Recent findings in fungus-growing ants: evolution, ecology, and behavior of a complex microbial symbiosis

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Summary. Fungus-growing ants (tribe Attini) exist in an obligate mutualism with the fungi they cultivate for food. This symbiosis has an ancient origin, likely beginning in the early Tertiary (50-60 million years ago). Both the ants and their mutualistic cultivars exhibit a series of adaptations for integrated coexistence with their respective partner, indicating substantial modification during a long co-evolutionary history. Ant-cultivar co-evolution culminates in the leaf-cutter ants, which derived from ancestors with a basic social system and evolved some of the most complex insect societies, characterized by enormous colony sizes, extreme worker polymorphism, and multiply-mated queens. Attine ants and their fungi have been intensively researched for over a hundred years, however, only recently has the underlying complexity of the attine ant-microbe association been realized. At least two additional microbial lineages occur in this symbiosis: the specialized fungus *Escovopsis* that parasitizes attine gardens, and a filamentous bacterium that produces antibiotics to suppress the growth of *Escovopsis*. The bacterium thus functions as a third mutualist in the system.

Social systems of the diverse ant lineages are influenced by this multi-partite microbial complexity and social systems in turn influence the evolution of all components of the ant-microbe symbiosis. Ant sociality therefore is embedded in and evolves within the microbial interactions between cultivar, parasite, and antibiotic bacterium. This new understanding of the microbial ecology of the diverse interacting partners opens novel research avenues on the evolution, ecology, and behavior of the attine ant-microbe symbiosis.

Introduction

The cultivation of fungi for food is extremely rare among animals. Within social insects, it is found in only three groups; in the ant tribe Attini, in the termite subfamily Macrotermitinae, and in the beetle subfamilies Scolytinae and Platypodinae. The transition from a hunter-gatherer existence to an agricultural life has resulted in both the fungus-growing ants and termites evolving into extremely successful insect societies, exhibiting substantial diversity in colony organization, widespread distributions, and large colony sizes. Fungus-growing ants and fungus-growing termites play dominant roles in the ecosystem, as foragers and decomposers, respectively. The evolutionary transition to fungus cultivation marks these social insects as particularly unique, and it is compelling to understand the interplay of the symbiont lineages within the attine ant-microbe symbiosis and how it fits within the larger framework of social insect research.

The most widely known group of fungus-growing ants is the leaf-cutters, being conspicuous and economically important (they are severe agricultural pests in Central and South America), though, leaf-cutters represent only a small component of the diversity of the fungus-growing ant tribe. The ants are composed of currently 13 recognized genera and over 200 species, although the basic ecology and behavior for many of these species has yet to be studied. Attine ants occur only in the New World, and are distributed from the northern United States to central Argentina, with the highest species and generic diversity in the wet tropical forests of Amazonian Brazil.

In this review we discuss the recent phylogenetic, ecological, and behavioral studies on the attine ant-microbe symbiosis and review the current hypotheses on the origin of fungus-growing behavior. In addition, we examine the risks and manifestations of disease in attine colonies, their effects on colony social structure, and the behavioral and chemical defense mechanisms used against attine pathogens. We highlight the complex symbiosis between the ants,' their fungal cultivars, parasitic fungi, and mutualistic bacteria, and finally, we discuss future attine research in light of the microbial complexity involved.

Ecology of the Ant-fungus mutualism

Attine ants and their fungal cultivars exist in an ancient and obligate mutualism. The fungus garden serves as the primary food resource to the ants, especially for the queen and larvae. In exchange, the ants' disperse the cultivar to new colonies, provide it with substrate for growth, protect it from pathogens, and enhance its' growth (Weber, 1972; North et al., 1997; Currie, 2001a; Currie and Stuart, 2001). When new colonies are established, the fungal cultivar is vertically transmitted from the natal nest to the newly founded nest (von Ihering, 1898; Weber, 1972; Hölldobler and Wilson, 1990). This is accomplished when newly emerged winged females carry small pieces of the fungus garden stored in the infrabuccal pocket (a pouch located beneath the opening of the esophagus, to the rear of the labium; Eisner and Happ, 1962) to new nests. After the nuptial flight a mated female excavates a nest chamber, spits out the stored fungal inoculums onto the chamber floor, and begins manuring the incipient garden with her feces. She tends the garden until the first workers are reared. Then, worker ants in the colony engage in multiple behaviors to further support the growth of the fungus garden.

The use of substrates to manure the fungus garden varies across attine genera. Most attine ants function as saprotrophs, using arthropod feces and various kinds of dead vegetative debris (e.g., flower parts, leaf debris, seeds, seed husks) as garden substrate. Leaf-cutters, in contrast, occupy the same ecological niche as herbivores, cutting and carrying large quantities of leaves and flowers as substrate for their fungus garden. With the exception of a small group of attine ants that cultivate yeast, the ants place small spherical pieces of substrate onto the garden, and then add fungal hyphae onto the new substrate to cultivate the garden. The yeast growers, a sub-group in the genus *Cyphomyrmex*, create small nodules of substrate by an alternating process of adding a mixture of crop and anal secretions onto insect body parts or feces, then allowing it to dry. Yeast is inoculated onto the new nodule by rubbing it against older nodules containing mature yeast growth (Murakami and Higashi, 1997).

The fungal cultivar provides a valuable diet for the ants, being rich in amino acids and carbohydrates and also containing lipids (Martin et al., 1969a; Quinlan and Cherrett, 1979; Febvay and Kermarrec, 1983). In some genera the fungi produce specialized spherical swellings called gongylidia, which hold the nutrients and are plucked from the surface of the garden and eaten (Möller, 1893; Wheeler, 1910; Hölldobler and Wilson, 1990). The fungus is fed primarily to the queen and larvae, while workers also acquire nourishment from nectar and plant sap (Littledyke and Cherrett, 1976; Quinlan and Cherrett, 1979; Murakami and Higashi, 1997). Fecal droplets, filled with enzymes, are added by the workers to the gardens for fertilization. The enzymes in these droplets are acquired from the cultivar by the ants in mature parts of the garden and redistributed to newly inoculated garden pieces (see review in Martin, 1992).

Attine ant systematics

The agricultural life history of the tribe Attini is believed to have evolved from a generalist forager ancestor in a rain forest of South America between 50-60 million years ago (Wilson, 1971; Mueller et al., 2001; Mueller, 2002). Attine ants (Myrmicinae) are a monophyletic group (Schultz and Meier, 1995) with approximately 220 described species (Table 1). These ants have historically been placed in two divisions. The higher attine ants are comprised of the leaf-cutter genera *Acromyrmex* and *Atta*, as well as three other genera, *Pseudoatta* (a highly derived social parasite), *Trachymyrmex* and *Sericomyrmex*. It is a monophyletic group that encompasses the more phylogenetically derived ants. The remaining attine genera comprise the lower attine ants, a paraphyletic group of phylogenetically basal fungus-growing ants (Weber, 1972; North et al., 1997). A new attine genus, *Mycetagroicus*, with three identified species, has been described from Brazil (Brandão and Mayhé-Nunes, 2001). It seems to belong to the higher attine group (probably near the genus *Trachymyrmex*), but little is know of its' biology and exact phylogenetic position within the Attini.

A small group of attine ants, a subset within the *Cyphomyrmex rimosus* species group (Kempf, 1965), cultivate their fungi as yeast (i.e., in a unicellular phase). Yeast cultivation has been assumed by some researchers to be the ancestral state of fungus growing in attine ants (Weber, 1972; Hölldobler and Wilson, 1990). However, based on a cladistic analysis of 44 morphological characters of 67 species of ant prepupae, with representatives of 11 attine genera, Schultz and Meier (1995) found that the *C. rimosus* group is not basal but is actually a derived group within the lower attine ants. This conclusion was further supported by molecular analyses using mitochondrial gene sequences (Wetterer et al., 1998). These data support the genus *Myrmicocrypta* as the most basal of attine ants (Schultz and Meier, 1995; Wetterer et al., 1998).

Several myrmicine genera have been proposed as sister group to the attine ants. Emery (1921, 22) and Wheeler (1985) hypothesized that the Asian ant, *Proatta,* may be the ant most closely related to the Attini, because *Proatta* larvae share significant morphological characters with some attine ants. And, Schultz and Meier (1995) suggested that *Blepharidatta* and *Wasmannia*, two exclusively Neotropical genera, may be the most likely candidates for sister groups to the fungus-growing ants. Future studies should compare the ecological and behavioral characters among several possible attine ant sister groups (Moffett, 1986; Diniz, 1998; Mueller et al., 2001).

Evolution of the fungal cultivars

Cultivar systematics

Studies using molecular techniques for phylogenetic reconstructions and genotyping of the fungal cultivars have provided exciting new insights into the diversity of cultivars and the host-specificity and co-evolution of the ants and cultivars. Traditional morphological taxonomy and systematics of the cultivars was hampered by the fact that they generally do not produce sexual structures in association with the ants or in pure culture (Hervey et al., 1977; Quinlan and Cherrett, 1978). It has long been recognized that the fungi cultivated by attine ants are in the order Agaricales (phylum Basidiomycota) (Möller, 1893; Weber, 1972). Chapela et al. (1994), Hinkle et al. (1994), and Johnson (1999) revealed that the fungal cultivars are polyphyletic and that most are in the family Lepiotaceae (Basidiomycota: Agaricales), belonging in the tribe Leucocoprineae. This tribe, cultivated by the majority of lower attines and all higher attine ants, is primarily composed of liter-decomposing species that are particularly abundant in wet tropical ecosystems (Dennis, 1952; Singer, 1986).

The majority of lower attine cultivars are polyphyletic within the family Lepiotaceae and belong in two clades (Mueller et al., 1998). The higher attine ants cultivate a derived monophyletic group of fungi in the lower attine-associated tribe Leucocoprineae (Chapela et al., 1994). A derived group of species within the lower attine genus *Apterostigma* cultivates distantly related fungi in the family Tricholomataceae (Chapela et al., 1994). It incorporates two sister clades of fungi, both closely related to the genera *Gerronema* and *Pterula* (Johnson and Vilgalys, 1998; Moncalvo et al., 2000; Munkacsi and McLaughlin, 2001; Villesen et al., in prep.). This remarkable association represents the only known cultivation of fungi not found in the family Lepiotaceae by attine ants.

Ant-cultivar co-evolution

Under a dispersal method of strict vertical cultivar transmission, where the ants and their fungal cultivars are descendants from parents that were associated in the previous generation, divergence in the cultivars should be associated with divergence in the ants. However, molecular phylogenetic studies exploring the co-evolution of the ants and their cultivars have demonstrated that these mutualists have not undergone strict co-speciation (Mueller et al., 1998; Green et al., 2001). Mueller et al. (1998) conducted an elaborate study examining the evolutionary history of fungi in the tribe Leucocoprineae, which is cultivated by the lower attine ants. An analysis of 553 isolates of cultivars from a diverse collection of attine genera and 309 free-living members of the tribe found that distantly related ants may cultivate the same fungal clone, and very closely related ant species may cultivate distantly related fungi (Mueller et al., 1998).

Green et al. (2001) provided further evidence of the incongruence between the ants and cultivars, establishing that lateral transfer of cultivars occurs frequently between two sympatric species of lower attine ants, *Cyphomyrmex costatus* and *C. muelleri*. A phylogenetic analysis of 72 cultivated fungi showed that in seven instances a clade of *C. costatus* cultivars was most closely related to a clade of *C. muelleri* cultivars. Similar patterns of lateral transfer of cultivar have been documented for sympatric species of the leaf-cutter genus *Acromyrmex* (Bot et al., 2001). A behavioral study involving the removal of gardens of *Cyphomyrmex muelleri* and *C. longiscapus* ants established evidence for between-nest transfer of cultivars. Colonies which had gardens experimentally removed were observed either to join a neighboring colony, steal part of a neighbor's garden, or usurp gardens of neighbor colonies (Adams et al., 2000a). Mueller et al. (1998) also found that some of the fungi cultivated by lower attine ants are very closely related to, or perhaps are even isolates of the same clones as free-living fungi. These findings indicate that fungal cultivar transfers occur between ant species, breaking down the strict co-speciation that would otherwise take place in the co-evolution between attine ants and their fungal cultivars. Thus, it seems that recent, and perhaps continuous, domestication of free-living fungi in the tribe Leucocoprineae might be an important aspect of the association between fungus-growing ants and their fungal cultivars.

These results have important implications for cooperation and conflict between the ants and the fungi they cultivate. Under a scenario of strict vertical transmission of fungi from parent to daughter nests by foundress queens (in the absence of within-nest cultivar diversity and in the absence of cultivar escape), we might expect that the reproductive interests of ants and their cultivars would largely (though not completely) overlap. Under a scenario in which cultivars are occasionally or frequently reacquired from both free-living sources and from other ant nests and multiple cultivar genotypes coexist and compete in the same nest, ant-cultivar conflict arises over the cultivars' investment into the fungus-fungus competition. Bot et al. (2001) argue that ants should be selected to keep their cultivars as monocultures and prevent the accidental introduction of novel cultivars into existing gardens, because fungal competition probably results in garden attrition.

The origin of attine fungiculture

Two models have been proposed for the evolutionary origin of attine fungiculture (Fig. 2). The traditional consumption-first model (e.g., Weber, 1958) hypothesizes successive evolutionary modification in the following three-stage sequence. First, non-symbiotic, saprophytic fungi grew accidentally in the walls or debris of ant nests (presumably constructed in leaf litter or in rotting wood), and these accidental fungal associates gradually became part of the ant diet. Second, the ants evolved behavioral repertoires to promote the growth of desirable fungi in their nests through the addition of substrate. Third, the ants evolved the ability to transmit fungi between nests via the mechanism of the infrabuccal pocket. An alternative model, transmission-first, outlined first by Bailey (1920) and recently reexamined by Mueller et al. (2001), reorders these evolutionary transitions and hypothesizes an initial stage in which the ants did not use fungi for food, but in which the fungi used the ants, specifically the ants' infrabuccal pellets, as vectors for dispersal.

During cleaning of their own integument and nests, as well as while straining solid particles out of their diet, all ants accumulate debris (including fungal spores and mycelium) in the infrabuccal pocket, a specialized filtering device in the bottom of the ant mouth (Eisner and Happ, 1962). The accumulated debris is expelled regularly as a pellet by workers on dump sites outside the nest, or at random places during foraging. The pellet, via the infrabuccal pocket, could serve as a dispersal vehicle for fungi, and attine fungiculture may possibly have arisen as a modification of some ancient ant-fungus vectoring system. Subsequent to an initial myrmecochorous (ant vectoring) stage of non-cultivated fungi, the fungi became part of the ant diet, and finally the ants evolved the fungicultural habits exhibited by extant attine ants.

The key difference between the two models is the timing of the evolutionary origin of fungal between-nest transmission relative to the origin of consumption and cultivation; specifically, whether fungal transmission evolved subsequent to fungal consumption (consumption-first), or whether transmission evolved prior to this stage because ants accidentally served as vectors for fungi (transmission-first; Bailey, 1920; Mueller et al., 2001). Under the transmission-first model, prior to a true fungicultural stage, the hypothesized cultivar ancestors may have evolved adaptations to promote their own dispersal: by presenting attractants to vectoring ants, such as enriched fungal tissue that matched the nutritional needs of ants, and by an enhanced ability to survive the passage through the infrabuccal pocket. This scenario parallels the hypothesized origin of other fungiculture in ambrosia beetles, which have been assumed for a long time for to have originated from systems of fungal vectoring by ancestral beetle hosts (Malloch and Blackwell, 1993; Farrell et al., 2001; Mueller and Gerardo, 2003).

Both the consumption-first and the transmission-first models are hypotheses in need of testing, and at this point neither is favored by compelling evidence (Mueller et al., 2001). Studies on non-attine ants and non-symbiotic fungi would support the view that the initial ant-fungus co-evolution was driven by the vectoring needs of fungi. Evidence in support of the transmission-first model may derive from studies of the infrabuccal pockets of ants, specifically determining if free-living, non-attine fungi are dispersed via the infrabuccal pellets. Furthermore, chemical and histological analyses of free-living fungi in the tribe Leucocoprineae may reveal specialized dispersal structures attractive to ants that are analogous to seed elaiosomes of many ant-dispersed plants. Documentation of such fungal features, adapted to meet the nutritional needs of non-attine ants, would indicate that ant-fungus co-evolution can occur in the absence of true fungicultural behavior.

Attine ant social systems

Colony social structure

Most attine genera have a relatively simple social structure, characterized by small colony size and minimal or no worker size polymorphism. These ants generally cultivate small gardens and are thought to be short-lived in nature. In contrast, the most derived genera, the leaf-cutters, have some of the most complex societies of social insects with thousands to millions of ants per nest and a high degree of worker age and size polymorphism (Table 1). The genus *Atta* possesses the largest colony sizes, made up of millions of workers tending potentially more than a thousand interconnected garden chambers (Weber, 1966, Hölldobler and Wilson, 1990). Leaf-cutter nests are long-lived, reaching from five to an upwards of 15 years in the wild and up to 17 years in the lab (Schade, 1973; Jonkman, 1980; Hölldobler and Wilson, 1990).

Mating frequencies

Queen mating frequencies across the generic diversity of attine ants have recently been investigated. So far, genetic evidence has disclosed that only the queens of leaf-cutter ants are polyandrous, while the queens of the remaining attine lineages are exclusively singly mated (Table 2). The average number of genetically detected fathers, which corresponds to the minimum number of matings per queen, for *Atta* colonies is around three. The range of fathers per colony is between one and five males (Fjerdingstad et al., 1998; Fjerdingstad and Boomsma, 2000; Murakami et al., 2000). *Acromyrmex* queens exhibit a larger range in the value of detected number of fathers per colony, between one and ten males, with the minimum queen mating frequencies between three and six (Reichardt and Wheeler, 1996; Boomsma et al., 1999; Fjerdingstad and Boomsma, 2000; Murakami et al., 2000). Ants in the lower attine genera *Myrmicocrypta*, *Apterostigma*, and *Cyphomyrmex* as well as in the higher attine genera *Trachymyrmex* and *Sericomyrmex* appear to be obligately monogamous (Murakami et al., 2000; Villesen et al., 2002).

One hypothesis explaining the evolution of multiple mating in social insects is the benefit obtained from an increase in genetic diversity and the potential augmentation of worker task specialization within the colony (Sherman et al., 1988; Shykoff and Schmid-Hempel, 1991; Boomsma and Ratnieks, 1996). An enhancement in the genetic diversity of the workers could be particularly beneficial for fungus-growing ants, because the ants and in particular the fungi they cultivate are susceptible to pathogens and parasites (Currie et al., 1999a; Currie, 2001a). Higher attine ants cultivate a clonal fungal lineage that has probably existed in tight association with the ants for millions of years (Chapela et al., 1994). This condition would lead to lower genetic diversity in the gardens of the higher attines and create a situation where the cultivar would be more susceptible to disease, requiring enhanced sanitary defenses in the ants. The lower attines likely acquire free-living, sexually reproducing fungi (Mueller et al., 1998), and the resulting increased genetic cultivar diversity would help prevent the establishment of devastating garden parasites.

This evolutionary scenario would explain the elevated mating frequency in the leaf-cutters, though it could not explain the single mating in the genera *Trachymyrmex* and *Sericomyrmex*, which are thought to cultivate clonal fungal lineages closely related to the leaf-cutter cultivars. In conjunction with the clonal versus non-clonal nature of the cultivated fungi, several other factors unique to leaf-cutters may help explain the evolution of polyandry, including larger colony size (in number of workers and in the size of the garden), longer-lived colonies, increased social complexity, and the use of fresh leaves and flowers as garden substrate (Villesen et al., 1999, 2002). Any of these aspects may be linked to the evolution of multiple mating in the leaf-cutters, and further research should aim at identifying the causal rather than the correlational factors explaining the transition to polyandry.

Social Parasites in attine colonies

Agropredation by Megalomyrmex *ants on attine gardens*

Nine ant species in the monophyletic *Megalomyrmex silvestrii* group (Myrmicinae, tribe Solenopsidini) are social parasites on fungus-growing ants and feed on the fungus gardens (Wheeler, 1925; Weber, 1941; Brandão, 1990; Adams et al., 2000b). Similar to attine ants, *Megalomyrmex* parasites ingest the mycelium of the fungus garden and also provision it to the parasite queen and brood. As far as is known, the parasite workers do not forage for substrate for the cultivation of gardens, and they are resultantly dependent on their attine hosts for much of the garden maintenance (Adams et al., 2000b).

The host parasite association differs between the attine host and the *Megalomyrmex* parasite species (Brandão, 1990; Adams et al., 2000b). There appear to be two distinct kinds of *Megalomyrmex*-attine associations: social parasitism where attine hosts maintain gardens for *Megalomyrmex* colonies that are non-nomadic and coexist with the attine hosts, and garden usurpation where attine ants are eliminated by nomadic *Megalomyrmex* colonies that are dependent on regular agropredation on new gardens. The former case of social parasitism is not well understood, but it is known that these parasite species may live lestobiotically in side-cavities of intact attine nests or cohabit in the fungus gardens together with their attine host, enslaving them and tolerating them for the maintenance of the fungus gardens (Adams et al., 2000b). In the latter case, the *Megalomyrmex* ants live alone in the attine nest after garden usurpation and displacement of the original attine occupants. In the best-studied case of the undescribed *M*. sp.nov., workers completely eliminate the attine hosts by expulsion or killing (Adams et al., 2000b). *M*. sp.nov., therefore, is not a true social parasite, but rather a garden thieve or "agropredator." Because *M*. sp.nov. workers do not maintain gardens, and because gardens become depleted with time in the absence of the expelled attine hosts, colonies of *M*. sp.nov. are forced to locate unparasitized attine nests and regularly usurp new gardens. *M*. sp.nov. is likely nomadic and, indeed, it possesses elaborate scouting and recruitment systems that enable the agropredators to move between attine nests (Adams et al., 2000b).

Ants in the genus *Megalomyrmex* are the most frequently collected agropredators of attine ants, but they are not the only ones. Usurpation of attine gardens was observed for the first time recently in the ponerine *Gnamptogenys hartmani* (Dijkstra and Boomsma, in prep.). Cases of garden usurpation by additional formicine, ponerine, and other ant species have been suspected (R.M.M. Adams, pers. comm.). Because of the phylogenetically diverse number of ant species preying on attine gardens, the cultivated fungi are presumably a suitable and attractive food resource for ants in general, with the possible exception of specialized arthropod predators such as army ants (army ants do not raid attine gardens, but only the attine brood; LaPolla et al., 2002; Sanchez-Peña and Mueller, 2002; Price et al., unpublished).

Social Parasitism by Attine Ants on Attine Hosts

As well as hosting unrelated social parasites, colonies of attine ants can also be infected by species of ants in the attine tribe that have evolved into having a social parasitic life history. Two such social parasites have been reported: species in the genus *Pseudoatta* (derived from *Acromyrmex*) and a nest invader appropriately named *Acromyrmex insinuator*. Interestingly, these social parasites have evolved from or are members of the genus *Acromyrmex* and only parasitize *Acromyrmex* ants. *Pseudoatta* is a genus containing only two known species: *P. argentina* which parasitizes *Acromyrmex lundi* (Santschi, 1926; Bruch, 1928; Gallardo, 1929), and a yet undescribed species which parasitizes *Acromyrmex rugosus* (Delabie et al., 1993). Species of *Pseudoatta* exhibit a highly derived form of social parasitism (see Hölldobler and Wilson, 1990): the worker caste seems to have been lost and the morphology of *Pseudoatta* queens and males is extremely diverged from the host (Schultz et al., 1998). *Acromyrmex insinuator* represents a more primitive form of social parasitism, still producing a worker caste (although limited) and possessing a similar morphology to that of its host, *Acromyrmex echinatior* (Schultz et al., 1998; Bekkevold and Boomsma, 2000). Future work comparing the ecology and life history of these social parasites will offer valuable insights into the evolution of social parasitism and attine parasite life history strategies. Particularly valuable will be studies exploring the mechanisms used by the social parasites to invade colonies (e.g., at the nest founding stage or at later stages) as well as the parasites' contributions to the establishment and maintenance of the fungus-garden.

The specialized garden pathogen *Escovopsis*

As in human agriculture, the 'crops' of attine ants are susceptible to serious diseases. Specifically, the gardens of fungus-growing ants are host to parasites in the microfungal genus *Escovopsis* (Ascomycota: Hypocreales) (Currie et al., 1999a; Currie, 2001a; Currie, 2001b) (Fig. 2). Escovopsis is a seemingly highly evolved parasite, having only been found associated with the gardens and dumps of attine colonies, from the most basal genera to the most derived (Currie et al., 1999a; Currie, 2001a; Currie 2001b). In addition, this parasite has the potential to be extremely virulent.

Escovopsis can have multiple effects on the fungus gardens of attine ants (Möller, 1893; Currie et al., 1999a; Currie, 2001b). Presence of this specialized parasite does not typically result in the destruction of gardens, but instead *Escovopsis* establishes persistent infections (Currie et al., 1999a). Currie et al. (1999a) found that field-infected nests maintained in the laboratory were still infected after ten months. In a follow up study, Currie (2001b) established that *Escovopsis*-infected gardens experience a significant reduction in the accumulation of new garden material and a decrease in the rate of colony production of new workers. In some cases, whole gardens have been observed to be rapidly overwhelmed by *Escovopsis*, both in colonies maintained in the laboratory as well as in the field (Currie et al., 1999a; C.R. Currie, pers. obs.; U.G. Mueller, pers. obs.). When this occurs, *Escovopsis* mycelia quickly grow over the entire surface of the fungus garden and then produce huge spore masses on the edges of the chambers. This typically forces the ants to abandon the nest, killing the ant colony indirectly through starvation if they are unable to obtain replacement cultivars. Even in cases where *Escovopsis* does not overgrow the garden, infection can still result in destruction of the colony. Colonies of the leaf-cutter ant *Atta colombica* experimentally infected with concentrated doses of *Escovopsis* spores suffered extremely high mortality, because the ants completely discarded the fungus garden, dumping it on refuse piles in an attempt to eliminate the pathogen (see below) (Currie et al., 1999a).

The exact mechanism of *Escovopsis* pathenogenicity is still unknown. It is not certain whether *Escovopsis* is a parasite of the fungal cultivar, consuming it for nutrients, or whether it is a highly evolved weed, competing with the ants' fungal mutualist for the substrate added to the garden. Preliminary results indicate that the parasite primarily consumes the fungus cultivated by the ants and not other nutrient sources in the garden, as *Escovopsis* grows rapidly on the ants' cultivar and does not seem to be able to utilize leaves as substrate for growth (Reynolds and Currie, unpublished).

It is now clear that Escovopsis modulates the ecology and evolution of the ant-fungus mutualism, influencing the nature of host-cultivar associations, the social structure of colonies, and even the behavior of ant workers. The complete loss of fungus gardens due to infections by *Escovopsis* might force colonies to actively acquire new cultivars, through stealing from neighboring colonies or collecting free-living strains (Mueller et al., 1998; Adams et al., 2000a). If this occurs, the ants, and consequently their descendents, would be associated with novel cultivar strains. *Escovopsis* might also be the selective force driving the evolution of multiple mating in the leaf-cutter queens. This hypothesis is supported by the observation that *Escovopsis* is more virulent in the leaf-cutters, especially the genus *Acromyrmex* (Currie et al., 1999a), which have queens that mate with multiple males. Additionally, as will be explored below, workers engage in specific behaviors to deal with infections of *Escovopsis* (Currie and Stuart, 2001). Finally, *Escovopsis* may shape worker task partitioning, both in regards to garden hygiene behaviors (Currie and Stuart, 2001; see below) and the removal of refuse to prevent re-infection of the garden by *Escovopsis* (Hart and Ratnieks, 2001, 2002).

Colony hygiene behaviors

Microbes, as agents of disease, are serious threats to ant colonies (Schmid-Hempel, 1998). To defend individuals in the colony from these pathogens, workers engage in grooming behaviors, both of their own body surface (autogrooming) and the surface of other workers (allogrooming) (Hölldobler and Wilson, 1990). Grooming behaviors occur at higher frequencies in attine ants compared to ants that do not cultivate fungi (Murakami, 1998). For example, in lower attine ants auto- and allogrooming behaviors represented over 15% of the total behaviors observed (Murakami, 1998). This frequency is an estimate ten times higher than that of *Pheidole* ants (Myrmicinae) (Wilson, 1976). There exist two possible but not mutually exclusive explanations for the high frequency of grooming behavior in attine ants: cultivation of fungi may expose workers to more microbial pathogens, and ant grooming is likely important for preventing workers from transporting alien microbes into and around the fungus garden.

In addition to auto- and allogrooming, fungus growing ants actively clean their environment, including leaves, other garden substrate, and the nest floor (Stahel and Geijskes, 1939; Autuori, 1941; Quinlan and Cherrett, 1977; Quinlan and Cherrett, 1978; Murakami, 1998, Currie and Stuart, 2001). Specifically, workers lick these surfaces, apparently removing the microbes that might otherwise invade the garden (Bass and Cherrett, 1994; Currie and Stuart, 2001). This licking behavior reduces the biomass of microbes coming in direct contact with the fungus garden, and hence serves as an important initial barrier defending the garden from microbial competitors and pathogens (Currie and Stuart, 2001).

Garden hygiene behaviors were experimentally examined in the leaf-cutter ant *Atta colombica* (Currie and Stuart, 2001). Fungus gardens were sprayed with spores of two types of fungi: a generalist pathogen in the genus *Trichoderma* and the specialized pathogen *Escovopsis*. The ants employ two main behaviors in response to the experimental introduction of pathogens. First, the ants groom spores from the surface of the garden in a behavior termed fungus grooming (Currie and Stuart, 2001), which is thought to prevent inadvertently introduced microbes from establishing an infection. Workers that carry out this task search for invading spores with bent antennae, grasp a piece of fungus garden with their maxillae and labia, and clean spores off of the cultivar by running the material through their mouthparts. The harmful spores are collected in the infrabuccal pocket and expelled from the garden. This behavior was extremely common in the presence of both *Trichoderma* and *Escovopsis*, but was rare in the control treatment using water. Workers even moved from other locations in the garden into infected regions to perform fungus grooming. The second hygiene behavior, weeding, is believed to eliminate already infected areas from the garden and prevent the spread of microbes within gardens once the spores have already germinated (Weber, 1957; Currie and Stuart, 2001). To remove infected garden material workers dislodge portions of the garden using their forelegs, midlegs and mandibles in a rocking motion. The substrate is then carried out of the nest and placed in the dump, typically by a different worker.

Compared to the experimental introduction of *Trichoderma* spores, *Escovopsis* spores triggered higher frequencies and longer durations of the fungus grooming and weeding behaviors (Currie and Stuart, 2001). The leaf-cutters were able to rapidly eliminate the presence of *Trichoderma* from the fungus garden, and conversely *Escovopsis* persisted in the nests despite the ants’ increased efforts. These data imply that *Escovopsis* has evolved some counteradaptation to the ants' sanitary behaviors. One possibility is that *Escovopsis* grows too quickly for the ants to muster an effective hygienic defense. Alternatively, Escovopsis may have evolved mechanisms of avoiding detections by the ants, preventing the workers from removing them for the garden.

A third mutualist

Most attine ants posses a white “bloom” on their cuticle, originally thought to be a crystalline material secreted by the ants (Weber, 1972). It is now clear that this material is a filamentous bacterium (actinomycete) probably in or closely affiliated with the genus *Streptomyces* (Currie et al., 1999b). The actinomycete has been isolated from all attine ant species examined (over 22 species in eight genera), but it is located in different places on the exoskeleton depending on the ant genus (Currie et al., 1999b; C.R. Currie, pers. obs.). In the lower attine ants the bacterium is concentrated under the forelegs of the propleura (structures on the ventral surface of the ant, under the head) while in the higher attine ants it is found in front of the forelegs on the laterocervical plates of the propleura (Currie et al., 1999b).

Blooms of the bacterium are found on other locations on the ants' integument, especially in the genera *Trachymyrmex* and *Acromyrmex*, where it can almost completely cover workers (Currie et al., 2003; C.R. Currie, pers. obs.). The actinomycete has been isolated from ants in the leaf-cutter genus *Atta*, but its exact location of growth is unknown (Currie et al., 1999b). As with the fungal cultivar, the bacterium is transferred vertically, from parent to offspring nest by nest-founding queens (Currie et al., 1999b).

Actinomycetes are potent producers of antimicrobial compounds, and most human pharmaceutical antibiotics are derived from them (Waksman and Lechevalier, 1962; Goodfellow and Cross, 1984). Fungus-growing ants employ actinomycetes in the same manner as humans, for the production of antibiotics (Currie et al., 1999b). These bacteria specifically target *Escovopsis* and evidently do not produce compounds with general antifungal properties. This was shown in bioassay challenges of the actinomycete against a diverse collection of general fungi and *Escovopsis*. The bacteria demonstrated no inhibition of the general fungi, yet challenges between the actinomycete and *Escovopsis* resulted in significant inhibition of *Escovopsis* (Currie et al., 1999b). Further evidence to support the role of the bacterium in suppressing *Escovopsis* was found *in vivo* within gardens of *Acromyrmex octospinosus* (Currie et al., 2003). The fungus gardens of sub-colonies experimentally infected with *Escovopsis* were larger when the bacterium was present on workers as compared to those when the actinomycete was physically removed. The prevalence of *Escovopsis* was also higher in sub-colonies where the actinomycete was removed from the ants, as compared to those sub-colonies with the bacterium present (Currie et al., 2003).

The *Streptomyces* bacterium has been established as a third mutualist in the attine ant-microbe symbiosis (Currie et al, 1999b, Currie et al., 2003). It effectively inhibits the growth of *Escovopsis* and is another line of defense in protecting the fungus garden from disease. The actinomycete is incorporated into the attine system in other ways, substantiating its position as a mutualist in the system. The bacterium may have the capacity to enhance the growth of the fungal cultivar. In laboratory bioassays using the cultivar and actinomycete associated with the ant genus *Apterostigma*, the cultivar grew significantly faster in the presence of chemicals produced by the bacterium than without them (Currie et al., 1999b). Also, the actinomycete may protect the ants themselves from diseases by acting as a cuticular obstruction, since this is the typical mode of entry for entomopathogens (Currie, 2001a; Currie et al., 2003). Finally, in the presence of *Escovopsis* the abundance of the bacterium increased on *Acromyrmex octospinosus* major workers (Currie et al., 2003), indicating that growth of the actinomycete can be induced by the ants, perhaps by facultative secretion of growth-promoting nutrients. In exchange for the benefits it provides, the actinomycete profits by being dispersed to new colonies and being provided with a unique habitat in which to flourish (Currie, 2001a).

As illustrated previously with the pathogen *Escovopsis,* the social structure of fungus-growing ants is shaped by, or in response to, the mutualistic actinomycete. In the leaf-cutter genus *Acromyrmex* the abundance of the actinomycete on individual workers is correlated with the worker caste (Currie et al., 2003). *Acromyrmex* major workers have an age-based division of labor, with the youngest individuals being involved in tending the garden, switching to foraging, and then tending the dump as they age (A.N.M. Bot, pers. comm.). Accordingly, young major workers have the largest biomass of the bacterium, while older major workers have only a small amount (Currie et al., 2003). Young major workers are typically covered with the bacterium and are more abundant at the bottom of the garden where *Escovopsis* is more prevalent (Currie, 2001b), whereas minor workers, with a relatively smaller abundance of actinomycete than major workers, are more common on the top of gardens (Poulsen et al., 2002a). These data suggest that workers with the greatest abundance of bacterium distribute themselves in locations within the garden with the highest prevalence of *Escovopsis* (Poulsen et al., 2002a). It has been hypothesized that the bacterium is maintained abundantly only on those workers involved in tending the garden to reduce overall energetic costs to the colony (Currie et al., 2003). This was experimentally investigated in *A. octospinosus* workers by removing the bacterium from the ants and measuring ant respiration rates. The respiration rates of the ants decreased when the bacterium was not present, signifying a substantial energetic cost to the ants to support the growth of the actinomycete (Poulsen et al., submitted).

Additional chemical defenses

While the actinomycete bacterium is a specialized garden defense mechanism, general chemical defense mechanisms, in the form of antimicrobial compounds secreted by the ants or the cultivars, can also have a sanitary function in attine fungus gardens. It has long been understood that the metapleural glands (located on the thorax) of *Atta* and *Acromyrmex* produce chemicals with antibiotic properties, including phenylacetic acid, 3-hydroxydecanoic (myrmicacin) acid and indoleacetic acid (Maschwitz et al., 1970; Schildknecht and Koob, 1970; do Nascimento et al., 1996). Current work has established, using gas chromatography and mass spectrometry, that the secretion from the metapleural glands in *Acromyrmex octospinosus* consists of more than 20 different compounds (Ortius-Lechner et al., 2000). Most of these compounds have effective antimicrobial properties and are thought to serve a broad antibiotic function within the colony, in protection of the ants from entomopathogens or in direct protection of the fungus garden from microbial parasites (Ortius-Lechner et al., 2000). In a study experimentally closing the metapleural glands in *A. octospinosus*, consequently preventing gland secretions, workers experienced higher mortality when exposed to the entomopathogen, *Metarhizium anisopilae* (Poulsen et al., 2002b). Ants with closed metapleural glands experienced lower rates of respiration, signifying a significant energetic cost to sustaining metapleural gland secretions (Poulsen et al., 2002b). To further demonstrate the potential importance of metapleural gland secretions in attine colonies, *A. octospinosus* and *A. echinatior* minor workers located on top of the fungus garden (Poulsen et al., 2002a), where microbes are initially introduced, possess relatively larger metapleural glands compared to major workers (which possess a greater proportion of the actinomycete bacterium) (Bot and Boomsma, 1996).

Biochemical studies on the fungus garden have offered some evidence to support the production of chemical defense compounds by the cultivar. Many attempts have been made to establish the function the cultivar plays in colony hygiene with negative results (Martin et al., 1969b; Weber, 1972; Hervey et al., 1977; Papa and Papa, 1982; Stradling and Powell, 1986). Conversely, a few studies have shown that the cultivars of leaf-cutter and lower attine ants produce chemicals in sanitary protection of the fungus garden (Hervey and Nair, 1979; Angeli-Papa, 1984; Wang et al., 1999). Hervey and Nair (1979) isolated the antibiotic compound lepiochlorin from *Cyphomyrmex costatus* cultivars and also suggested the possibility that additional unidentified antimicrobial compounds were acting in the fungus garden along with lepiochlorin. Bioassay challenges of isolated chemicals from the cultivars of leaf-cutters against several bacterial strains showed slight to strong inhibition against the bacteria (Angeli-Papa, 1984).

Most recently, Wang et al. (1999) have discovered that the yeast-growing ants *Cyphomyrmex rimosus* and *C. minutus* produce large quantities of diketopiperazines, compounds that can have antifungal properties. Diketopiperazines have been isolated from fungi other than attine cultivars (Chen, 1960; Kodaira, 1961; Trigos et al., 1995; Arnone et al., 1996; Kozlovskii et al., 1997) and are therefore thought to be a general defense mechanism, eliminating garden parasites or protecting the ants from entomopathogens. Diketopiperazines may also have antibacterial (Arnone et al., 1996) and antiviral properties (Tomassini et al., 1996).

Conclusions and future directions

Within the Hymenoptera, attine ants are unique for having evolved an obligate mutualistic association with fungi they cultivate for food. The transition to dependence on fungiculture imposes a major cost for the ants because pathogens of the fungal cultivar are also indirect pathogens of the ants. Death of the entire ant colony is a likely outcome of a pathogen completely devastating the fungus garden. The pathogenic threat to the fungus garden is in turn significant since microbes are continuously introduced into the nest on new garden substrate and the ants' bodies. In addition, the fungal cultivars of many ants are presumed to have low genetic diversity.

Fungus-growing ants have evolved multiple defenses to protect the fungus garden. When infected by *Escovopsis*, the ants use behavioral responses, such as fungus grooming and weeding (Currie and Stuart, 2001), and chemical defenses, such as antibiotics produced by the actinomycete bacterium (Currie et al., 1999b; Currie, 2001a; Currie et al., 2003). Additional defense mechanisms against microbes have been identified but are less well established (see Currie, 2001a). The ants not only lick and groom the garden but also perform auto- and allogrooming behaviors, presumably to prevent the establishment and spread of microbes. Chemical defenses, such as metapleural gland secretions, secretions from other glands, and compounds produced by the cultivar may have a function in defending the ants and the fungus garden against a diverse array of microbes. As explored above, the social structure of colonies within the higher attines is apparently shaped in part to optimize the energetic costs or effectiveness of some of these defense mechanisms. This may be the case for the lower attines, as well. Finally, it is probable that other aspects of fungus-growing ants' social structure, such as the evolution of multiple mating and task allocation in the removal of colony refuse, have evolved as defense mechanisms,

The attine ants' transition to fungiculture has resulted in additional symbiont lineages evolving associations with the ants and their fungal cultivars. The attine ant-microbe symbiosis entails, at the very least, the presence of both parasitic and mutualistic microbes. Future work is needed to identify additional microbes, including other potential pathogens of the fungus garden and specialized parasites of the ants. If new microbial pathogens are discovered, the ants may have evolved other defense mechanisms against these invaders.

The ability to cultivate fungus for food and the resulting complex interactions with microbes in the attine system has resulted in fascinating levels of complexity within these insect societies. Experimental studies to test the link between microbial complexity and social complexity can de done within the Attini, using ant genera with differing degrees of social systems. Broad phylogenetic tests can be done by comparing fungus-growing termites and beetles with fungus-growing ants and additionally, fungus-growing ants with closely related non-attine ants. Fungus-growing ants are engaged in a complex microbial symbiosis, and this system is therefore a distinctive model for understanding the study of species interactions in general, and specifically the evolution of insect social systems.

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Figure captions

Figure 1. The two main models for the successive evolution of the behavioral elements of attine fungus cultivation (see text for explanation) (figure adapted from Mueller et al., 2001).

Figure 2. The complex relationships in the quadripartite attine ant-microbe symbiosis. This illustrates the mutualism between the ants, the fungal cultivars and filamentous bacteria, and parasitism of the fungus garden by the microfungal genus *Escovopsis* (from Currie, 2001a).

Table 1. Known ecological characteristics of the attine genera, placed from the most phylogenetically basal to the most derived.

Attine genera	No. described species	Mature colony size (no. workers)	Degree of worker polymorphism	Garden substrate
Lower attines				
Myrmicocrypta	24	60-1600	none	dry plant debris, flower parts, arthropod feces, woody material, seeds, seed husks
Mycocepurus	5	50-200	none	dry plant debris, flower parts, arthropod feces, seeds, seed husks
Apterostigma	47	20-135	none	woody material, arthropod feces
Mycetarotes	4	50-150	none	dry plant debris, likely arthropod feces
*Mycetosoritis**	5	20-150	none	dry plant debris, dry flower parts, arthropod feces, seeds, seed husks
Cyphomyrmex (mycelium cultivar)	38	50-300	none	arthropod feces, flower parts
C. rimosus group (yeast cultivar)	22	50-4100 (occasionally)	none	yeast nodules grown on the ants' crop contents and fecal secretions
*Mycetophylax**	6	50-100	none	dry plant debris, grass fragments
Higher attines				
Mycetagroicus	3	unknown	unknown	small plant fragments and seeds
Trachymyrmex^	40	50-1000	slight	dry plant debris, arthropod feces
Sericomyrmex	19	200-3000	slight	dry plant debris, fruit, arthropod feces
Acromyrmex	27	500-100,000	moderate	dry and fresh leaves and flowers, dry plant debris
Atta	16	1-7 million	strong	fresh leaves and flowers, dry plant debris

Footnote 1: Weber, 1966; Wilson 1971; Weber, 1972; Schade, 1973; Jonkman, 1980; Hölldobler and Wilson, 1990; Bolton, 1995; Lattke, 1997; N.M. Gerardo, pers. comm.; A.G. Himler, pers. comm.; U.G. Mueller, pers. obs.; T. Murakami, pers. obs.; T.R. Schultz, pers. obs.

Footnote 2: Garden substrate is listed in approximate order of usage.

Footnote 3: Values for mature colony size are approximate.

*probably a polyphyletic genus, with some lineages relatively basal and others relatively derived among the lower attines (Schultz, unpublished).

^*Trachymyrmex* is a paraphyletic genus (Schultz and Meier, 1995; Schultz, unpublished). One set of *Trachymyrmex* species have closer affinities with *Sericomyrmex*, a second set has closer affinities with the leaf-cutters (genera *Atta* and *Acromyrmex*), and a third possible set may represent basal lineages within the higher attines.

Table 2. The genetically detected number of fathers of 17 attine species, including the range in detected number of fathers and between-worker relatedness. Based only on genetic methods, the average number of detected fathers reflects the minimum mating frequency of females.

Attini species	**Average number of detected fathers**	**Range in detected number of fathers**	**Worker-worker relatedness**
Lower attines			
Myr. ednaella[1,2]	1[1,2]	1[2]	0.70[1], 0.71-0.73[2]
Ap. collare[2]	1	1	0.72-0.74
Ap. mayri[1]	1	1	0.71
C. costatus[1]	1	1	0.73
C. longiscapus[2]	1	1	0.73-0.74
C. rimosus[1]	1	1	0.69
Higher attines			
T. isthmicus[1]	1	1	0.78
T. zeteki[3]	1	1	0.68
T. cornetzi sp.1[3]	1	1	0.66
T. cornetzi sp.2[3]	1	1	0.69
S. amabilis[1,3]	1[3], 2[1*]	1[3]	0.52[1*], 0.69[3]
S. cf. *amabilis*[3]	1	1	0.65
Ac. octospinosus[1,4]	6.1[4], ~3[1]	4-10[4]	0.45[1], ~0.33[4]
Ac. echinatior[5]	2.53	1-4	~0.47
Ac. versicolor[6]	~3	3-4	NA
At. colombica[1,7]	2.64[7], ~3[1]	1-5[7]	0.41[1], 0.52[7]
At. sexdens[8]	2.7	2-3	0.44

[1] Murakami et al., 2000 (methods: CAP-PCR fingerprinting, detected number of fathers inferred); [2] Villesen et al., 1999 (methods: microsatellite analysis); [3] Villesen et al., 2002 (methods: microsatellite analysis, worker-worker relatedness are minimal values, *S. amabilis* and *S.* cf *amabilis* may be the same species); [4] Boomsma et al., 1999 (methods: microsatellite analysis); [5] Bekkevold et al., 1999 (methods: microsatellite analysis); [6] Reichardt and Wheeler, 1996 (methods: RAPD analysis); [7] Fjerdingstad et al., 1998 (methods: microsatellite analysis); [8] Fjerdingstad and Boomsma, 2000 (methods: microsatellite analysis)

*later determined to be singly-mated, with low worker relatedness caused by multiple queens (Villesen et al., 2002)