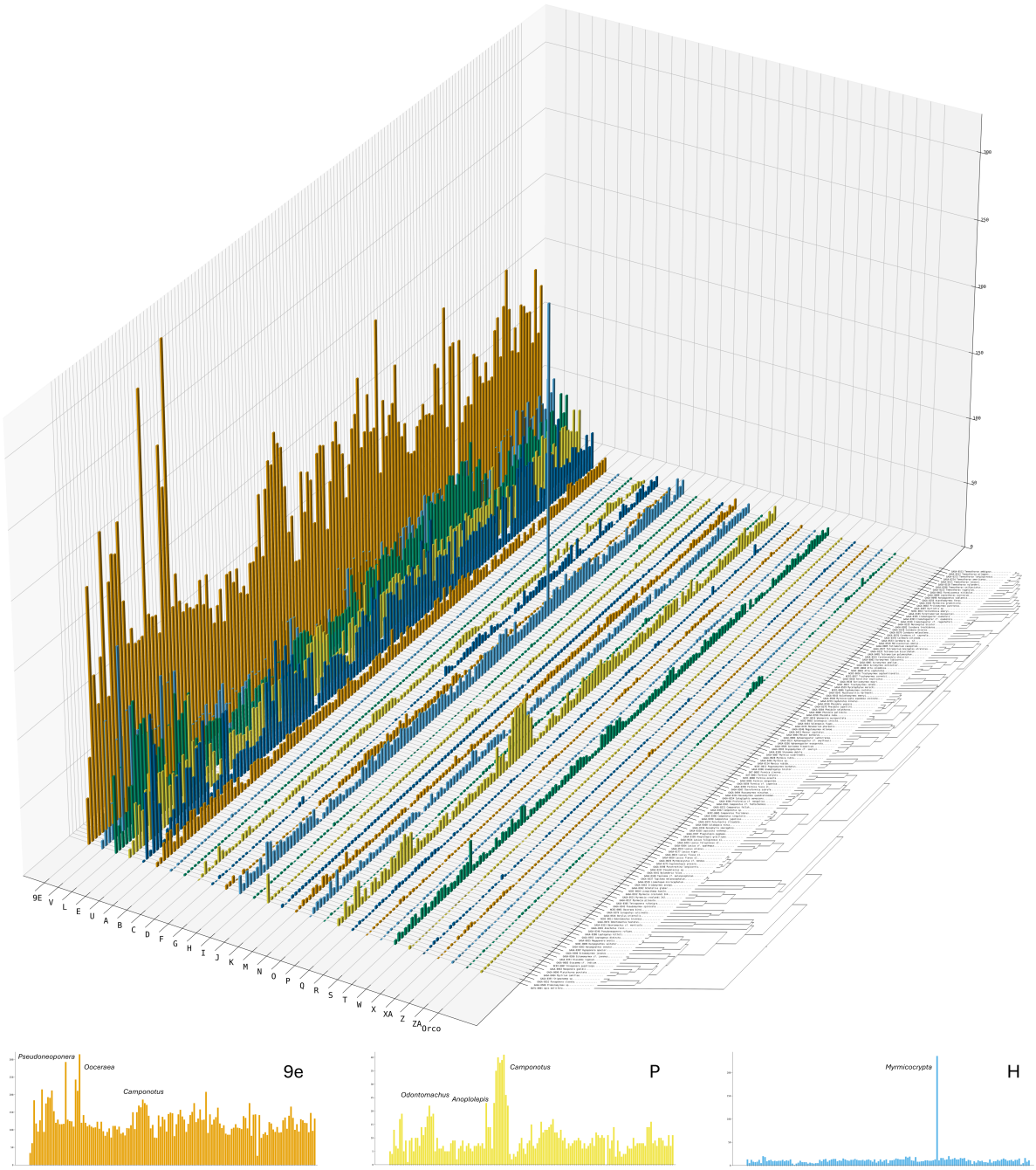


Figure 2: Subfamily composition of the OR superfamily across the ant phylogeny. The plotted data include only complete ORs, and exclude the species with low-quality genomes.

Correlation of OR gene numbers with social and ecological traits

The elaboration of sociobiological traits may require an extended OR repertoire to enable more sophisticated chemical communication in ant colonies. Therefore, we predicted a correlation between the number of ORs and traits such as colony size or worker polymorphism. We also expected that adaptation for different diets may be associated with the OR gene numbers. Therefore, we tested for an association between the total number of ORs or the size of OR subfamilies and specific traits of interest using a PGLS analysis (Figure 3A and Supplementary Table S2). We observed an expansion of the 9e OR subfamily in species with larger colony size (*q*-value = 0.012), while another large subfamily, V, showed a significant contraction with bigger colony size (*q*-value = 0.012). Our study confirms previously described significant contractions of the OR gene family associated with the evolution of social parasitism (Schrader et al. 2021; Jongepier et al. 2022; Vizueta et al. 2025). This contraction was in the total number of ORs (*q*-value = 0.000015) and specifically in the 9e (*q*-value = 0.000027) as well as several other OR subfamilies. The only exception is subfamily N that was expanded in social parasites (*q*-value = 0.070). We further focused the analysis on inquilines, the more extreme form of social parasitic species that live in the nest of another ant species. Inquilines show an even stronger contraction (*q*-value = 0.000000027), which is particularly striking in *Tetramorium atratulum*, a workerless inquiline species which retained only 90 complete ORs, 26 of which are in the 9e subfamily compared to an average of 120 9e ORs in other ant species (Figure 3 and Supplementary Table S1). Contrary to our expectations, we found no significant correlation between OR gene numbers and worker polymorphism, worker reproduction, or diet.

A)

ORs subfamily	Average colony size	Social parasitism	Inquilinism	Worker polymorphism	Diet	Worker ovary	Worker spermatheca	Worker sterility
All ORs	0.145	1.49E-05	2.67E-08	0.878	0.852	0.935	1	0.997
9-exon ORs	0.0119	2.67E-05	2.67E-08	0.878	0.302	1	1	0.997

B)

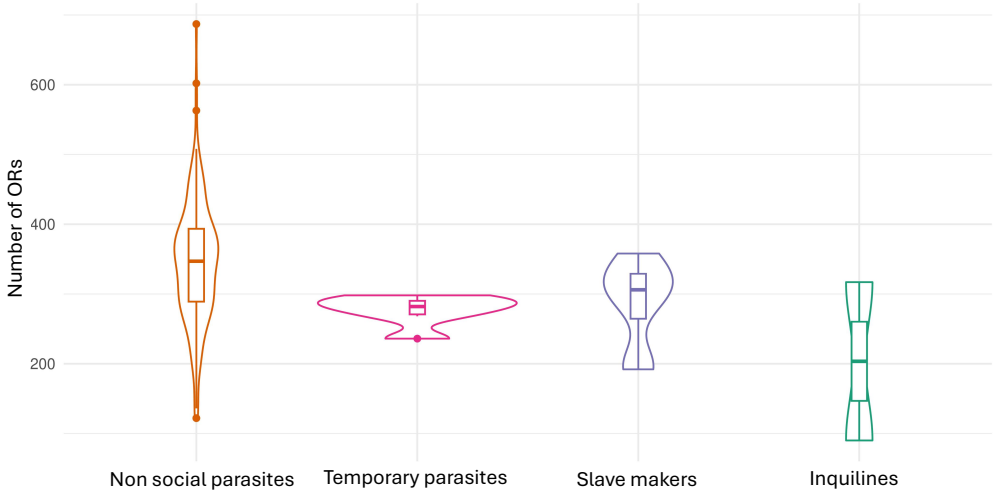


Figure 3: A) Phylogenetic Generalized Least Squares (PGLS) tests for association of gene family expansion or contraction with chosen traits, for the whole OR superfamily and for the 9-exon subfamily. Results are presented after correction for multiple testing (*q*-values). B) Number of ORs for species that are either not socially parasitic, temporary parasites, slave makers or inquilines.

Positive selection on ORs

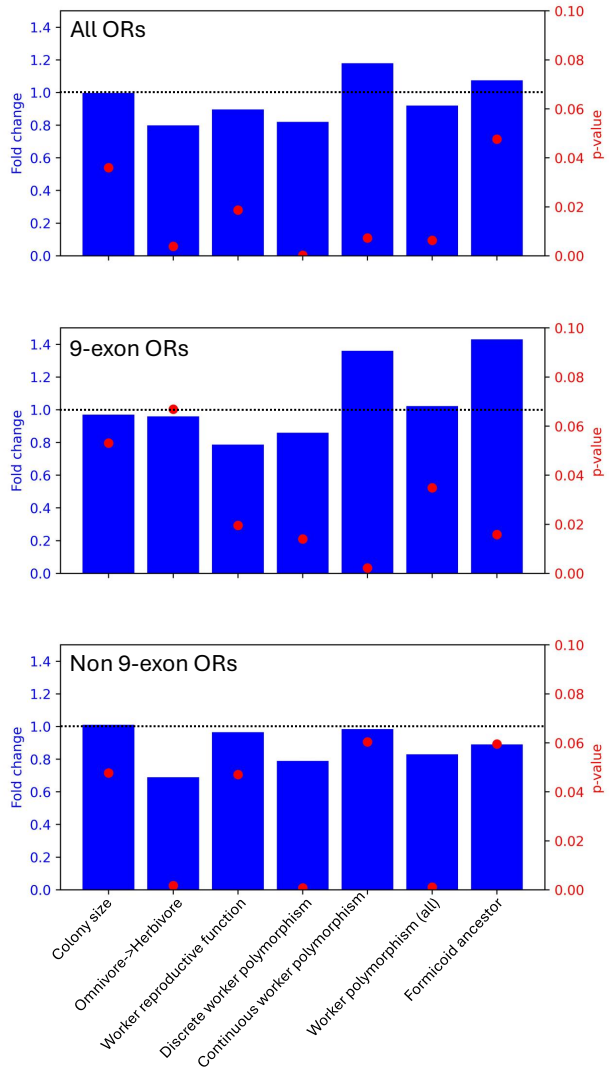
We used the branch-site test for positive selection to infer adaptive evolution of OR protein sequences in individual branches of the OR gene tree. We then evaluated whether species tree branches were enriched or depleted in positive selection on ORs (see Methods). We focused this analysis on subsets of the species phylogeny that allowed testing branches where we observed shifts in our chosen target traits ("transition branches") based on ancestral trait reconstruction: colony size, worker reproduction, worker polymorphism, and diet (marked by shapes in Figure 1). This analysis involved testing branches of the gene trees of each OR subfamily for each subset of species, 249 gene trees in total. Out of a total of 89,802 gene tree branches tested, positive selection was detected in 652 branches (0.7%), with a false discovery rate of 10% after correction for multiple testing.

We pooled the results for transition branches showing the same trait change. For example, we pooled the ten different branches that showed a convergent increase in colony size. We generally observed depletion in positive selection associated with most trait transitions (Figure 4). Despite being statistically significant, the depletion seen for increased colony size has a negligible effect size (0.998-fold depletion, *p*-value=0.036). The evolution of continuous worker polymorphism was the exception, with a significant enrichment of positive selection (1.18-fold enrichment, *p*-value=0.007). Also the ancestral branches of the formicoid clade were enriched for positive selection (1.075-fold enrichment, *p*-value=0.0476).

We were especially interested in positive selection in the 9e subfamily because it was suggested to be involved in social communication. We found that the enrichment in the formicoid ancestor was driven by the 9e subfamily (1.43-fold enrichment, *p*-value=0.016; Figure 3), while non 9e ORs were depleted for positive selection. A similar pattern was observed for the evolution of continuous worker polymorphism (1.36-fold enrichment in the 9e ORs, *p*-value=0.0022). In contrast, the shift to discrete polymorphism showed an opposite effect, with a significant depletion (0.863-fold depletion, *p*-value=0.014). In general, the enrichment or depletion in the 9e ORs mostly follows the pattern for total ORs but shows a stronger effect. The exception is the depletion associated with changes in diet, which was most pronounced in the non 9e ORs.

In addition to the pooled results, we also inspected individual transition branches. We found significant enrichment of positive selection in 11 branches and depletion in 23 branches (*q*-value < 0.10; Supplementary Table S3). For the three ancestral formicoid branches, the two more ancient branches were enriched and the most recent one was depleted for positive selection. Some branches display especially strong depletion or enrichment for positive selection. For example, the common ancestor of *Wasmannia* and *Pheidole*, where increased colony size was inferred (Supplementary Figure S1), was strongly depleted for positive selection (0.534-fold depletion, *q*-value=0.03262). Out of the 14 branches in which discrete polymorphism evolved (Supplementary Figure S3), four were enriched and ten depleted for positive selection. Among these, the common ancestor of the clade including *Oecophylla* and *Camponotus* was strongly depleted (0.477-fold depletion, *q*-value=0.03262) of positive selection. The common ancestor of

Cardiocondyla and *Tetramorium*, showing a loss of functional ovaries in workers (Supplementary Figure S4), was strongly depleted for positive selection (0.595-fold depletion, *q*-value=0.02461).



Trait	Normalized foreground gene tree branches [a]	Number of positive foreground gene tree branches	p-value	Fold change of normalized positive branches
Colony size	3362	138	0.0360	0.998
Omnivore->herbivore	2215	80	0.0039	0.799
Worker reproductive function	2522	109	0.0187	0.896
Discrete worker polymorphism	5422	197	0.0002	0.82
Continuous worker polymorphism	2165	112	0.0073	1.18
Worker polymorphism (all)	7587	309	0.0063	0.92
Formicoid ancestor	916	57	0.0476	1.075
Colony size	1219	61	0.0531	0.97
Omnivore->herbivore	718	38	0.0669	0.959
Worker reproductive function	821	36	0.0196	0.787
Discrete worker polymorphism	1945	88	0.0140	0.86
Continuous worker polymorphism	890	64	0.0022	1.36
Worker polymorphism (all)	2835	152	0.0349	1.023
Formicoid ancestor	299	26	0.0158	1.43
Colony size	2137	77	0.0477	1.011
Omnivore->herbivore	1501	42	0.0017	0.69
Worker reproductive function	1705	73	0.0471	0.965
Discrete worker polymorphism	3472	109	0.0008	0.789
Continuous worker polymorphism	1268	48	0.0604	0.985
Worker polymorphism (all)	4740	157	0.0011	0.83
Formicoid ancestor	617	31	0.0595	0.89

Figure 4: Enrichment for positive selection on ORs associated with trait evolution. Results of the hypergeometric test for enrichment of branches under positive selection (considering Godon's p-values under 0.05 as positive) for the pooled branches exhibiting the same change in trait.
a: Length-normalized number of gene tree branches mapped on the species tree branch that is tested.

Discussion

The high quality of the GAGA genomes and wide coverage across the ant phylogeny, coupled with an annotation pipeline specifically tailored for ant ORs, resulted in 50,657

410 ORs across 143 species, a more comprehensive and complete dataset for the study of
411 the evolution of ant ORs than any previously generated dataset. We used this
412 unprecedented gene set to infer ancestral OR repertoire sizes, reconstruct gene trees for
413 all ant OR subfamilies, and test for adaptive evolution of ORs that may be associated with
414 important sociobiological and ecological traits of ants.
415
416 Our tests found only 0.7% of branches under positive selection, which is low compared
417 to previous studies that tested for positive selection in ant ORs (Zhou et al. 2015; Saad
418 et al. 2018). This may be because we focused mainly on relatively recent events and
419 short branches in the ant phylogeny, whereas previous studies analysed only a few
420 species representing different ant subfamilies, making the tested branches much longer.
421 It could also be because we tested many more branches, forcing us to be more stringent
422 in our FDR correction.
423
424 Our results confirmed previous analyses, finding evidence of an ancestral expansion of
425 the OR gene family in ants (C.D. Smith et al. 2011; Engsontia et al. 2015; Mier et al.
426 2022), and especially the 9e subfamily. We also observed further expansion in the
427 formicoid ancestor, consistent with the further elaboration of social traits inferred in this
428 lineage. Genome-wide analyses also inferred significant expansions in the common
429 ancestor of ants in gene families implicated in chemical communication, including
430 chemoperception and synthesis of cuticular hydrocarbons (Vizueta et al. 2025). The
431 ancestral expansion of the OR gene family, likely playing a major role in the evolution of
432 sociality in ants, has been reversed in socially parasitic species (Schrader et al. 2021;
433 Jongepier et al. 2022; Vizueta et al. 2025), and this results also occurs in the 9e subfamily.
434
435 Previous studies identified an enrichment in positive selection on the branch of the
436 ancestor of all formicoid ants (Romiguier et al. 2022; Vizueta et al. 2025), but these were
437 genome-wide analyses limited to single-copy genes, and did not include large gene
438 families such as ORs. This is because the study of adaptive evolution in multi-copy gene
439 families requires complex reconciliation of gene trees with the species tree, to account
440 for gene duplications and losses (Page and Charleston 1998). Here we put in first
441 evidence for enrichment of positive selection on the OR gene family in the formicoid
442 ancestor. This pattern is driven by the 9e subfamily of ORs that is highly enriched for
443 positive selection, while non 9e ORs are actually depleted. This is in-line with the
444 hypothesis that 9e ORs are responsible for social communication functions in ants,
445 including their putative role in nestmate recognition, which is supported by their specificity
446 for cuticular hydrocarbons and their expression in workers (Engsontia et al. 2015; Zhou
447 et al. 2015; McKenzie et al. 2016; Pask et al. 2017; Slone et al. 2017). We propose that
448 as the ancestor of formicoid ants evolved various elaborate sociobiological traits such as
449 larger colonies and greater specialization of the non-reproductive castes, they evolved
450 additional complexity in their chemical communication, including new 9e ORs that
451 respond to chemical signals like CHCs shaped by positive selection.
452
453 Overall, we observed more depletions in positive selection than enrichments in the
454 transition branches we tested, especially in the most recent ones, and the 9e subfamily
455 seems to be the major driver of these changes. We observed depletion of positive

selection associated with increased colony size, complementing the findings by Vizueta et al. (2025) of genome-wide relaxation of purifying selection in species with increased colony size. Colony size was suggested to be a major driver in the evolution of ants, with many other social traits depending on it, including worker polymorphism (Bell-Roberts et al. 2024; Vizueta et al. 2025). The genome-wide analysis by Vizueta et al. revealed intensified selection associated with the recent loss of worker ovaries in multiple formicoid lineages, but predominantly relaxation of selection associated with the evolution of worker polymorphism. Our analysis found depletion of positive selection on ORs associated with the loss of worker reproductive functions. While we found that evolution of discrete polymorphism shows a depletion in positive selection, we detected enrichment of positive selection associated with the evolution of continuous polymorphism. This intriguing pattern would be interesting to investigate further, as it could imply that the evolution of different forms of worker polymorphism involved different mechanisms of chemical communication. The differences between our results and the analyses by Vizueta et al. may be attributed to the very different nature of the genes analysed: while we studied the fast-evolving multi-gene OR family, their analyses were limited to conserved single-copy gene families. Enrichment or depletion of positive selection on ORs is most strongly represented in the 9e subfamily for all tested sociobiological traits. It is interesting to note the exception of transition to herbivorous diet, which was depleted for positive selection. This depletion was most pronounced in the non 9e ORs, a result that is in line with the hypothesis that non-social olfactory functions are mediated by other OR subfamilies. Overall, our results indicate that only the evolution of certain worker traits involved a detectable signature of positive selection on ORs, whereas the elaboration of other sociobiological traits was associated with less adaptive evolution of ORs.

Conclusion

ORs expanded in the ant ancestor, mainly driven by the 9e subfamily that is associated with social communication. Our analyses detected enrichment of positive selection on ORs in the ancestor of the formicoid ants, mostly in the 9e subfamily, which was accompanied by a further expansion of OR gene numbers. These results support a central role for 9e ORs in the evolution of this large and diverse ant clade. Enrichment of positive selection in the 9e ORs was also associated with the evolution of continuous worker polymorphism, providing additional evidence for the role of this subfamily in social evolution. Our study demonstrates the potential of molecular evolutionary analyses of the OR gene family for explaining the evolution of social traits in ants. Our analyses provide new insights into the role of ORs in the social evolution and diversification of ants and raise new questions about specific aspects of OR evolution in the context of specific sociobiological traits and in specific lineages. It would be interesting to investigate the underlying causes for the depletion in positive selection in more recent branches, and what explains the differences observed between discrete and continuous worker polymorphism. Future studies including more species as well as functional genomic data could shed light on these evolutionary processes.

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Data availability

All the data generated in this study was submitted to the zenodo repository (10.5281/zenodo.16753530) including the OR annotations in GFF and FASTA format, multiple sequence alignments, gene trees, and generax results.

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