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## Opinion piece

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One contribution of 12 to the Special Feature '50 Years on: the legacy of William Donald Hamilton' organized by Joan Herbers and Neil Tsutsui.

## Evolutionary biology

# Dissecting ant recognition systems in the age of genomics

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Hamilton is probably best known for his seminal work demonstrating the role of kin selection in social evolution. His work made it clear that, for individuals to direct their altruistic behaviours towards appropriate recipients (kin), mechanisms must exist for kin recognition. In the social insects, colonies are typically comprised of kin, and colony recognition cues are used as proxies for kinship cues. Recent years have brought rapid advances in our understanding of the genetic and molecular mechanisms that are used for this process. Here, I review some of the most notable advances, particularly the contributions from recent ant genome sequences and molecular biology.

## 1. Introduction

The defining feature of societies is the recognition of group members by others within the society, and the associated exclusion of non-members [1]. As recognized by Hamilton [2,3], social insect colonies are typically comprised of relatives, and thus colony recognition (often referred to as 'nest-mate recognition') can serve as a proxy for kin recognition. Since Hamilton's work, social insects have proved to be fruitful models for illuminating some aspects of kin recognition, whereas other processes, such as within-colony kin recognition and nepotism, have found less or mixed support (e.g. [4,5]). Recognition at the between-colony level has been demonstrated in many taxa and, in some cases, the specific mechanisms involved in colony recognition are becoming clear.

In recent years, there have been rapid advances in genetics and genomics, for both social insects and model organisms. Draft genome sequences have been published for a number of eusocial insects, including seven ant species [6–11], revealing myriad candidate genes for signal production and signal perception in colony recognition systems. At the same time, functional genetic tools offer powerful approaches to test hypotheses about the structure and function of recognition systems. Here, I discuss some of the ways that these recent advances may inform studies of recognition systems.

## 2. The production of colony recognition signals

Cuticular hydrocarbons (CHCs) are molecules produced by insects that typically function as a barrier to desiccation and/or to prevent microbial infection [12,13]. The additional function of CHCs as the cues used for colony recognition has been demonstrated for many species of ants (e.g. [14,15]). Because of this crucial role in regulating social structure, ant CHCs have been the focus of intense study, and about 1000 different CHCs have been described from ants [16]. However, nearly all of these studies have been descriptive or correlational, and the biosynthetic pathways that underlie production and the mechanisms for generating molecular variation are not well understood. In fact, much of what we know about CHC synthesis in social insects relies on extrapolation from other, more well-studied, non-social insects, particularly species of Diptera [17–20].

A wealth of studies has pointed to CHCs, typically alkanes and alkenes, as the chemical cues that ants use for colony mate recognition (e.g. [21–23]). The specific chemicals that comprise the CHC profile vary within and among species [24], and dozens of different CHCs can be present on the cuticle of an individual ant [21]. In insects, long-chain CHCs are synthesized when fatty acyl precursors are converted into long-chain acyl-CoA thioesters through a series of steps that are catalysed by desaturases, elongases and fatty acid synthases [13,25]. Acyl-CoA reductases then convert the acyl-CoA thioesters into aldehydes that are, in turn, converted into hydrocarbons (HCs) by a cytochrome P450. The synthesis of CHCs occurs in the insect's oenocytes [26], and the products are then transported to the cuticle (and other tissues) by lipophorin proteins [27].

Some ants appear to use alkenes for colony mate recognition (e.g. [28,29]). In these cases, molecules may vary in length, the number and location of double bonds and, occasionally, the number and location of methyl groups. Desaturases have been well established as enzymes that catalyse the formation of double bonds in CHCs. Although little is known about the role of specific desaturase genes in ant CHC production, in *Drosophila*, the *desat1* gene is known to play a role in alkene biosynthesis [30,31]. In three of the sequenced ant genomes, annotation revealed 11–33 complete or fragmentary  $\Delta 9$  desaturase genes [6–8]. Of these, one gene in both *Atta cephalotes* and *Pogonomyrmex barbatus* was identified as a clear orthologue of the *Drosophila desat1* gene, and two such orthologues were discovered in *Linepithema humile* (the Argentine ant). If function is conserved in both *Drosophila* and ants, these genes hold promise as key players in the biosynthesis of alkenes used in ant colony mate recognition.

In other ant species, methyl-branched alkanes have been implicated as the primary chemical recognition cues. In Argentine ants, for example, between-colony variation in specific methyl-branched long-chain HCs is correlated with the presence or the absence of aggression among workers [32,33], and manipulation of CHC profiles using pure, synthetic versions of these CHCs has been shown to induce aggression among nest-mates [22]. Moreover, behavioural studies using synthetic versions of CHCs have also shown that methyl group presence and location, and overall carbon backbone length, are key features that are recognized by Argentine ant workers [34]. Similarly, in the carpenter ant, *Camponotus herculeanus*, treatment with a synthetic dimethyl alkane has been shown to elicit intraspecific aggression [23]. Although these functional tests of specific HCs are powerful approaches for identifying recognition cues, the synthesis of pure candidate HCs can be challenging.

Although many of the genes and gene networks underlying the production of and variation in methyl-branched alkanes are yet to be discovered, studies from *Drosophila* (and others) have implicated elongase genes as key regulators of overall carbon chain length. In the ant genomes, cursory analyses reveal many candidate elongase genes, but they have yet to be closely annotated and analysed.

Some clues to the evolution of CHCs biosynthesis have recently been revealed in large comparative studies of CHC profiles across the ant phylogeny [16,24]. A recent study of 58 ant species showed that, in contrast to the saltational evolutionary patterns reported for other insect pheromones [35,36], ant CHCs (largely analysed from workers) appear to have evolved more gradually [24]. In this study, ancestral state reconstruction revealed that the presence of alkenes

and dimethyl alkanes is ancestral in ants, whereas the production of alkadienes and trimethylalkanes in CHC profiles is more derived (in the lineages that express them). Interestingly, although some taxa have lost one or the other of the more ancestral structural types (either alkenes or dimethyl alkanes), no taxon has lost both. This pattern may reflect a fundamental, widespread role for these structural classes in colony mate recognition, and/or a conserved requirement for at least one of these structural classes in physically stabilizing the CHC mixture by, for example, reducing brittleness and maintaining flexibility [24].

Although the final biosynthetic step of CHC synthesis, the decarbonylation of long-chain fatty aldehydes to HCs, has long been believed to be catalysed by a P450 enzyme [18], the specific protein that performs this function has only recently been identified [20]. In *Drosophila*, oenocyte-directed RNA interference (RNAi) was used to suppress gene expression of *Cyp4g1* and (separately) *NADPH-cytochrome P450* reductase (CPR) [20]. In both cases, treated insects experienced high levels of mortality, and flies that did survive were characterized by radically altered CHC profiles. *In vitro* assays using CYP4G-CPR fusion proteins confirmed the role of CYP4G as an aldehyde oxidative decarbonylase, and indicated that both gene products are required to produce the final HC products.

Clearly, there is much more to be done, particularly in clarifying the basic biochemistry of CHC synthesis and expression of recognition cues in ants. Moreover, the diversity of these cues at multiple levels—within colonies, among colonies and among species—hints at a richer array of biological functions and processes that still await discovery.

### 3. The perception of colony mate recognition signals

Just as ants possess a diversity of glands that produce a bewildering array of chemicals, their chemosensory systems have diversified to enhance their detection of the chemical world around them. This is mostly clearly exemplified in the number of odourant receptor genes (*Ors*) that occur in the ant genomes: 399 in *P. barbatus* (55 of which are apparent pseudogenes) [7], 337 in *L. humile* (30 pseudogenes) [8], 377 (30 pseudogenes) in *Harpegnathos saltator* [37] and 407 (55 pseudogenes) in *Camponotus floridanus* [37]. For comparison, the genome of the honeybee, *Apis mellifera*, contains only 174 *Or* genes (11 pseudogenes) [38], and the jewel wasp, *Nasonia vitripennis* has 301 (76 pseudogenes) [39]. In part, these differences represent a loss of *Or* genes in *A. mellifera* and *N. vitripennis*, but birth-and-death analysis also suggests that each of these four ant lineages has also experienced net expansions in *Or* gene number [37].

Interestingly, one *Or* gene subfamily, characterized by a 9-exon gene structure, has undergone a remarkable diversification in ants, hinting that some of these genes may code for the colony mate recognition cue receptors [7,8,37]. In the Argentine ant, for example, this subfamily has expanded to 136 genes [8], and the red harvester ant (*P. barbatus*) genome harbours 169 9-exon *Or* genes [7]. Consistent with the use of these receptors for colony mate recognition, recent transcriptomic analysis of *C. floridanus* and *H. saltator* has revealed that the vast majority of these genes are expressed at higher levels in (female) workers, who display colony mate

recognition behaviours, but not in males, who do not perform colony-mate recognition [37]. The same transcriptomic analysis of antennally expressed *Or* genes showed that 40 and 120 of these genes (respectively) are differentially expressed between workers and males, and the vast majority of these differentially expressed genes are enriched in workers, consistent with a potential function as receptors for colony-mate recognition cues [37]. In both species, this pattern of worker-biased expression was particularly pronounced for genes in the 9-exon *Or* subfamily.

Gustatory receptors (Grs), which are used largely for contact chemoreception, are also quite diverse in some of the ant genomes and may also harbour genes that are used by ants for the detection of colony-mate recognition cues. In *Drosophila*, for example, Grs have been implicated in the detection of female CHCs by male flies during courtship [40].

Of the four ant species in which the *Gr* gene family has been closely annotated, the number of *Gr* genes varies widely. In *H. saltator*, the most basal of the four species, only 21 *Gr* genes have been found, four of which are apparent pseudogenes. In the more derived taxa, however, this gene family has undergone a remarkable expansion: the *L. humile* genome harbours 117 *Gr* genes (20 of which are apparent pseudogenes), *C. floridanus* has 63 (17 pseudogenes) and *P. barbatus* has 73 (12 pseudogenes) [7,8,11,37]. In comparison to the gene expression patterns observed for *Or* genes in *H. saltator* and *C. floridanus*, however, there appears to be little differential gene expression of Grs between workers and males in these two species [37]. Although the Argentine ant genome project revealed two expansions of *Gr* subfamilies, neither is as extreme as that seen in the 9-exon *Ors* [8].

Finally, the ionotropic glutamate receptor-related chemosensory receptors ('ionotropic receptors'; IRs) are a third family of genes that may include chemoreceptors for colony-mate recognition cues. The *IR* gene family is an ancient one, as *IR* genes are distributed across the wide swath of animals in the Protostomia (including molluscs, nematodes and molluscs) [41]. Unlike *Ors* and *Grs* (and the canonical ionotropic glutamate receptors), IRs are ligand-gated ion channels that function as transmembrane chemoreceptors. However, as seen in ORs and GRs, IRs are more abundant in ants (23–32 genes) than in *Apis* or *Nasonia* (10 *IR* genes in both).

The relatively modest total number of *IR* genes in ants suggests that colony-mate recognition is unlikely to be mediated solely by genes in this family, but it is possible that some of the IRs contribute to the detection of chemical signals used in colony-mate recognition.

## 4. Looking forward

Although the complexity of signal production and the diversity of chemosensory genes in ants pose significant challenges, it is clear that ongoing advances in functional genetic technology will lead to rapid progress in identifying the specific players in ant recognition systems. In the near term, focused attention to functional screening of ORs and GRs may be fruitful for linking particular chemical cues to the specific receptors that detect them. In *Drosophila*, for example, the coupling of single-sensillum recording and engineered  $\Delta$ halo mutants has allowed researchers to quantify the breadth of receptor tuning to a large number of chemical stimuli (e.g. [42]). Similarly, recent advances have been made in model organisms using genome editing with engineered nucleases [43], such as transcription activator-like effector nucleases [44,45] and zinc finger nucleases [46]. Although these powerful genetic tools have not yet been developed for ants, the rapid pace of development in model organisms suggests that these techniques may soon find their way into studies of social insect biology. Finally, functional genetic techniques using RNA interference (RNAi) hold great promise for near-term analyses of colony-mate recognition systems. Just as RNAi-mediated gene silencing has provided new insights into the molecular mechanisms that underlie chemical signalling in *Drosophila* (e.g. [20]), the application of these techniques to ants (and other social organisms) is likely to produce a wealth of new information about both the signal production and signal perception involved in colony-mate recognition. These powerful new tools offer exciting opportunities to advance our understanding of Hamiltonian dynamics in the natural world, as we can now closely examine the interaction of the specific molecular players that are the phenotypic targets of kin-selected behaviours.

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