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Phylogenetic and Transcriptomic Analysis of Chemosensory Receptors in a Pair of Divergent Ant Species Reveals Sex-Specific Signatures of Odor Coding

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Abstract

Ants are a highly successful family of insects that thrive in a variety of habitats across the world. Perhaps their best-known features are complex social organization and strict division of labor, separating reproduction from the day-to-day maintenance and care of the colony, as well as strict discrimination against foreign individuals. Since these social characteristics in ants are thought to be mediated by semiochemicals, a thorough analysis of these signals, and the receptors that detect them, is critical in revealing mechanisms that lead to stereotypic behaviors. To address these questions, we have defined and characterized the major chemoreceptor families in a pair of behaviorally and evolutionarily distinct ant species, Camponotus floridanus and Harpegnathos saltator. Through comprehensive re-annotation, we show that these ant species harbor some of the largest yet known repertoires of odorant receptors (Ors) among insects, as well as a more modest number of gustatory receptors (Grs) and variant ionotropic glutamate receptors (Irs). Our phylogenetic analyses further demonstrate remarkably rapid gains and losses of ant Ors, while Grs and Irs have also experienced birth- and-death evolution to different degrees. In addition, comparisons of antennal transcriptomes between sexes identify many chemoreceptors that are differentially expressed between males and females and between species. We have also revealed an agonist for a worker-enriched OR from C. floridanus, representing the first case of a heterologously characterized ant tuning Or. Collectively, our analysis reveals a large number of ant chemoreceptors exhibiting patterns of differential expression and evolution consistent with sex/species-specific functions. These differentially expressed genes are likely associated with sex-based differences, as well as the radically different social lifestyles observed between C. floridanus and H. saltator, and thus are targets for further functional characterization. Our findings represent an important advance toward understanding the molecular basis of social interactions and the differential chemical ecologies among ant species.

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Introduction

The family of insects commonly known as ants (family Formicidae) originated during the Cretaceous period, approximately 140 million years ago [1]. Since that time, they have established a global presence, with only the most remote locations lacking ant species [2]. Indeed, in some cases, such as lowland tropical rainforest canopies, ants have come to dominate the biomass [3,4]. Their ecological success is reflected in the number and diversity of ants, of which there were 283 known genera [5].

There is a wide diversity in the behavior and morphology of different ant subfamilies that includes both the level and complexity of social organizations. For instance, Camponotus floridanus (the Florida Carpenter Ant), is a Formicine ant from the South-Eastern United States which belongs to one of the most globally prevalent ant genera [6]. These ants feature a rigid caste structure, with strict division of labor between the reproductive queens and the non-reproductive workers that is primarily regulated through pheromones [7,8,9]. Workers have a high threshold to lay eggs, and regulation of their reproduction through aggressive interactions does not occur [10]. Furthermore, the worker caste is divided into two classes: minor workers and major workers, which differ in size and morphology [2,6]. On the other hand, Harpegnathos saltator, a predatory species of Ponerine ant endemic to India and Sri Lanka is characterized by a more flexible reproductive system. H. saltator colonies are relatively small (averaging 65 to 225 individuals, depending on season and region) [11], and queen to worker dimorphism is weak [11,12]. When a H. saltator colony loses its queen, one or more of the workers will begin laying eggs and become functional reproductives (referred to as...
Author Summary

Chemical communication is an important factor in the regulation of social interaction in animals. The family of eusocial insects commonly known as ants offers an almost unique opportunity for examining the genetic basis for the chemosensory pathways that underlie ant sociality. In order to address this issue, we have manually and comprehensively reannotated the chemoreceptor repertoire in a pair of evolutionarily and behaviorally divergent ant species, <i>Camponotus floridanus</i> and <i>Harpegnathos saltator</i>. In addition, we have used next-generation RNA sequencing to examine the chemosensory receptor transcriptome between males and females within these species. Our analysis demonstrates rapid gene birth-and-death for the ant odorant and gustatory receptor gene families, as well as clear differences in the expression of particular subsets of chemoreceptor genes between males and females. Finally, we have begun to examine the odor space within these discrete social units by heterologous characterization of the first <i>C. floridanus</i> odorant receptor that also shows sex-specific differential expression. Taken together, our results provide a foundation for future studies of the genetic basis for the chemical signaling and chemical ecology underlying the dramatically different social lifestyles exhibited by these and other species of ants.

required for proper function of tuning Ors [29,31]. Rather than playing a role in odorant specificity, Orco forms an essential part of a heteromeric ion channel in cooperation with a tuning Or that is gated by its cognate odor ligand [32,33,34,35,36].

In contrast with the Ors, Grs are highly expressed in gustatory organs [20,21,22], and a large portion of these receptors respond to soluble tastants [37,38,39] and pheromones [40,41,42], leading to the “gustatory” designation for this group of chemoreceptors. However, there are some exceptions; for example, one unusual group of Grs respond to the volatile chemical carbon dioxide [43,44], demonstrating that members of this receptor family are not necessarily limited to gustatory or pheromonal responses. This is further supported by the expression of some Grs in non-gustatory organs such as the arista and Johnston’s organ [45].

Irs are homologous to ionotropic glutamate receptors (iGluRs) and thus are evolutionarily unrelated to Ors and Grs [24,26]. The role of IRs as chemosensory receptors has recently been uncovered based on multiple lines of evidence, including their divergence from conventional iGluRs at sequence level and the expression of several Irs in chemosensory neurons [24]. While Irs are generally thought to mediate responses to acids and amines [25], members of this family of chemosensory receptors may also sense other classes of chemicals.

We hypothesize that the striking contrast between <i>C. floridanus</i>, with its strict queen-worker dimorphism and largely pheromone-regulated reproduction, and <i>H. saltator</i>, with its flexible reproductive system that is associated with behavioral and pheromonal regulation of reproduction, is correlated with distinctive semiochemical and chemoreceptor profiles, which in turn generate differences in their chemical ecologies. The same is likely to be true of caste- or sex-based differences in behavior within each species.

To test these hypotheses, we first developed a custom gene annotation pipeline to comprehensively describe the chemosensory receptor repertoires of <i>C. floridanus</i> and <i>H. saltator</i>. We then investigated the evolutionary patterns (e.g. gene gain-and-loss) of these chemosensory receptor genes, in order to gain insight on their functional diversification. Furthermore, we performed RNAseq analyses of caste- and sex-specific antennal transcriptomes to identify chemoreceptors that are differentially expressed between males/females and between species. We found multiple clades of chemosensory receptor genes that show differential expansion/contraction among ant species. In addition, a large number of chemosensory receptor genes exhibited sex-specific expression or male/female-enrichment. These chemosensory receptor genes exhibiting interesting evolutionary and expression patterns may have potentially contributed to the different chemical ecology between sexes/species. We also successfully identified agonists for two Or genes to further validate these annotations. The findings of this study inform us as to the genetic basis for the differences in chemical ecology between <i>C. floridanus</i> and <i>H. saltator</i>, as well as the potential role of chemosensory receptors in the biology and evolution of eusociality in ants.

Results

Annotation of <i>C. floridanus</i> and <i>H. saltator</i> chemosensory receptor genes

The automated genome annotations of <i>C. floridanus</i> and <i>H. saltator</i> revealed about 100 Or and about 10 Gr genes [46], which is substantially fewer than the number of Or and Gr genes in two other sequenced ant genomes (e.g. argentine ant: <i>Lampropelta humile</i> [47], and harvester ant: <i>Pogonomyrmex barbatus</i> [48]; Figure 1). These low numbers were not surprising because the annotation of Or/Gr genes in other insect genomes has been difficult and usually
The number of Ir loci (all chemosensory receptor genes annotated in this study are available in Dataset S1). The number of intact genes, which is significantly higher than the 17 intact Ir genes found in H. saltator (Figure 1). Moreover, all three families of chemosensory receptor genes exhibited high degrees of sequence divergence among family members (Table S1).

To examine the genomic sequences revealed two principal mechanisms apparently leading to these fragmented gene models: 1) the presence of multiple frame-shift mutations and premature stop-codons, suggesting that they represent pseudogenes; and 2) their locations around undetermined genomic regions (e.g. edges of contigs/scaffolds), indicative of incomplete assembly as expected from a draft genome. The latter mechanism explains about 80% of the incomplete gene models.

Furthermore, similar to other insects [28,47,48,49,50,51], most chemosensory receptor genes are tandemly arrayed in the C. floridanus and H. saltator genomes. In both cases, about 75% of Or genes are located in gene clusters of 4 to about 40 genes, and these occur in 24 and 20 Or gene clusters (n=1) in C. floridanus and H. saltator, respectively (Figure S1). Although to a lesser degree than the Or, half of the Ir and Gr genes in both ants have at least one neighboring homolog.

**Phylogenetic analysis**

To better understand the evolutionary history of chemosensory receptor genes in the two ant species, we performed Hymenoptera-wide phylogenetic analysis on each of the Or, Gr, and Ir gene families. Additional analyses including D. melanogaster and Tribolium castaneum showed that most relationships among hymenopteran and non-hymenopteran sequences were not resolved within the Or and Gr families (see below). In this study, while they are generally categorized as belonging to the same receptor superfamily [22], we elected to analyze the Or and Gr families separately due to their high level of divergence.

**OR family.** Our phylogenetic analysis of hymenopteran Or genes revealed a highly dynamic evolutionary history of this gene family featuring rapid gene birth and death (Figure 2A). Due to the rapid divergence of Or genes (average amino acid distance = 2.56; overall protein sequence identity = 19.45%), most deep relationships in the OR phylogeny lacked support (see Figure S2 and Dataset S2 for the full version of OR phylogeny with gene names and bootstrap values). In spite of this, we found 24 well-supported clades (referred to as subfamilies; A-V, Orco, and 9-exon in Figure 2), each potentially representing one Or gene copy in the common ancestor of Hymenoptera (also see Figure S3 for the OR phylogeny with D. melanogaster and T. castaneum sequences). These subfamilies exhibited vastly different patterns of expansion/contraction, which can be divided into three types (Figure 2A, 2B): 1) strict single-copy representation in each of the six analyzed hymenopterans was

|    | OR | Gr | IR |
|----|----|----|----|
| N. vitripennis | 225 / 301 | 47 / 58 | 10 |
| A. mellifera | 163 / 174 | 10 / 13 | 10 |
| H. saltator | 347 / 377 | 17 / 21 | 23 |
| L. humile | 337 / 367 | 97 / 117 | 32 |
| C. floridanus | 352 / 407 | 46 / 63 | 31 |
| P. barbatus | 344 / 399 | 61 / 73 | 24 |

Figure 1. Annotation of C. floridanus and H. saltator chemosensory receptor genes. Number of Or, Gr, and Ir gene predictions in six hymenopteran species. For Or and Gr genes, the number to the right is the number of all gene models (coding for proteins longer than 300 aa in C. floridanus and H. saltator, or 200 aa in other species), while the number to the left is the number of seemingly intact gene models. doi:10.1371/journal.pgen.1002930.g001

Analysis of Ant Chemosensory Receptomes

requires extensive manual efforts [47,48]. In order to address this potential discrepancy and comprehensively elucidate the genomic repertoire of chemosensory receptor genes in C. floridanus and H. saltator, we rigorously re-annotated Or, Gr, and Ir genes in these two ant species using a custom automated pipeline followed by careful manual inspection.

To maximize the sensitivity of our re-annotation, we collected reported Or, Gr, and Ir gene sequences from other sequenced Hymenoptera and insect relatives of C. floridanus and H. saltator, including Apis mellifera, Acrystothorax pisum, Drosophila melanogaster, Nasonia vitripennis, L. humile, and P. barbatus. These insect chemosensory receptor genes were used to identify putative Or/Gr/Ir coding regions within the C. floridanus and H. saltator genomes and to guide homology-based gene prediction. As a result, we discovered a large number of previously unannotated chemosensory receptor genes and corrected several previously reported gene models [46]. All these annotations were manually inspected in multiple sequences alignments to identify and correct for potential errors (e.g. missing exons, unrelated sequences). This analysis indicates that C. floridanus contains 407 putative Or coding loci, of which 352 loci encode intact Or genes, which is similar to those newly annotated in H. saltator, with 377 loci in total and 347 intact loci (all chemosensory receptor genes annotated in this study are available in Dataset S1). The number of Ir predictions is also similar between the two ants, with 31 Ir genes in C. floridanus and 23 in H. saltator. On the other hand, C. floridanus contains 46 intact Gr genes, which is significantly higher than the 17 intact Gr genes found in H. saltator (Figure 1). Moreover, all three families of chemosensory receptor genes exhibited high degrees of sequence divergence among family members (Table S1).

In addition to the chemosensory receptor genes listed above, we also found a large number of incomplete gene models in these two ant genomes. For example, in C. floridanus and H. saltator, there are respectively ~100 and ~80 Or gene models encoding proteins shorter than 300 amino acids. In parallel to the difference in intact Ir genes, only three fragmented Ir gene models were found in H. saltator, while C. floridanus has ~30 short Ir genes. Close examination of their genomic sequences revealed two principal mechanisms apparently leading to these fragmented Or/Gr gene models: 1) the presence of multiple frame-shift mutations and premature stop-codons, suggesting that they represent pseudogenes; and 2) their locations around undetermined genomic regions (e.g. edges of contigs/scaffolds), indicative of incomplete assembly as expected from a draft genome. The latter mechanism explains about 80% of the incomplete gene models.

Furthermore, similar to other insects [28,47,48,49,50,51], most chemosensory receptor genes are tandemly arrayed in the C. floridanus and H. saltator genomes. In both cases, about 75% of Or genes are located in gene clusters of 4 to about 40 genes, and these occur in 24 and 20 Or gene clusters (n=1) in C. floridanus and H. saltator, respectively (Figure S1). Although to a lesser degree than the Or, half of the Gr and Ir genes in both ants have at least one neighboring homolog.

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**OR family.** Our phylogenetic analysis of hymenopteran Or genes revealed a highly dynamic evolutionary history of this gene family featuring rapid gene birth and death (Figure 2A). Due to the rapid divergence of Or genes (average amino acid distance = 2.56; overall protein sequence identity = 19.45%), most deep relationships in the OR phylogeny lacked support (see Figure S2 and Dataset S2 for the full version of OR phylogeny with gene names and bootstrap values). In spite of this, we found 24 well-supported clades (referred to as subfamilies; A-V, Orco, and 9-exon in Figure 2), each potentially representing one Or gene copy in the common ancestor of Hymenoptera (also see Figure S3 for the OR phylogeny with D. melanogaster and T. castaneum sequences). These subfamilies exhibited vastly different patterns of expansion/contraction, which can be divided into three types (Figure 2A, 2B): 1) strict single-copy representation in each of the six analyzed hymenopterans was
observed for the Orco subfamily, which is the only Or gene with clear orthologous relationships throughout insects [15,28,50,51,52,53,54] (Figure S3); 2) 11 subfamilies showed either gene loss only, or a limited number of gene duplication events (e.g. B and C); 3) the remaining 12 subfamilies had experienced substantial expansions within and/or shared by hymenopteran lineages (e.g. A and D).

In particular, the most dramatic expansion was found in the subfamily composed of Or genes with 9 exons (Figure 2A, 2B). This 9-exon subfamily encompasses more than 30% of the entire repertoires of Or genes in the six hymenopterans, which is in agreement with previous observations in other ants [47,48]. Furthermore, our analysis revealed highly dynamic Or evolution within these subfamilies; subclades were often differentially expanded and/or contracted in different species and rapid expansions were usually accompanied by frequent gene losses (Figure S4).

As described above, most Or genes in C. floridanus and H. saltator were found in tandem arrays in their respective genomes. Our phylogenetic results provided further evidence that these clustered Or genes were derived from tandem whole-gene duplication events. Moreover, more than 60% of all tandem duplicates in the two ants were due to lineage-specific expansions, while the others were generated during or even before the divergence of Hymenoptera. For example, the neighboring Or genes on C. floridanus scaffold538 and H. saltator scaffold105 belonged to four different subfamilies, suggesting that these two gene clusters were established before the divergence of the six hymenopterans, and underwent further expansion within each lineage (Figure S5).

Although Hymenoptera-wide orthology of Or genes may have been obscured by rapid gene gain and loss, we were still able to identify several clear 1-to-1 relationships among Formicidae. In total, we found 35 ant-specific clades that are composed of a single copy of Or gene from each of the four ants. Among these, genes in 30 clades are located in gene clusters while all others occur as singletons. A chi-square test showed that neither tandem duplicates nor singletons are significantly enriched in the 35 orthologous gene clades (p-value = 0.05).

**GR family.** Similar to the OR family, the GR phylogeny provided evidence for birth-and-death evolution in this family (Figure 3; see Figure S6 and Dataset S3 for the full version of GR phylogeny with gene names and bootstrap values). Within the Gr phylogeny, 13 well-supported subfamilies were found, most of which were likely generated by ancestral duplications before the divergence of Hymenoptera (although the precise relationships among them remained unresolved). Within Hymenoptera, ancestral duplications and/or lineage-specific expansions were found in most subfamilies, except for the GR1 and GR2 subfamilies. Indeed, significant lineage-specific expansions of ant Gr genes include the GR3 (L. humile) and GR8/9 (C. floridanus) subfamilies. The most dramatic expansion was observed in an ant-specific subfamily which underwent multiple rounds of amplifications.
before and after the separation of H. saltator, especially within C. floridanus and L. humile. In contrast to the other three ants, H. saltator specific duplication was only observed once (in the GR4 subfamily), which explains the low number of Gr genes in this species. Moreover, our GR phylogeny showed that the formation of Gr gene clusters was likely due to tandem duplication, highlighting the importance of this duplication mechanism in the evolution of chemosensory receptor genes.

Among the Grs, orthologs of the known sugar receptor genes (Gr1 and Gr2) [55,56,57,58] and another insect-wide conserved Gr, D. melanogaster GrE3a (Gr3) [28,53,54], were observed in all of the species examined (also see Figure S7 for the GR phylogeny with D. melanogaster and T. castaneum sequences). However, no orthologs of the well-described dipteran carbon dioxide (CO2) receptor genes [43,59] were found (Figure S7), consistent with the proposed loss of dipteran CO2 receptors in the ancestor of Hymenoptera [60]. Interestingly, it is known that the ability to perceive CO2 is present in ants [61], suggesting that different receptor genes are involved.

**IR family.** Unlike Ors and Grs, Ir genes have maintained relatively stable copy numbers during ant evolution (Figure 4; see Figure S8 and Dataset S4 for the full version of IR phylogeny with gene names and bootstrap values). While multiple duplications are likely to have occurred in the ancestor of Formicidae, unambiguous orthology among H. saltator, C. floridanus, L. humile, and P.
barbatus genes has been maintained across most IR clades. The only lineage-specific expansion of ant Ir genes occurred in the IR317 subfamily, in which the number of C. floridanus genes increased from 1 to 7, partially due to tandem duplications. The evolutionary history of Ir genes across Protostomia (e.g. nematodes, arthropods, and molluscs) has been described, where Ir genes are classified into “antennal IRs”, which are more conserved, and “divergent IRs”; of the seven antennal IRs, one (IR21a) was only found in N. vitripennis [26]. Nevertheless, orthologs of the other 6 antennal IRs, including IR8a, IR25a, and IR76b (which are thought to code for Ir co-receptors, that may play similar roles as the Orco Or coreceptor) [24,25], as well as IR68a, IR75u/f, and IR93a,—were found in ants (also see Figure S9 for the IR phylogeny with D. melanogaster and T. castaneum sequences). In addition, there were 13 other subfamilies of divergent IRs. Of these divergent IRs, no ortholog is present in the genome of N. vitripennis and only one is found in A. mellifera, which could be due to ant specific duplications and/or preferential retention of these divergent IRs occurred in ants.

Evolutionary dynamics

To further understand the evolutionary dynamics of chemosensory receptor genes, we quantified the gene birth and death events and estimated the number of ancestral gene copies in each family using both the maximum-likelihood (ML) and the parsimony based methods implemented in CAFE [62] and Notung [63], respectively. For all three families, the ML method suggested relatively high copy numbers in the ancestor of Hymenoptera (Figure 5). For instance, it estimated a repertoire of 266 Or genes in the hymenopteran ancestor, which was expanded in all ant lineages, but significantly contracted in both N. vitripennis and A. mellifera. A similar pattern was also observed in both the GR and IR families. Moreover, the ML analysis suggested
that the low number of Or genes in *H. saltator* is due to a significant gene loss in this lineage.

On the other hand, the parsimony approach gave conservative estimates of ancestral copy numbers and showed that many more gene-gain events occurred during later stages of hymenopteran evolution. According to the parsimony analysis, the number of Or genes increased from 25 in the last common ancestor of Hymenoptera to about 200 in *N. vitripennis* and *A. mellifera*, and more than 300 in all four ants (Figure 5A). Most notably, the repertoire of Or genes increased by three-fold in the ancestor of ants (from 51 to 204 copies), after the separation of *A. mellifera*, and continued to expand greatly along each ant lineage. Interestingly, although to a lesser degree, the ML method also identified significant expansion on the branch leading to the ant ancestor. In addition to the large number of gene gains, substantial gene losses also occurred in all ants. On the other hand, most duplications of ant Gos occurred in *C. floridanus*, *L. humile*, and *P. barbatus*, while there were only one gene gain and four gene loss events on the lineage to *H. saltator* (Figure 5B). Similar to the Or and GR families, the number of Ir genes also doubled in the ancestor of ants after its separation from other Hymenoptera (Figure 5C). Subsequent increase of Ir gene number was only observed in *C. floridanus* and *L. humile*.

Overall, the ML and parsimony analyses gave different estimates of the ancestral copy numbers and gene gain and loss events. The ML method assumes a random gene birth and death process [64], which is significantly violated by both the Or and GR families (p-values<0.01). On the other hand, the parsimony approach aims to minimize the number of gene gain and loss events, and thus might underestimate the number of ancestral copies. Nonetheless, both analyses support the hypothesis that chemosensory genes have distinct evolutionary dynamics in ant lineages in comparison to the other two hymenopterans.

Antennal expression profiles of ant chemosensory receptor genes

In insects, most Ors and some Gos/Irs are expressed in antennal ORNs [18,24,49,65]. As best illustrated in studies of the *Drosophila* olfactory system, each ORN expresses a single tuning Or which is responsible for the odorant response profile and all the ORNs expressing that singular tuning Or send axonal connections to a single antennal lobe glomerulus thereby providing a mechanistic basis for the initial stages of odor coding [18]. Therefore, we analyzed antennal transcriptomes of workers and males for both *C. floridanus* and *H. saltator*, to identify chemosensory receptor genes that are differentially expressed between castes (minors and majors in *C. floridanus*) and between different sexes, and which might play salient roles in social communication (see Table S2 for information on transcriptome datasets).

We performed pairwise comparisons between males and females within *C. floridanus* and *H. saltator* (Dataset S5). At the whole transcriptome level, there was a very high similarity between major and minor worker of *C. floridanus* (r² = 0.99; Figure S10A), while greater diversity was found between workers and males (r² values around 0.85 for all comparisons), largely due to mild up-regulation of many genes in males (Figure S10B, S10C).

Similar trends were also observed for chemosensory receptor genes (Figure S10D).

**OR family.** In both sexes of *C. floridanus* and *H. saltator*, the ortholog of Orco was consistently the most highly expressed Or gene. It accounted for ~15%–20% of all the Or gene expression in *C. floridanus* and ~6%–8% in *H. saltator*. For the repertoire of tuning Ors within each species, almost all of them were expressed in workers at levels above the medians of their respective antennal transcriptomes (which was used as the criterion for expression versus non-expression of chemosensory gene in the present study). In contrast, only one third of the tuning Or genes were expressed in males of both ants. These comparisons identified almost 40 Ors in *C. floridanus* and 120 Ors in *H. saltator* that displayed significant differential expression between workers and males (Table 1, Dataset S5). Interestingly, ~95% of these genes were enriched in workers, almost all of which had below-median expression levels in males. In addition, we found 13 Or genes that were differentially expressed between major and minor workers of *C. floridanus* (Table 1, Dataset S5). However, the log2 fold-changes of these genes (less than 1.5) were much lower than those of the genes (greater than 3) revealed in worker vs. male comparisons.

To investigate the relationship between evolutionary relatedness and expression regulation of Or genes, we mapped results of worker vs. male comparisons to the phylogeny of *C. floridanus* and *H. saltator* Or genes. As shown in Figure 6A, there are multiple examples where Or genes in one ant species showed sex-specific-enrichment patterns similar (or opposite) to closely related homologs in the other ant species. Notably, the 9-exon Or subfamily illustrates both situations described above (Figure 6B, 6C). In the three basal clades, *C. floridanus* genes were mostly enriched in male, while all but one *H. saltator* gene had higher expression levels in workers (Figure 6B). In contrast, all the remaining Or genes formed a well-supported monophyletic clade and almost all of them were enriched in workers for both *C. floridanus* and *H. saltator* (Figure 6C).

We further examined the expression patterns of (co-)orthologous genes in the two ant species. Using bootstrap values of 70 as threshold, we delineated 98 orthologous groups of *C. floridanus* and

**Table 1. Significantly differentially expressed *C. floridanus* and *H. saltator* chemosensory receptor genes revealed by analysis of antennal transcriptomes.**

| Species   | Comparison          | OR  | GR  | IR  |
|-----------|---------------------|-----|-----|-----|
| *C. floridanus* | Major worker vs. Male | 38 (1) | 0 (0) | 1 (1) |
|           | Minor worker vs. Male | 42 (1) | 0 (0) | 1 (1) |
|           | Major vs. minor worker | 13 (0) | 2 (1) | 0 (0) |
| *H. saltator*    | Worker vs. Male     | 120 (4) | 1 (0) | 2 (2) |

Significantly differentially expressed genes were identified by using Cuffdiff (q-value<0.05). Number in bracket indicates the number of genes with higher expression level in male (or minor worker in the major worker vs. minor worker comparison).

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H. saltator Or genes, of which 41 groups included at least one gene from each ant species being differentially expressed by at least two-fold between males and females. C. floridanus and H. saltator genes were enriched in the same sex in 29 of the 41 groups and in different sexes in other 10 groups (Table S3). The remaining 2 groups showed conflicting expression patterns within species.

**GR family.** Unlike Or genes, only a portion of Gr genes within each species were expressed in workers (less than 25% for C. floridanus and ~35% for H. saltator) and males (less than 15% for both ants) (Figure 7A). Furthermore, worker vs. male comparisons revealed only one H. saltator Gr gene that was differentially expressed between worker and male (HsGr7), and none in C. floridanus. While two C. floridanus Gr genes were found to have differential expressions between major and minor workers (CfGr9 and CfGr54), their absolute expression values were close to or below the median of their respective transcriptomes.

**IR family.** 50% or less of Ir genes of each species were expressed in any given sex (Figure 7B), and almost all expressed Ir genes are conserved “antennal IRs”. We identified only one C. floridanus Ir gene and two H. saltator Ir genes that have differential expressions between workers and males. Interestingly, all of these Ir genes were enriched in male. The ortholog of IR8a, encoding one of the Ir co-receptors [24,25], was differentially expressed in both C. floridanus and H. saltator, and was also the most highly expressed Ir gene in males of both ants, while another “antennal IR” (HsIR75u.2) was also found to be more highly expressed in H. saltator males than in workers.

**Identification of a ligand for a differentially expressed ant odorant receptor**

In order to validate our bioinformatic annotations and in an attempt to link functional data to the antennal expression data, we have cloned a small subset of 14 C. floridanus and H. saltator Or genes, drawn from 6 subfamilies in the Or phylogeny (D, E, H, L, V, and 9-exon). These include four genes (CfOr263, HsOr212, HsOr213, and HsOr279) that display significant differential expression in our transcriptome analysis (see Methods and Materials for full list). This allowed us to carry out deorphanization studies to decipher the odorant response profiles of these receptors through the use of two-electrode voltage clamp recordings in Xenopus oocytes heterologously expressing ant Ors [44,66]. After first confirming that the C. floridanus and H. saltator Orco proteins showed coreceptor function in combination with a previously

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**Figure 6. Diversified expressions of evolutionarily related Or genes.** (A) Expression profiles of C. floridanus and H. saltator Ors shown along with the phylogeny of Or genes. In the phylogenetic tree, C. floridanus Ors are labeled by red and H. saltator Ors by blue. In the heat-map, red color indicates higher expression level in worker and green indicates higher expression level in male. The inner circle shows the relative expressions of C. floridanus Ors between major worker and male (comparison between minor worker and male not shown because of the highly similar expression profiles of C. floridanus major and minor workers); the outer circle shows the relative expressions of H. saltator Ors between worker and male. FPKM stands for Fragments Per Kilobase of exon per Million fragments mapped. (B) Expression levels of Ors belonging to the three basal clades in the 9-exon subfamilies. C. floridanus Ors had significantly higher expression in male (p-value<0.05; Wilcoxon ranked-sum test), while H. saltator Ors had significantly higher expressions in worker (p-value<1e-4; Wilcoxon ranked-sum test). (C) Expression levels of the remaining Ors in the 9-exon subfamilies. For both C. floridanus and H. saltator, Ors had significantly higher expressions in worker (p-value<1e-15; Wilcoxon ranked-sum test). Short lines indicate median expression levels for each gene set. For both panels (B) and (C), genes expressed below the medians of their respective transcriptomes were labeled by grey.

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Figure 7. Expression levels of Gr and Ir genes. (A) The GR family. (B) The IR family. Short lines indicate median expression levels for each gene set. In both panels, genes expressed below the median of their respective transcriptomes were labeled by grey.

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deorphanized mosquito tuning Or (Figure 8A, 8B), candidate ant tuning Ors were screened against a panel of 73 unitary and complex stimuli (Table S4). These stimuli consisted of a variety of general odorants, as well as hydrocarbons known to be produced by H. saltator or C. floridanus.

Out of the 14 tuning Ors initially screened, CfOr263 (from OR subfamily D; Figure 2), which is highly expressed in workers as compared to males (Figure 6A), produced specific and dose-dependent responses to 2,4,5-trimethylthiazole (Figure 8D, 8F), a naturally occurring odorant found in cooked beef and pork [67] found in the library of general odorants that we screened. An additional Or from H. saltator, HsOr55 (from OR subfamily L; Figure 2), showed a dose-dependent response to another odorant from our general odorant library, 4-methoxyphenylacetone (Figure 8E, 8G), which is a naturally occurring odorant found in anise essential oil [68]. However, this particular Or has not been shown to be differentially expressed between males and females. It should also be noted that, as is the case for most ant Ors, both receptors have multiple closely related homologs that may possess similar chemosensory functions (Figure 6A).

Discussion

Expanded ant chemosensory receptor repertoire

We have developed and used a dedicated annotation scheme to comprehensively elucidate the repertoire of chemosensory receptor genes in both C. floridanus and H. saltator. Through exhaustive homology search and careful manual curation, we significantly improved upon previous studies to identify roughly equivalent numbers of Or/Gr/Ir genes in the genomes of C. floridanus and H. saltator as compared to two other sequenced ant genomes [47,48], providing a solid foundation for subsequent study.

It is striking that, in general, ants have the most expanded repertoire of chemosensory receptor genes in Hymenoptera (Figure 1). The numbers of ant OR and IR family members are much greater than those of the other two hymenopteran genomes currently available. Indeed, thus far, ant genomes have the largest number of Or genes among all insects [69]. Furthermore, although the number of the Gr genes varies greatly among hymenopterans and also within ants, L. humile carries the largest Gr family; it has about 2- and 10-fold more Grs than N. vitripennis and A. mellifera, respectively. Interestingly, although ants and honey bees are both social insects, ants have much larger repertoires of all three chemosensory receptor gene families than honey bees, possibly indicative of a more sophisticated communication system relying on chemicals [70].

Our phylogenetic analyses of hymenopteran chemosensory receptor genes reveal distinct evolutionary patterns among gene families. Among chemosensory receptors, the OR family shows the most dramatic birth-and-death evolution, with many OR subfamilies displaying diversified patterns of gene gain-and-loss. For example, the 9-exon subfamily and others have experienced rapid gene duplications at almost all stages of Hymenoptera evolution, followed by numerous losses of duplicates. In contrast, there are 35 subclades that have only one ortholog in all four ants. Further, the IR family has maintained relatively stable copy numbers in ants; lineage-specific expansion only occurred in C. floridanus and L. humile for two of the 13 “divergent IRs”. In between these extremes is the GR family that has expanded moderately in N. vitripennis and three of the four ants.

Recent studies of chemosensory receptors in mammals and Drosophila, as well as other genes with important regulatory and physiological functions, have suggested a possible correlation between functional requirements and the variations of gene numbers [52,71,72]. Genes with conserved roles tend to have relatively stable copy numbers while those with diversified functions have higher rates of birth-and-death, although the degrees of copy number changes are somewhat random. Our results suggest that this pattern could also hold true for the evolution of the hymenopteran chemosensory receptor genes. For example, as an obligatory co-receptor for all other Ors [29], Orco is the most conserved insect Or gene and also the only one that has maintained unambiguous orthology in all insects studied to date, including ants [69]. Similarly, orthologs of most “antennal Ir”
Analysis of Ant Chemosensory Receptomes

A

CfOrco
AgOr10

-4 logM
VUAA1

-4 logM
BA

500 nA
50 s

B

HsOrco
AgOr10

-4 logM
VUAA1

-4 logM
BA

500 nA
50 s

C

Water-injected

-4 logM
VUAA1

-4 logM
BA

500 nA
50 s

D

CfOrco
CfOr263

-4 logM
TMT

-4 logM
4MPA

-5 logM
TMT

-4 logM
TMT

-3 logM
TMT

100 nA
100 s

E

Water-injected

-4 logM
TMT

-4 logM
4MPA

-5 logM
TMT

-4 logM
TMT

-3 logM
TMT

100 nA
100 s

F

HsOrco
HsOr55

-4 logM
4MPA

-4 logM
TMT

-5 logM
4MPA

-4 logM
4MPA

-3 logM
4MPA

100 nA
100 s

G

Water-injected

-4 logM
4MPA

-4 logM
TMT

-5 logM
4MPA

-4 logM
4MPA

-3 logM
4MPA

100 nA
100 s
[26] have also maintained strict single-copy in Hymenoptera. It has been proposed that these conserved “antennal IRS” represent the earliest insect chemosensory receptors and perform functions important for all insects [26]. Therefore, we suggest that the chemosensory receptor genes that have constant copy numbers in ants (e.g. the 35 single-copy tuning Ors) are likely to carry out important functions common for all ants.

On the other hand, prevalent rapid expansions in chemosensory receptor gene families could allow for diversification in ligand specificity/sensitivity among duplicated receptor genes. Such functional divergences would offer tremendous opportunities for organisms to explore different chemical niches, thus facilitating the adaption to new environments and/or the evolution of novel life styles such as sociality. In all three gene families, we found either a single expansion or a partial expansion in the ancestor of ants (Figure 5), which might have contributed to the success and subsequent diversification of this group.

In addition, there are many cases of unbalanced expansions/contractions among lineages in specific sub-families, suggesting that the chemosensory receptor repertoire has been differentially exploited among ants, which might shed light on the evolution of different lifestyles of ants. For example, our results indicate expansions of Gs in C. floridanus, L. humile, and P. barbatus, but not H. saltator, which are likely to reflect differences in their feeding behaviors. In this view, scavengers like C. floridanus might require a highly expanded repertoire of taste receptors to discriminate nutritious food sources from spoiled, contaminated, or poisoned substrates. In contrast, H. saltator workers likely rely more on visual cues to track down prey, as suggested by their large eyes and expanded number of ommatidia [73]. Furthermore, Gs which act as contact chemoreceptors would be far less useful for identifying and capturing prey. In fact, ponerine ants in general rarely use liquid food sources, since they normally lack the ability to exchange liquids stored in their crop [74] which further reduces the potential benefit of a large Gr repertoire.

Another intriguing possibility is that Gs are involved in the contact chemosensation of species-specific, nonvolatile CHCs (e.g. queen pheromone, nestmate recognition signals, etc.), and that C. floridanus has more Gs precisely because they utilize a greater number and variety of pheromones to support their more rigid and complex social lifestyle. Presumably, these Gs would be in addition to the large number of worker-enhanced Ors that are likely to be involved in the same process. Furthermore, C. floridanus has expansions in multiple GR subfamilies, including 5 homologs of the DmGr43a/AmGr5 gene, which has been recently shown to be a fructose receptor [73]. Taken together, our results indicate a correlation between the expanded GR family and the more complex chemical ecology of C. floridanus.

Diversified expression of chemosensory receptor gene

The antenna is perhaps the most important chemosensory organ for ants, where a variety of ant species have been observed to closely inspect their environment and each other by touching their antennae in a process known as antennation [2]. This makes it likely that most of the behaviorally important chemosensory neurons (and their corresponding chemosensory receptors) are located in this organ. Our comparative analysis of antennal transcriptomes of workers and males in both C. floridanus and H. saltator reveal differential expressions of chemosensory receptor genes both within and between species, providing important clues on their functional divergence.

One major pattern revealed by our results is the substantial sexual dimorphism in chemosensory receptor gene expression in ants. For both C. floridanus and H. saltator, almost all Ors were expressed in workers, but only one third were expressed in male. Similarly, workers consistently had more expressed Gs and IRS than males. In contrast, expression of chemosensory receptor genes was highly similar between major and minor workers in C. floridanus. Previous studies have shown that the antennal lobes of males from both C. floridanus and H. saltator lack a large subset of glomeruli relative to workers [76,77,78], which may explain the low number of chemosensory receptor genes expressed in males. Given that the number of glomeruli in insects generally correlates with the number of functional odorant receptors [18,65], it is likely that most of the Ors that are only expressed in C. floridanus and H. saltator workers project to these female-specific glomeruli. Furthermore, it has been shown in another Camponotus species (Camponotus japonicus) that females exclusively possess the olfactory sensilla necessary to detect non-nestmate CHCs, [79,80]. It is therefore likely that the CHCs receptors are encoded by some of the worker-specific Ors in C. floridanus. In particular, the 9-exon subfamily represents the largest expansion of Ors in all ants and it harbors close to 100 worker-specific Ors in both C. floridanus and H. saltator. These results strongly support previous hypothesis that members of the 9-exon subfamily are likely candidates for ant CHCs receptors [47,48]. These Ors are potentially involved in detecting CHCs involved in worker-to-worker or worker-to-queen intracolonal social communication.

Interestingly, we also noticed discrepancies between the overall number of Ors and the number of glomeruli in the adults of these two ant species. H. saltator workers and males both have far more expressed Ors than the number of glomeruli in the adult antennal lobe (approximately 78 in the adult male and 178 in the adult worker [77]). The discrepancy in H. saltator could possibly be the result of co-expression of multiple tuning Ors in the same ORN and/or the projection of ORNs expressing different, but related tuning Ors to the same glomerulus, which have both been observed for a small number of Ors/ORNs in D. melanogaster [81,82,83,84].

However, given that the number of expressed Ors is about twice the number of observed glomeruli, this would mean that each glomerulus received input from, on average, two odorant receptors. Although co-expression of tuning Ors has not been observed to such a broad extent in any insect olfactory system studied to date, it should be noted that many of the receptor pairs that are co-expressed in Drosophila appear to be the result of tandem duplication events [84]. Therefore, it is possible that the extensive tandem duplication of H. saltator Or genes may also result in the co-expression of closely related odorant receptors from the same clusters. All of these are highly interesting hypotheses that may be examined in future studies.
In contrast to *H. saltator*, *C. floridanus* has approximately 80 fewer *Ors* than the number of adult worker glomeruli (about 434 [76]). In this instance it is possible that many of those glomeruli receive projections from *Or* and *Ir* expressingORNs, as there is precedence for this in *Drosophila* [24,43] and the number of predicted *Ors* and *Irs* would be enough to fill the gap. Moreover, it could be that several *Ors* have been missed by the current analysis due to incomplete genome assembly; some of the fragmented *Or* gene models might represent genuine genes, and further genomic/transcriptomic data would help address this possibility.

Although chemosensory receptor genes in general had higher expression in workers, our studies have nevertheless identified a single *Or* (*CfOr267*, in subfamily 9-exon) and a single *Ir* (*CfIR8a*) in *C. floridanus*, as well as 4 *Ors* (*HsOr32, HsOr35, and HsOr37*, in subfamily L; and *HsOr224*, in subfamily E) and 2 *Irs* (*HsIR8a* and *HsIR75a.2*) in *H. saltator* that were significantly male-enriched. The male-enrichment of a receptor gene could be due to elevated expression of the gene in ORNs of males relative to workers, and/or increased number of ORNs expressing the gene in males. No matter which of the possibilities is indeed the case, our results indicate higher overall abundances of these chemosensory receptor genes in male antennae. These genes are viable candidates for receptors that are specifically tuned for male-specific social cues, including queen pheromones. In fact, at least one male-specific honeybee odorant receptor that responds to a queen-specific pheromone has already been revealed through microarray analysis and subsequent functional characterization in *Xenopus* oocytes [85]. It would not be surprising to see that similar results will be found with the male-enriched ant *Ors*.

In insects, the co-receptors *IR8a* and *IR25a* are the two most conserved *Irs* [26]. Although a systematic profiling of sexual dimorphic *Ir* expression is still lacking, a previous study has shown that the *Anopheles gambiae* orthologs of both *IR8a* and *IR25a* have higher expression in female than male [49]. Interestingly, *IR8a* was the most male-enriched *Ir* in both *C. floridanus* and *H. saltator*. While *IR25a* also displayed higher expression in *C. floridanus* male, it was not expressed in the male of *H. saltator*. These results could possibly indicate a functional divergence of *IR8a* and *IR25a* between *Diptera* and *Hymenoptera*. In addition, the high expression of *IR25a* in males of *C. floridanus*, but not *H. saltator*, suggests that *IR25a*-mediated signaling might have contributed to the more expanded roles for males within the colony of the former species. It may be that *C. floridanus* males are more involved in intracolonial interactions than *H. saltator* males, since males from other *Camponotus* species are known to participate in food exchange in the colony [86], which has not observed in *H. saltator* males.

We have also found diversified expression of closely related *Ors* within and between species. For example, in the basal clades of the 9-exon OR subfamily, closely related *C. floridanus* and *H. saltator* showed opposite sexual dimorphism in their expression (Figure 6B). Although the well-supported monophyletic clade within the 9-exon OR subfamily mostly consists of worker-enriched genes, it also harbors a few genes that are highly enriched in male (Figure 6C). Thus, while our expression results are generally (and strongly) consistent with the idea that members of the 9-exon OR subfamily are involved in the detection of CHCs by workers [47], a subset of these receptors have apparently been adapted for use in males, possibly for detecting queen mating pheromones.

Taken together, these results indicate that ant *Or* genes have experienced not only extensive gain-and-loss, but also rapid changes in their expression, once again highlighting the highly dynamic nature of chemosensory receptor gene evolution. Our phylogenetic and transcriptomic analyses, in combination, have identified ant chemosensory receptor genes that exhibit evolutionary and expression patterns indicative of species-sex-specific functions. Ultimately, deorphanzization of these receptors will greatly facilitate our understanding of the chemical ecology of social lifestyle in ants.

**Heterologous characterization of differentially expressed *C. floridanus* *Ors***

In our heterologous studies of ant tuning *Ors*, we have identified chemical agonists for a single receptor from each of the two species analyzed. These data provide conclusive validations for our bioinformative-based annotations. Although a honeybee odorant receptor has been previously shown to respond to the queen substance 9-oxo-2-decenonic acid [85], we believe that this represents the first published report of ligand activators for odorant receptors from ants.

In these studies, *HsOr55* from *H. saltator*, display significant responses to 4-methoxyphenylacetone, a naturally occurring odorant found in anise essential oil [60]. Since anise essential oil has been shown to have a repellent and/or insecticidal effect on at least some species of insects [87,88], 4-methoxyphenylacetone might represent a general insect repellent, with *HsOr55* acting as the detector for this repellent in *H. saltator*. Whatever *HsOr55*'s role may be, it is likely to be a very general one, since *HsOr55* transcripts do not appear to be differentially expressed between workers and males.

The other odorant receptor characterized in this study, *CfOr263* from *C. floridanus*, displayed sensitivity to 2,4,5-trimethylthiazole, a naturally occurring odorant found in cooked beef and pork [67] that has been previously shown to induce strong responses in the CPG neuron of the maxillary palp in the mosquito *Anopheles gambiae* [44]. While the relevance of this chemical to *C. floridanus* remains unclear, the fact that *CfOr263* transcripts are enriched in workers relative to males suggests that this odorant may be an important volatile semiochemical for *C. floridanus* workers. Regardless, the successful identification of odors that activate *CfOr263* and *HsOr55* strongly validates the role of ant *Ors* as chemosensory receptors. Furthermore, the large differential expression of *CfOr263* between workers and males indicates that it is detecting a sex-specific signal that is relevant to workers but not to males, and testing a broader panel of odorants in the future will provide a better understanding of what that signal might be.

**Conclusions**

We have revealed a greatly expanded repertoire of chemosensory receptor genes for a pair of divergent ant species, including about 400 *Ors* and an order of magnitude smaller number of *Irs* and *Irs*. Phylogenetic analysis of these newly annotated genes indicates that there are likely to be vast differences in the importance of particular chemoreceptor families and subfamilies between the four ant species examined, which is likely to reflect the variety of ecological and social demands experienced the members of each species. These analyses also reveal high rates of gene birth-and-death evolution among the olfactory and gustatory receptor genes, suggesting that some factor (such as changes in the complex CHC profiles that control ant social behavior) is driving rapid evolution in their chemical response profiles. The large repertoire of ant chemosensory genes might be either due to preferential retention of ancestral genes or rapid expansions in the ant ancestor and during later stages of ant evolution. To further complement these phylogenetic results, we have generated and analyzed antennal-specific RNAseq expression data to identify ~40 *C. floridanus* and ~120 *H. saltator* chemosensory receptors that exhibit significant sexual dimorphism in expression. This expression data has, in turn, informed studies towards the identification of odorant...
ligands for socially relevant receptors, a process that we have already successfully accomplished in a heterologous system for one of the differentially expressed C. floridanus Ors. Taken together, our evolutionary analysis, transcriptome profiling, and heterologous characterization provide new insights into the roles of the chemosensory receptors in inter-sex behavioral and social differences of ants.

**Materials and Methods**

**Gene annotations**

The assemblies of C. floridanus (version 3.5) and H. saltator (version 3.5) were downloaded from the Hymenoptera Genome Database [89]. Protein sequences of reported chemosensory gene were also collected from Apis mellifera, Atherigona piscum, Drosophila melanogaster, Nasonia vitripennis, L. humile, and P. barbatus [15,26,28,47,48,50,54]. An in-house bioinformatics pipeline was developed to identify candidate chemosensory genes in C. floridanus and H. saltator. First, all collected chemosensory gene sequences were searched against the two ant genomes using TBBLASTN [90] with an e-value cutoff of 1e-5. Resulting high-scoring Segment Pairs (HSPs) were sorted by their blast bit-scores, and an average bit-score of the top 75% HSPs were calculated. Any HSPs with a bit-score less than 25% of the average was discarded. Chains of HSPs were than created from retained HSPs. Two HSPs were chained together if the following criteria were met: 1) they are derived from the same query; 2) they are located within 3 kb on the same strand of a scaffold/contig; and 3) the corresponding query region of the upstream HSPs must also be N-terminal to that of the downstream HSPs. The third criterion was applied to avoid artificial concatenation of neighboring chemosensory genes. Genomic regions covered by HSPs chains were considered putative chemosensory gene coding regions. For each putative gene, we then selected the query corresponding to the highest scoring HSPs at that region as reference sequence for homology-based gene prediction using GeneWise (version 2.2.0) [91]. All predictions were sorted by ORF length and the lowest 25% was filtered. This pipeline was iterated by adding results of previous run to input until no additional genes were found.

Multiple sequence alignments (MSAs) of predicted OR/GR/IRs were constructed using MUSCLE (version 3.8) [92] and manually inspected. Attempts to improve annotations were made whenever an obvious problem was identified (e.g. missing exon, incorrect exon-exon junction). In addition, in the OR and GR families, we observed many fragmented gene models, likely due to pseudogenization and incomplete genome assembly. For the convenience of subsequent analyses, a minimum size cutoff of 300 amino acids was used for the ORs and GRs. For IRs, we screened all predicted protein sequences with InterProScan (V4.8) [93] and filtered the ones without characteristic domains of IR (PF10613 and PF00060) [26].

**Phylogenetic analysis**

We included in our phylogenetic analysis chemosensory receptor genes in six hymenopteran species, including A. mellifera, C. floridanus, H. saltator, N. vitripennis, L. humile, and P. barbatus. For each of the OR/GR/IR families, all family members were firstly aligned at once using MUSCLE (version 3.8) and a preliminary phylogenetic tree was built using RAxML (version 7.2.8) [94]. Sequences were then divided into groups corresponding to highly supported clades in the preliminary phylogeny. Groups were aligned individually using PROALIGN (version 1.4) [95] and then combined together using the profile alignment function of MUSCLE. The complete alignment were further manually inspected and adjusted using GeneDoc (version 2.6) [96]. In addition, poorly aligned regions in the alignment were removed using trimAl (version 1.4) [97]. The final maximum-likelihood tree was constructed using RAxML with Le-Gascuel (LG) substitution model [90] and GAMMA correction for rate variation among sites. Reliability of tree topology was evaluated by 100 bootstrap replicates. To estimate the number of gene gain and loss events, we used a maximum-likelihood based approach implemented in CAFÉ (version 2.2) [62] with default settings. As an alternative approach, we also used the parsimony based “modified reconciliation method” [99]; we first collapsed branches with bootstrap support lower than 70 in phylogenies of OR/GR/IR families and then reconciled condensed trees with known organismal relationships using Notung (version 2.6) [63].

**Analysis of ant antennal transcriptome**

Samples originated from C. floridanus colonies that had been founded in the Liebig lab from queens captured in southern Florida between 2002 and 2009 and from H. saltator colonies collected in Karnataka, India between 1993 and 1999. Antennae were collected from each of five groups of adult ants: H. saltator workers and males and C. floridanus major workers, minor workers, and males. Whole ants were flash-frozen in liquid nitrogen and kept on dry ice as 100 antennae from each group were removed with forceps. Antennae were placed directly into RNA later (Ambion) that had been pre-chilled on dry ice in a conical, ground-glass, tissue homogenizer. RNA later was replaced with 1 ml Trizol (Invitrogen), in which antennae were homogenized. Total RNA was isolated following Trizol manufacturer instructions; briefly, after addition of 200 µl of a chloroform/isoamyl alcohol mixture (24:1), each sample was mixed vigorously and the RNA-containing aqueous layer was isolated with centrifugation. RNA was further purified and DNAs-treated with the RNasy MiniPrep kit (Qagen). After ethanol precipitation, the RNA pellet was resuspended in 30 µl nuclease-free water. Male samples were sequenced using Illumina HiSeq2000 at the NYULMC Genome Technology Center, generating ~33 million 50 bp single-end reads for C. floridanus male and ~164 million 51 bp single-end reads for H. saltator male. All worker samples were sequenced at Hudson Alpha, generating more than 20 million 30 bp paired-end reads for each sample (sum of two technical replicates).

**Analysis of ant antennal transcriptome**

Reads of C. floridanus male sample were trimmed to 34 bp (3 bp trimmed from both ends) to remove low-quality positions. In addition, for all worker datasets, we treated each paired-end read as two single-end reads. Therefore, all datasets in our subsequent analyses consist of only single-end reads. Alternative strategies for data processing led to highly similar estimations of gene expression values (Table S5). For each dataset, reads were mapped to the corresponding ant genome using TopHat (version 1.3.3) [100] with default settings. Gene annotations for C. floridanus (version 3.5) and H. saltator (version 3.5) were downloaded from the Hymenoptera Genome Database and used in combination with our annotation of chemosensory genes to guide the reads mapping. Gene expression levels (in FPKM values) and differentially expressed genes were determined using Cuffliff v1.3.0 [101] with frag-bias-correct, multi-read-correct, and upper-quartile-norm options turned on.

**Heterologous analysis of ant odorant receptors**

Predicted Or coding sequences were amplified, by PCR, from H. saltator and C. floridanus worker antennal cDNA samples obtained...
from colonies established at Arizona State University (Tempe, AZ). The PCR-amplified sequences were then TOPO cloned into the Gateway Entry vector pENTER/D-TOPO (Life Technologies), followed by an additional cloning step into a destination vector derived from pSP64T. To obtain cRNA for each Or, the pSP64T vector containing the appropriate coding sequence was linearized by restriction digest and used as a template for cRNA synthesis using the mMessage mMachine Sp6 Kit (Ambion). Heterologous expression of ORs was accomplished as described previously [66]. Briefly, mature oocytes were surgically extracted from Xylocopa levis adult females, treated with 2 mg/mL collagenase II in 1× Ringer’s solution (96 mM NaCl, 2 mM KCl, 5 mM MgCl₂, and 5 mM Hepes, pH 7.6) for 30–45 minutes at room temperature, and then injected with 27.6 nL of a 1:1 mixture (by mass) of a given tuned Or in combination with the appropriate Orco ortholog (either H. levis Orco or C. floridanus Orco). After injection, oocytes were stored in Incubation Medium (10% dialyzed horse serum in 1× Ringer’s solution) at 18C for 3–7 days before testing. Responses to odorants were measured by recording whole-cell currents in Clampex 10.2 (Molecular Devices) using a two-electrode voltage-clamp setup (OC-725C, Warner Instruments) maintained at a holding potential. Odorants were first dissolved in DMSO, and then further diluted into Ringer’s solution before being introduced to the oocyte recording chamber using a perfusion system. For the hydrocarbons that were tested, 0.01% Triton X-100 (Sigma) was added to the Ringer’s solution to aid in dissolving the odorant. 0.01% Triton X-100 (Sigma) was also added to the Ringer’s solution to aid in dissolving the odorant. The following odorant receptors were tested with the odorants also added to the Ringer’s solution to aid in dissolving the odorant.

Supporting Information

Dataset S1 | Details of C. floridanus and H. saltator chemosensory receptor genes annotated in this study. The genome location and predicted protein and transcript sequences are provided for each annotated gene. (XLSX)

Dataset S2 | Phylogenetic relationships of Hymenoptera Or genes shown in newick format. Bootstrap values are shown for all nodes. See Figure 2A and Figure S2 for graphical presentation of the same phylogeny. (TXT)

Dataset S3 | Phylogenetic relationships of Hymenoptera Gr genes shown in newick format. Bootstrap values are shown for all nodes. See Figure 3 and Figure S6 for graphical presentation of the same phylogeny. (TXT)

Dataset S4 | Phylogenetic relationships of Hymenoptera Ir genes shown in newick format. Bootstrap values are shown for all nodes. See Figure 4 and Figure S8 for graphical presentation of the same phylogeny. (TXT)

Dataset S5 | Complete results of antennal transcriptome comparisons for all chemosensory receptor genes. Four pairwise comparisons are presented, including major worker vs. minor worker (C. floridanus), major worker vs. male (C. floridanus), minor worker vs. male (C. floridanus), and worker vs. male (H. saltator). (XLSX)

Figure S1 | C. floridanus and H. saltator OR genes are mostly distributed in tandemly arrayed gene clusters. (A) C. floridanus OR genes. (B) H. saltator OR genes. (EPS)

Figure S2 | Phylogenetic relationships of Hymenoptera Or genes. The same tree as in Figure 2A is shown with gene names. Only bootstrap values ≥50 are shown. Supported subfamilies are indicated by brackets. (EPS)

Figure S3 | Phylogenetic relationships of Or genes in representative insects. A maximum-likelihood tree of Or genes from D. melanogaster, T. castaneum, and six hymenopteran species. The topology is estimated by using RAxML with Le-Gascuel (LG) model. Reliability of internal nodes was evaluated by 100 bootstrap replicates. Only bootstrap values ≥50 are shown. Subfamilies that are delineated based on hymenoptera OR phylogeny are indicated by brackets. All D. melanogaster and T. castaneum genes are highlighted in blue. Confidently resolved relationships among hymenopteran and non-hymenopteran Or genes are indicated by red. (EPS)

Figure S4 | Phylogeny of selected OR clades exhibiting distinct modes of gene birth-and-death: (A) constant single-copy in all ants; (B) gene gain in P. barbatus only; (C) gene loss in H. saltator, but multiple gene gains in other ants; and (D) lineage-specific expansions in all ants. (EPS)

Figure S5 | Tandemly arrayed ant OR genes were generated by duplications at multiple stages of ant evolution. Evolutionary relationships and genomic arrangements of selected C. floridanus and H. saltator OR genes were shown. M, N, O, and P indicate four well-supported OR subfamilies, each likely representing one OR gene in the ancestor of Hymenoptera. C. floridanus OR genes belonging to the cluster on scaffold338 were labeled by red. H. saltator OR genes belonging to the cluster on scaffold105 were labeled by blue. (EPS)

Figure S6 | Phylogenetic relationships of Hymenoptera Gr genes. The same tree as in Figure 3 is shown with gene names. Only bootstrap values ≥50 are shown. Supported subfamilies are indicated by brackets. Subfamilies showing interesting evolutionary patterns are named after the orthologs in N. vitripennis and A. mellifera. The other subfamilies are named as A–H. (EPS)

Figure S7 | Phylogenetic relationships of Gr genes in representative insects. A maximum-likelihood tree of Gr genes from D. melanogaster, T. castaneum, and six hymenopteran species. The topology is estimated by using RAxML with Le-Gascuel (LG) model. Reliability of internal nodes was evaluated by 100 bootstrap replicates. Only bootstrap values ≥50 are shown. Subfamilies that are delineated based on hymenoptera Gr phylogeny are indicated by brackets. Subfamilies showing interesting evolutionary patterns are named after the orthologs in N. vitripennis and A. mellifera. The other subfamilies are named as A–H. All D. melanogaster and T. castaneum genes are highlighted in blue. Confidently resolved relationships among hymenopteran and non-hymenopteran Gr genes are indicated by red. The clade of Grs encoding carbon dioxide receptor is indicated by blue. (EPS)
Figure S8  Phylogenetic relationships of Hymenoptera Ir genes. The same tree as in Figure 4 is shown with gene names. Only bootstrap values ≥50 are shown. Supported subfamilies are indicated by brackets, and named after the orthologs in L. humile and P. barbatus.

Figure S9  Phylogenetic relationships of Ir genes in representative insects. A maximum-likelihood tree of Ir genes from D. melanogaster, T. castaneum, and six hymenopteran species. The topology is estimated by using RAxML with Le-Gascuel (LG) model. Reliability of internal nodes was evaluated by 100 bootstrap replicates. Only bootstrap values ≥50 are shown. Subfamilies that are delineated based on hymenoptera IR phylogeny are indicated by brackets, and named after the orthologs in L. humile and P. barbatus. All D. melanogaster and T. castaneum genes are highlighted in blue. Confidently resolved relationships among hymenopteran and non-hymenopteran Ir genes are indicated by red.

Figure S10 Pairwise comparisons of whole transcriptome between castes for C. floridanus and H. saltator. Chemosensory receptor genes were highlighted in red.

Table S1  Sequence divergence of chemosensory receptor genes.

Table S2  Summary of ant antennal transcriptome data sets and mapping results.

Table S3  Expression patterns of (co-)orthologous Or genes in C. floridanus and H. saltator.

Table S4  List of the 73 odors screened in this study.

Table S5  Alternative strategies for bioinformatic processing of ant transcriptomes do not significantly affect read mapping.

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Author Contributions

Conceived and designed the experiments: X Zhou, JD Slone, A Rokas, LJ Zwiebel. Performed the experiments: X Zhou, JD Slone. Analyzed the data: X Zhou, JD Slone. Contributed reagents/materials/analysis tools: J Liebig. Wrote the paper: X Zhou, JD Slone, A Rokas, SL Berger, J Liebig, A Ray, D Reineberg, LJ Zwiebel.

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