Insect Symbioses: A Case Study of Past, Present, and Future Fungus-growing Ant Research*

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ABSTRACT Fungus-growing ants (Attini: Formicidae) engage in an obligate mutualism with fungi they cultivate for food. Although biologists have been fascinated with fungus-growing ants since the resurgence of natural history in the modern era, the early stages of research focused mainly on the foraging behavior of the leaf-cutters (the most derived attine lineage). Indeed, the discovery that the ants actually use leaf fragments to manure a fungus did not come until the 1800s. More recently, three additional microbial symbionts have been described, including specialized microfungal parasites of the ant's fungus garden, antibiotic-producing actinobacteria that help protect the fungus garden from the parasite, and a black yeast that parasitizes the ant–actinobacteria mutualism. The fungus-growing ant symbiosis serves as a particularly useful model system for studying insect-microbe symbioses, because, to date, it contains four well-characterized microbial symbionts, including mutualists and parasites that encompass micro-fungi, macro-fungi, yeasts, and bacteria. Here, we discuss approaches for studying insect–microbe symbioses, using the attine ant–microbial symbiosis as our framework. We draw attention to particular challenges in the field of symbiosis, including the establishment of symbiotic associations and symbiont function. Finally, we discuss future directions in insect-microbe research, with particular focus on applying recent advances in DNA sequencing technologies.

KEY WORDS Attini, *Escovopsis*, *Leucoagaricus*, *Pseudonocardia*, symbiosis

The history of life on earth has been mostly microbial. Since the origin of life ≈3.8 billion years ago, microbes have evolved into the most abundant and phylogenetically diverse life forms on the planet. Microbes also played key roles in biogeochemical processes, which helped make the biosphere more hospitable to other life forms, and they continue to drive mineralization and nutrient cycling (Borneman et al. 1996, Pace 1997, Dawson and Pace 2002, Baldauf 2003). In addition to shaping life on earth, the importance of microbes is exemplified by their crucial role as symbionts with plants and animals. Parasitic symbionts cause virulent diseases and are a significant factor driving biological diversification (Price et al. 1986, Ewald 1994, Jaenike and Perlman 2002). At the opposite end of the spectrum, mutualisms, once regarded as rare and of limited importance, are now recognized as having strongly influenced the evolution of diverse life forms (Margulis 1970, Boucher 1988, Hibbett et al. 2000, Lutzoni et al. 2001, Sanders 2002, Moran 2006). The most striking example is perhaps the symbiotic origin of eukaryote organelles (Mereschkowsky 1905, Margulis 1981, Tomitani et al. 1999, Moreira et al. 2000).

As is the case with other major groups of eukaryotes, symbiotic microbes have a major impact on the biology of insects. At one end of the symbiotic continuum, entomopathogenic microbes, the most intensively studied group of insect symbionts, are a significant source of mortality in insects and have been explored for their potential application as biocontrol agents (Pedigo and Rice 2006, Douglas 2007, Thomas 2008). At the other end, the pervasiveness of beneficial insect symbioses is also becoming increasingly clear (Currie et al. 2006, Moran 2006, Janson et al. 2008), and the fitness increase insects receive from the associations include nutrition and protection from predators, parasitoids, and pathogens (Currie et al. 1999b, Bourtzis and Miller 2003, Vega and Blackwell 2005, Bourtzis and Miller 2006, Moran 2006, Haine 2008). Considering the diversity of insects and microbes on the planet, their mutual abundance and co-occurrence in virtually every terrestrial and fresh water habitat and their shared ancient evolutionary histories, it is likely that the biology of every insect species on the planet is influenced by microbial symbionts. There is already considerable support for this suggestion. For example, it is well established that entomopathogenic fungi infect diverse insects (Hajek and St. Leger 1994, Clarkson

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and Charnley 1996, Shah and Pell 2003, Vega and Blackwell 2005), and recent estimates suggest that the endosymbiont *Wolbachia* infects ≈66% of insect species (Hilgenboecker et al. 2008). Furthermore, microbes are important in aiding digestion in many insects, especially those that feed on plant biomass (Ohkuma 2003, Hongoh et al. 2005, Warnecke et al. 2007), although this is mostly recognized in termites and cockroaches (Cruden and Markovetz 1987, Dillon and Dillon 2004, Geib et al. 2008). We believe that an emerging frontier in insect biology research in the coming decade will be the identification of insect-symbiotic associations and further characterization of how these interactions shape insect biology.

In this review, we use our experience working with the fungus-growing ant–microbe symbiosis to discuss approaches and challenges in studying insect–microbe symbioses. The context of this discussion is a review of the progress made, as well as future directions of research on fungus-growing ant–microbe symbiosis. Specifically, we highlight key considerations in the field, including establishing the presence of symbiotic associations, determining coevolutionary patterns, and the application of new DNA sequencing technologies to the study of insect symbioses.

The Early Era of Fungus-growing Ant Research

For centuries, humans have been fascinated by fungus-growing ants (Attini: Formicidae), especially the leaf-cutters. The conspicuous trails of leaf-cutter ants carrying small leaf fragments are mentioned by the ancient Mayans in their creation myth, The Popul Vuh, and early western colonists of the New World noted them in reports sent back to Europe (Weber 1972). Early research on fungus-growing ants focused on the macroorganism, i.e., the ants. For example, Fabricius (1804) and others focused on classifying the ants, and Linnaeus (1758), the "Father of Taxonomy," even described several attine species in his classic book *Systema Naturae.* The foraging behavior of leaf-cutters also received a lot of early scientific study (e.g., Bates 1863), a trend driven by the status of these ants as agricultural pests in the Neotropics and the salient nature of their microbial symbionts. Interestingly, despite receiving significant attention from early biologists, the ant–fungus symbiosis was not established until more than a century after Linnaeus first described several species. In 1874, Belt discovered that leaf fragments transported by the ants are not directly consumed, but instead are used as manure to grow a mutualistic fungus. Möller (1893) followed up on Belt's discovery, conducting the first mycological studies on the mutualism and describing the first signs of coevolution between the ants and their cultivated fungi. Specifically, he discovered that the fungi cultivated by leaf-cutters produce specialized swellings at the hyphal tips, later termed gongylidia, which are consumed by the ants. Based on the discoveries of Möller (1893) and his own observations, Wheeler (1910) suggested that fungus-growing ants exhibit an evolution toward more complex agriculture. This idea was based on the observation that some fungus-growing ant genera have more worker castes and substantially larger colony sizes, in comparison to seemingly more basal lineages. In addition, the social structure of ant colonies follow a pattern of increasing complexity, with monomorphic worker size in basal genera and strong worker size polymorphism in the more phylogenetically derived genera, especially *Acromyrmex* and *Atta.*

In the latter half of the 20th century, the focus of attine research moved further from being mostly myrmicocentric to include studies on the interactions between the ants and their mutualistic fungus. This included several decades of studies by Weber describing, in great detail, the natural history of ant-fungus interactions (reviewed in Weber 1966, 1972). Weber and others focused their research on the ants' active promotion and optimization of the conditions for fungus growth, including studies on foraging ecology and plant preference (Cherrett 1968, 1972; Rockwood 1975; Littledyke and Cherrett 1978; Bowers and Porter 1981), substrate preparation (Quinlan and Cherrett 1977), and pruning of the fungal cultivar to maintain high productivity (Bass and Cherrett 1994). Subsequent studies showed that worker polymorphism is a reflection of within-colony division of labor and task specialization (Wilson 1980a, b; Hölldobler and Wilson 1990; Wetterer 1999), with the smallest workers (minors) primarily being involved in fungus garden maintenance and brood care (Weber 1972, Wilson 1980a, Bass and Cherrett 1994). In contrast, young major workers are generally involved in garden tending, whereas older majors perform foraging and waste management tasks outside the colony (Weber 1972, Wilson 1980a).

An early discovery in the ant–fungus association was that the fungus is vertically transmitted between host ant generations (Ihering 1898, Autuori 1956). A colony-founding queen collects a pellet of fungus from her natal nest before leaving for her mating flight; and this pellet is stored in a pouch in the oral cavity (the infrabuccal pocket). After mating, she selects and excavates a suitable nest site and uses the fungus from her infrabuccal pocket as the inoculum for a new garden (Ihering 1898, Autuori 1956, Mueller et al. 2001, Fernández-Marín et al. 2004). This finding led to the expectation that the cultivar symbionts of fungus-growing ants represent an ancient, clonally propagated lineage of fungi that tightly evolve in parallel with their ant hosts, a finding that has shaped a number of later studies in host-symbiont specificity, codiversification, and coevolution (see below).

For most of the 20th century, progress toward a deeper understanding of the ant–fungus mutualism was slowed by a dearth of mycology-trained investigators and a lack of methods for studying the cultivated fungi. As we describe below, an increase in microbiologically trained investigators studying the attine system, and newly developed molecular methods, have led to the identification of additional microbial symbionts in the symbiosis, and the ability to tackle more sophisticated questions of host-symbiont

population dynamics and coevolution. This movement toward a deeper understanding of the bipartite mutualism parallels research in other model systems of insect symbiosis (Aanen et al. 2002, Klepzig and Six 2004, Six and Klepzig 2004) and capitalizes on the advances made in the areas of molecular ecology and phylogenetics in the 1990s.

Contemporary Fungus-growing Ant Symbiosis Research

Symbiont Community Complexity. Beginning in the 1990s, our paradigm of the fungus-growing ant system began shifting away from a bipartite mutualism toward that of a multipartite symbiosis characterized by a continuum of symbionts, ranging from mutualists to antagonists. Historically, fungus-growing ants were assumed to maintain their fungus gardens in monocultures free of parasites (e.g., Möller 1893). This view persisted until Currie et al. (1999a) established that the ants' fungus gardens host specialized microfungal parasites in the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales). *Escovopsis* is a mycotrophic parasite that directly targets and exploits the ants' cultivar (Reynolds and Currie 2004) through chemical attraction to the cultivar (chemotaxis) (Gerardo et al. 2006b). On contact with the cultivar, *Escovopsis* secretes compounds that degrade the host cells and subsequently absorbs the released nutrients (Reynolds and Currie 2004, Gerardo et al. 2006b). All evidence thus far indicates that *Escovopsis* is only found associated with ant colonies, is horizontally transmitted, and has the potential to be virulent (Currie et al. 1999a, 2001b). Although only two species of this pathogen have been formally described (*E. weberi* [Muchovej and Della Lucia 1990] and *E. aspergilloides* [Seifert et al. 1995]), it is clear that additional species parasitize attine ant fungal gardens (Currie et al. 2003a, Gerardo et al. 2006a; C.R.C., unpublished results).

Escovopsis illustrates a challenge associated with establishing that a particular microbe has a symbiotic association with a host. The symbiotic relationship between attine ants and the fungus they cultivate is quite obvious as the cultivar can be seen with the naked eye. In contrast, *Escovopsis* is much less conspicuous and often requires several rounds of culture isolations or molecular probing of the fungus garden to detect it. For example, Currie et al. (1999a) sampled microfungi from the fungus gardens of attine ants through microbiological isolation by placing small garden pieces on petri plates containing microbiological media. The result was the isolation of a large number of *Escovopsis* cultures, second only to the fungal mutualist. Using this approach, they showed a high prevalence of *Escovopsis* in the gardens of fungus-growing ants, thus providing the first solid evidence of the presence of an additional symbiont within the attine ant–microbe association.

Fungus-growing ants, like all insects, do not occur in isolation. Instead, they occupy niches full of microbes, and thus at any particular time are likely in contact with a multitude of microbes, of which only a subset can be considered symbionts. By definition, symbiosis is "the living together of unlike named organisms" (de Bary 1879), and it is generally accepted in the symbiosis community that transient microbes are not symbionts. Thus, although the isolation of microbes can be an important method for establishing an insect–microbe symbiosis, it is important to note that mere isolation of a bacterium or fungus from a host does not establish it as a symbiont. For example, numerous other fungi have been detected in the ants' fungus gardens using isolation (Currie et al. 1999b, Rodrigues et al. 2008) or molecular methods (Abril and Bucher 2007); however, it is likely that most of these are spores or inactive fungal mycelium present inside plant leaves in the soil or brought into the garden by workers. Thus, an additional challenge is to distinguish symbionts from the plethora of transient microbes that are obtained through culturing, as shown by *Escovopsis* being isolated several times before it was established as a symbiont (Möller 1893, Seifert et al. 1995, Fisher et al. 1996). For a summary of methodological approaches useful for identifying "resident" microbial symbionts from "tourists" see Ciche and Goffredi (2007).

Once a microbe is established as a symbiont, it is important to characterize its ecological role. The role of *Escovopsis* was determined by using Koch's postulates (Currie et al. 1999a), a rigorous test for determining if a microbe is a pathogen. Koch's postulates require that the microorganism of interest be found in diseased organisms but not in healthy ones. The microorganism must be isolated in pure culture and confirmed to cause disease when introduced to a healthy organism. Last, the microorganism must be reisolated from the inoculated, diseased experimental host (Agrios 1988). These postulates provide an invaluable tool for establishing the pathology of symbionts; however, establishing the ecological function of less obvious microbial interactions, such as commensalisms, may require more subtle observations of host response (see Ciche and Goffredi 2007).

Coinciding with the establishment of *Escovopsis* as a parasite of the ant–fungus mutualism, an additional symbiont was discovered. What was previously dubbed a "waxy bloom" growing on the cuticle of the ants (Weber 1972) was found to be actinobacteria in the genus *Pseudonocardia* (Currie et al. 1999b, Cafaro and Currie 2005). These bacteria secrete secondary metabolites with antifungal properties that inhibit the growth of *Escovopsis* (Currie et al. 1999b, 2003a). The chemical structure of the antifungal responsible for the inhibition of *Escovopsis* from a *Pseudonocardia* symbiont of *Apterostigma dentigerum* (Wheeler) has recently been characterized (Oh et al. in prep.). The bacteria, which are housed in specialized cuticular modifications on the ants' body, likely receive nutrients through integumental pores connected to specialized bicellular glands within the ants (Currie et al. 2006). The discovery of the mutualistic association of *Pseudonocardia* with fungus-growing ants highlights another important aspect of insect symbiosis: the use

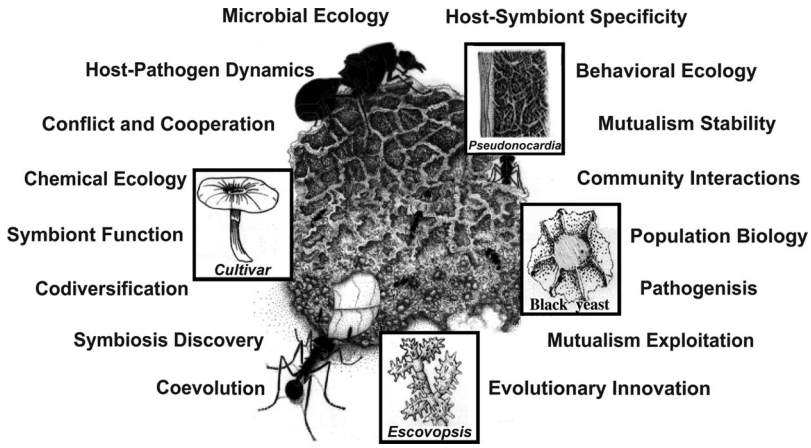

Fig. 1. The fungus-growing ant symbiosis is a model system for studying the ecology and evolution of symbiotic interactions. The symbiosis currently contains five identified and characterized symbionts: Attine ants, the fungi that they cultivate for food, cultivar-attacking microfungi in the genus *Escovopsis*, antibiotic-producing bacteria in the genus *Pseudonocardia*, and a black yeast parasitizing the ant–*Pseudonocardia* mutualism. The diversity of interactions in this model system provides a wealth of opportunity for scientific inquiry, particularly beyond bipartite interactions.

of microbes by hosts as a form of evolutionary innovation. Through these symbiotic associations, insects can gain access to the specialized physiological capabilities of microbes. In the ant–*Pseudonocardia* case, the ants gain access to secondary metabolites with antimicrobial properties. Insect use of symbiotic bacteria to derive antibiotics is now known to occur beyond the fungus-growing ant symbiosis (Kaltenpoth et al. 2005, Scott et al. 2008).

In addition to using *Pseudonocardia*-derived antifungal compounds, ant workers play an important role in maintaining fungus garden hygiene. In the presence of alien fungal spores, the leaf-cutter *Atta colombica* (Guerin-Meneville) engages in two behaviors: (1) grooming of fungal spores from the garden and (2) removal of infected garden substrate (Currie and Stuart 2001). These two behaviors, coined "fungus grooming" and "fungus weeding," respectively, are effective at eliminating the aggressive general pathogen *Trichoderma* (Currie and Stuart 2001). Interestingly, the fungus-growing ant *Trachymyrmex* cf. *zeteki* (Weber) combines the use of behaviors and symbionts to control *Escovopsis* (Little et al. 2006). The spores of the parasite are removed through fungus grooming and are subsequently stored in the infrabuccal pocket, where *Pseudonocardia* is also present. When the contents of the infrabuccal pocket are later regurgitated, the *Escovopsis* spores are no longer viable (Little et al. 2006). In addition, fungus-growing ants have paired metapleural glands, which produce antimicrobial compounds, and these secretions help protect ants from generalist insect pathogenic fungi (do Nascimento et al. 1996; Poulsen et al. 2003, 2006). By passing their forelegs across the surface of the gland, and subsequently passing them through their mouthparts (termed metapleural gland grooming), workers actively apply metapleural gland-derived compounds (Fernández-Marín et al. 2006). Metapleural gland grooming occurs during garden maintenance and is up-regulated during parasite infection, leading to the speculation that this behavior allows application of compounds directly to the fungus garden (Fernández-Marín et al. 2006). Although it remains to be fully established whether this behavior plays a role in defending the mutualistic fungus, these findings show how behavioral observations, chemical analyses, and infection experiments are important and powerful tools for research in insect symbiosis.

Further advances in microbiological and molecular techniques have resulted in the recent identification of another microbial symbiont in the fungus-growing ant system (Fig. 1). As *Escovopsis* parasitism shows, mutualisms are prone to exploitation by parasites (Bronstein 2001). It is therefore not surprising that, just as the attine ant–fungus mutualism is parasitized, so is the ant–bacteria mutualism. A black yeast (Ascomycota; *Phialophora*) grows on the same locations on the ant cuticle as the ant-associated *Pseudonocardia* (Little and Currie 2007). This black yeast parasitizes the fungus-growing ant system by acquiring nutrients from the bacteria and thereby indirectly reduces the ability of *Pseudonocardia* to suppress *Escovopsis* growth (Little and Currie 2008). The finding of a relatively inconspicuous fourth symbiont associated with attine ants shows some of the potential challenges researchers face in insect symbiosis. The presence of the black yeast was discovered only through the use of specialized culturing techniques and polymerase chain reaction (PCR), and its antagonistic role was only established through combined infection experiments with the garden parasite (Little and Currie 2007). In addition, because the black yeast significantly alters the dynamics of the other four symbionts, this finding illustrates the importance of discovering additional associates within symbiotic communities.

Host-symbiont Phylogenetics and Coevolution. A more complete understanding of the ant–fungus mutualism has been greatly hindered by the lack of in-

	Experimental tools	Focus and discoveries
Past	•Behavioral observations •Microbiological techniques •Electron microscopy	•Ant identification •Ant behavior (foraging) •Discovery of ant-fungus mutualism •Role of fungus in the association •Bipartite mutualistic symbiosis
Present	•Allozyme and enzyme work •PCR •Population biology •Tree building •Theories of codiversification •Chemical ecology	•Dynamics in bipartite interactions •Discoveries of additional symbionts •Population biology and phylogenetics •Codiversification and coevolution •Understanding symbiont interactions •Chemical mediation of symbiosis
Future	•Metagenomics •Functional metagenomics •Genomics •Phylogenomics •Target genes •Metabolic pathways	•Identification of additional symbionts •Identification of symbiont role •Identification of coevolutionary change •Host-symbiont genetic interactions •Symbiosis-relevant genes •Symbiosis-relevant metabolism

Fig. 2. The history of research in the fungus-growing ant system and anticipated future directions. As with many biological study systems, research efforts have been shaped largely by the tools available for scientific inquiry. Past research was focused on the ant host, using mostly observational approaches, whereas more contemporary research has used molecular genetic tools to address more in-depth questions in ecology and evolution. We predict that future directions will seek to make use of the recent genomics revolution.

formation regarding the evolutionary history and taxonomic placement of the fungal cultivars. Traditionally, fungal taxonomy and systematics were based solely on the morphology of fruiting structures. However, the fungi cultivated by attine ants rarely, if ever, produce these structures, either in association with the ants or in pure culture (Möller 1893, Hervey et al. 1977, Muchovej et al. 1991). The development of molecular phylogenetic techniques in the late 20th century facilitated the reconstruction of the evolutionary history of the ants' cultivar and reliably established their taxonomic placement (Chapela et al. 1994, Mueller et al. 1998). This has greatly advanced our understanding of the ant–fungus mutualism by providing new insights into the origin of the mutualism (see Mueller et al. 2001) and the phylogenetic diversity of the cultivated fungi. Importantly, it has also allowed for a more rigorous examination of ant–fungus coevolution.

The ants and their mutualistic fungi exhibit broad-scale phylogenetic congruence (Chapela et al. 1994, Mueller et al. 1998, Bot et al. 2001, Richard et al. 2007, Schultz and Brady 2008) and can be divided into five agricultural systems, each involving distinct lineages of ants and fungi (Schultz and Brady 2008). First, in "lower attine agriculture," the most basal form of ant fungiculture, ants associate with a paraphyletic group of parasol mushrooms in the tribe Leucocoprinae (Mueller et al. 1998). In the second agricultural system, "coral fungus agriculture," a group of lower attines in the genus *Apterostigma* secondarily switched to cultivating fungi in the family Pterulaceae (Agaricales) (Munkacsi et al. 2004). *Cyphomyrmex* ants in the rimosus group cultivate their Lepiotaceous fungi in a yeast form, termed "yeast agriculture" (Mueller et al. 1998). Finally "higher attine agriculture," which includes "leaf-cutter ant agriculture," is a system where the cultivated fungus apparently no longer exists outside the ant mutualism (Chapela et al. 1994, Mueller et al. 2001). Higher attine agriculture is the most recent transition in ant fungiculture and marks the origin of the two leaf-cutting ant genera, *Atta* and *Acromyrmex*, the only lineage with the ability to use fresh leaf material for cultivar substrate (Schultz and Brady 2008).

The application of molecular phylogenetics has also facilitated the reconstruction of the evolutionary history of the garden parasite. *Escovopsis* parasitism had a single and early evolutionary origin in the symbiosis (Currie et al. 2003b). Despite being horizontally transmitted between colonies (Currie et al. 1999a), phylogenetic evidence indicates that the parasite is divided into four major lineages, each of which is associated with a corresponding group of fungus-growing ants and their mutualistic fungi (Currie et al. 2003b). These groups correspond broadly to the major agricultural systems identified above (Currie et al. 2003b), although *Escovopsis* has not been found infecting yeast agriculture. Thus, at deeper levels, the phylogenies of the ants, cultivars, and *Escovopsis* are highly congruent, suggesting that the ant–microbe symbiosis is the product of tripartite coevolution (Currie et al. 2003b). One exception to this pattern is a switch in *Escovopsis* host use across one ant agricultural system, the coral fungus group (Gerardo et al. 2006a), which is not surprising considering that this corresponds to the ants' switch from cultivating fungi in the family Lepiotaceae to fungi in the Pterulaceae. At finer phylogenetic levels, where interactions on ecological time scales become increasingly important, related strains of *Escovopsis* are known to switch between closely related ant species, so that cophylogenetic patterns are disrupted within each of the agricultural systems (see below).

Broad-scale patterns of codiversification between fungus-growing ants and *Pseudonocardia* are expected

from the default vertical transmission of *Pseudonocardia* between host ant generations (Currie et al. 1999b), and, as predicted, signatures of codiversification have been found at deeper evolutionary time scales. A combined 16S rDNA and elongation factor-Tu phylogeny of *Pseudonocardia* indicates ant–*Pseudonocardia* codiversification and broad-scale matching in the evolutionary history of *Pseudonocardia* with those of the ants, cultivar, and *Escovopsis* (Cafaro et al. in prep.). This pattern is, as with the fungal cultivar and *Escovopsis*, disrupted by horizontal transmission of bacterial strains between ant species and genera at finer phylogenetic time scales (Poulsen et al. 2005). Further support for coevolution between the ants and *Pseudonocardia* is derived from the discovery that attine ants have elaborate morphological and physiological modifications to maintain the bacteria on the ants' cuticle (Currie et al. 2006). This has been documented in representative ant species and genera spanning most of the evolutionary history of fungus-growing ants, and it seems that these structures have undergone significant modifications over the course of the evolutionary history of the association (Currie et al. 2006). These findings are disputed by Mueller et al. (2008), who maintain that there is no pattern of codiversification, hence coevolution, between the ant and *Pseudonocardia.* However, this is based on inferences from 16S rDNA sequencing alone (Mueller et al. 2008), which provides only a coarse level of resolution that is typically not be appropriate for revealing patterns of codiversification at evolutionary time scales (Cohan 2006, Staley 2006; see Cafaro et al. in prep.).

Phylogenetic information for the black yeast is, as of yet, limited (Little and Currie 2007). As mentioned above, this symbiont has only been isolated from *Apterostigma.* However, its presence has been detected in other lineages using PCR amplification (Little and Currie 2007), suggesting an early origin of the black yeast within the symbiosis and the potential for coevolution with the other four symbionts. Sequencing of more informative genes is needed to determine whether this is the case. Selecting the appropriate molecular marker for a given study is largely an issue of scale. As questions move into more recent ecological time scales and into the realm of population biology, it becomes increasingly important to use more variable markers, such as DNA microsatellites and other types of fragment length–based genotyping.

Population Aspects of Symbiosis. Understanding population level dynamics of symbiotic interactions is essential to understanding long-term coevolutionary trajectories. In particular, the mode of symbiont transmission plays an important role in shaping coevolutionary dynamics. For example, hosts and symbionts that are closely tied to one another across generations through vertical transmission (i.e., from parent to offspring) are better equipped to withstand stochastic evolutionary forces and are more likely to retain their associations over large evolutionary time scales. In contrast, horizontally transmitted associations are predicted to be more diffuse, barring sustained selection pressures holding them together. Furthermore, parasite transmission mode is a critical factor driving the evolution of virulence (Ewald 1994). Thus, an important first step in understanding the population level dynamics of symbiotic interactions is the characterization of symbiont life histories. This can be accomplished through direct observation; however, identifying deviations from life history strategies is of equal importance and is often more difficult. For example, *Wolbachia* are endosymbiotic bacteria that reside in the reproductive organs of insects and are transmitted vertically into the cytoplasm of unfertilized eggs. Although this transmission method seems quite strict, molecular genetic studies have shown that horizontal transmission of *Wolbachia* is rampant (O'Neill et al. 1992; Werren et al. 1995; Schilthuizen and Stouthamer 1997; Heath et al. 1999; Vavre et al. 1999; Huigens et al. 2000, 2004; Noda et al. 2001).

In the fungus-growing ant system, vertical transmission of fungal cultivars is similarly punctuated by horizontal transfer events. In the lower attines, amplified fragment length polymorphism (AFLP) genotyping has shown that two closely related species in the genus *Cyphomyrmex* share cultivar genotypes, indicating that fungal cultivars are regularly exchanged between them (Green et al. 2002). A study by Adams et al. (2000) has proposed a potential mechanism for how such horizontal transfer events might occur. In the laboratory, when *Cyphomyrmex* ants have their fungal gardens artificially removed (mimicking natural garden loss), they obtain a new fungus garden either by fusing with, or stealing fungus from, another colony. Another potential mechanism of cultivar switching in lower attines is through parabiosis, the merging of con- or allospecific ant colonies (Sanhudo et al. 2008). Similar examples of cultivar switching are seen in the higher attines. Multiple leaf-cutter species from the two most derived attine genera (*Atta* and *Acromyrmex*) share genetically similar cultivar types based on AFLP (Bot et al. 2001, Richard et al. 2007) and microsatellite markers (Mikheyev et al. 2007). However, considering the ultimate importance of horizontal host switching versus strict vertical transmission on long-term coevolutionary stability, we should point out several problems inherent to the empirical nature of several of these molecular bases studies. First, in the absence of lineage sorting, resolving gene trees with species trees becomes difficult, if not impossible. Similarly, if symbiont genotypes have not undergone sorting events, their associations with hosts (or host genotypes) may seem diffuse despite high specificity (Page and Charleston 1998). This problem becomes exaggerated when phylogenies are wide and have relatively short branch lengths (Maddison et al. 1997), as is often the case with studies at the interface of population genetics and phylogenetics.

The dynamics of cultivar host switching are further complicated by the potential for recombination among cultivar strains. A recent study found indirect evidence of recombination among leaf-cutter cultivars, suggesting that all leaf-cutter cultivars comprise a single biological species (Mikheyev et al. 2006). As of yet, rates of sexual versus clonal reproduction have

not been recorded, although the evolutionary implications are important (Halkett et al. 2005), particularly in microbes (Whitaker and Banfield 2006). If sexual reproduction is frequent, this will disrupt the physical linkage between genes directly involved in symbiosis and other genes in the genome. This emphasizes the critical task of identifying genes directly involved in symbiotic interactions, because these are the genes that will show the strongest patterns of specificity and coevolution with hosts.

Despite the presence of cultivar diversity within populations and lateral transfers of cultivar strains between colonies, individual ant colonies seem to associate with a single fungus strain (Poulsen and Boomsma 2005). Genetic monocultures are maintained through both ant-mediated exclusion of nonnative fungal strains entering colonies (Bot et al. 2001, Viana et al. 2001, Mueller 2002) and by the resident fungus through the expression of incompatibility mechanisms (Poulsen and Boomsma 2005). In leafcutters, these incompatibility mechanisms do not seem to operate during the first few days of colony formation, likely because queens do not feed on the fungus during this early stage of garden founding (Poulsen et al. in prep.). Thus, this is a stage in the colony life cycle where horizontal transfer events are most likely to occur. Maintaining a single fungal strain within a colony at any given point in time prevents potential competition between genetically different cultivars, which is both in the interest of the ants and the cultivar as the presence of multiple cultivar genotypes is predicted to reduce productivity (Frank 1996, 2003). At the same time, single strain rearing reduces potential conflict in the association, because rearing multiple fungal strains means that genetically different fungi share ant host resources and transmission (with gynes) to future generations (Frank 1996, 2003; Poulsen and Boomsma 2005).

In contrast to vertical transmission of the fungal cultivar, *Escovopsis* is not found in newly established ant nests, indicating that the parasite is acquired horizontally from other ant colonies (Currie et al. 1999a). Although the transmission mechanism of *Escovopsis* is unclear, it has been suggested that other arthropods (inquilines) associated with the fungus gardens might transmit *Escovopsis* (Currie 2001a). Candidates for such transmission include parasitoid wasps that infect attine larvae, which are regularly present in fungus gardens and refuse dumps of attine ants (Fernández-Marín et al. 2006). Thus, it is perhaps not surprising that, similar to ant-cultivar specificity, *Escovopsis* host switching is a regular occurrence among leaf-cutter ant species (Taerum et al. 2007). Contrasting these relatively diffuse associations against patterns of phylogenetic congruence at deeper time scales (Currie et al. 2003b) begs the question of how host-symbiont specificity is maintained at one scale but not another. Gerardo et al. (2006b) identified a potential mechanism for maintaining broad-scale specificity between the cultivar and *Escovopsis.* Using three genetically distinct cultivar types associated with three species of *Apterostigma*, they showed that *Escovopsis* strains are attracted to native cultivar types through chemotaxis. However, when *Escovopsis* strains were artificially switched to non-native cultivars, parasite growth was strongly inhibited, possibly selecting against parasite host switching across ant agriculture systems (Gerardo et al. 2006b). Interestingly, although this study provides a mechanism for maintaining cultivar–*Escovopsis* associations among species within the genus *Apterostigma*, this type of host-genotype tracking is not in place within species (Gerardo and Caldera 2007).

Transmission patterns of the bacterial mutualist *Pseudonocardia* seem similar to those of the fungal cultivar. In species with visible cover of *Pseudonocardia* on the cuticle, foundress queens depart from parent nests with an inoculum of the symbiotic bacteria growing on the cuticle, thus transmitting the bacteria vertically (Currie et al. 1999b). Furthermore, similar to the presence of horizontal host switching among leaf-cutter ant species (Mikheyev et al. 2006), Poulsen et al. (2005) showed that two species within the leafcutter ant genus *Acromyrmex* also share similar *Pseudonocardia* genotypes. Cafaro et al. (in prep.) pointed out that switches across agriculture systems may be more common in *Pseudonocardia* than in the fungal cultivar or *Escovopsis* and also documented examples of acquisitions of free-living *Pseudonocardia.* Although more detailed studies are needed to fully understand the mechanisms controlling host specificity in *Pseudonocardia*, our current view is that horizontal transfer of *Pseudonocardia* is a phenomenon that is likely to occur more frequently than cultivar exchanges at ecological time scales and thus becomes a question of population biology. Despite a seemingly greater degree of horizontal transfer in *Pseudonocardia*, individual ant colonies of *Acromyrmex*, the only ant genus in which this question has been addressed, seem to associate with only a single *Pseudonocardia* strain (Poulsen et al. 2005). This ability may be governed by the behavior of the ants, because they can distinguish among *Pseudonocardia* strains (Zhang et al. 2007), a behavior that is likely useful in reducing within-colony conflict between different strains (Poulsen et al. 2007).

A rich history of theoretical and empirical work in population genetics has provided a solid framework for understanding how factors such as genetic diversity, population structure, mutation, and recombination shape evolutionary trajectories. For symbiotic interactions, these concepts have been applied most readily to the coevolutionary dynamics of adaptation in host-parasite systems (Lively 1999). Within this framework, Gerardo and Caldera (2007) examined the population genetic structure and interactions of fungal cultivars and *Escovopsis* parasites associated with *Apterostigma dentigerum* across Central America using AFLP genotyping. Parasite populations were less structured than hosts (indicative of higher gene flow), but host population genetic structure was also relatively low. Moreover, the parasites did not seem locally adapted to particular locations or host genotypes; rather, they were successful at infecting almost all

cultivars. These empirical findings are in contrast to traditional views of host–parasite dynamics, where a host (usually a macroorganism) houses a horizontally transmitted parasite that generally harbors more evolutionary potential in the form of a larger population size, shorter generation time, and higher mutation rate. The predicted outcome of this interaction is that the parasites become locally adapted to their hosts (Hamilton et al. 1990). These differences are perhaps not surprising, for several reasons. While *Escovopsis* harbors the evolutionary "advantage" of horizontal transmission, contrasting the asexually propagated cultivar host, the parasite is actually evolving in response to the effective populations of the ants and the bacterial mutualist, both of which are arguably capable of imposing equally if not stronger selection pressures. These dynamics are further complicated because the presence of the black yeast can potentially relax these selection pressures. Moving forward, one of the challenges in the system is that few theoretical and empirical population genetic studies have focused on more than two symbionts in any system (but see Stanton 2003, Strauss and Irwin 2004), leaving little insight from other multi-partite symbiotic systems.

Contemporary attine research is characterized by the movement toward greater focus on microbiology of the system, including the identification of additional symbionts and symbiont interactions, as well as exploring patterns of coevolution. Furthermore, the last 5 years has seen a shift toward new types of questions being addressed, including those spanning different spatial and temporal scales and fields of study (Fig. 2). As these new questions become increasingly complex, new challenges will arise, and there is a need for increasingly sophisticated tools. New approaches, driven by technological advances such as genomics, will be able to help address some of these new questions. Additionally, just as contemporary research has broadened in perspective (e.g., from phylogenetics to population genetics), future studies will continue this trend as we start to explore interactions at the genetic and chemical levels.

Postgenomics and the Attine Ant Symbiosis: Future Directions

The genomics revolution, made possible through the rapid advances in DNA sequencing technology of the last 10 years, has dramatically altered the way biological research is conducted, and the field of insect–microbe symbiosis is no exception. High-throughput molecular techniques make it possible to identify new symbionts and study symbiotic interactions at a much faster pace. We believe greater integration of genomic approaches will make the next decade of insect–microbe symbiosis research especially fruitful, providing deeper insight into symbiosis at a number of different levels, including the genetic, molecular, and chemical scales. Below, we discuss how some of these advances are moving the field of insect-symbiosis forward. We will discuss how these techniques can be used to further our understanding of the fungus-growing ant symbiosis, partly by highlighting recent work in other insect-symbiotic systems.

Metagenomics: Identifying Microbial Symbionts. Over the last 15 years, our understanding of the ant–fungus symbiosis has expanded to include *Pseudonocardia*, *Escovopsis*, and the black yeast parasite. As discussed above, one of the challenges and emerging frontiers in insect biology is in identifying microbial symbionts. Traditionally, the majority of the known microbial symbionts have been identified because they can be cultured or are readily observed in the system. However, it is likely that there are a number of yet undiscovered symbionts that play significant roles in these systems. Metagenomics is a relatively new technique capable of identifying the microbial diversity in a system by either sequencing well-studied genetic identifiers, such as 16S or 18S rDNA, or by sequencing more specific genes in the system. In 16S/18S metagenomics, the microbial diversity of a sample is estimated by applying high-throughput sequencing approaches such as 454 sequencing (Margulies et al. 2005). A single 454 sequencing run, which takes an afternoon, generates hundreds of thousands of sequences, orders of magnitude more compared with traditional sequencing approaches, such as Sanger. Coupled with the development of specialized databases, such as the Ribosomal Database Project (Cole et al. 2007) and Greengenes (DeSantis et al. 2006), it is also possible to rapidly attach putative microbial identity to a given sequence. As a result, a much larger view of a system's microbial diversity can be rapidly assessed, potentially providing insight into new symbionts.

The main challenge in using 16S/18S metagenomics data is to determine which microbes are symbionts and which are not. The application of functional metagenomics can help predict the function of the microbial community by sequencing all DNA isolated from a system. In this way, a snapshot of many of the genes that exists within a sample can be obtained, regardless of their origin. This data can be used to infer the types of physiological pathways supported by the whole community and thereby provide insight into the biology of the insect and its associated microbes. Furthermore, putative symbiotic interactions can be predicted without knowledge of the specific symbiont. For example, the presence of multiple copies of a specific amino acid biosynthesis pathway could point to a nutritional deficiency in the diet of the insect and the presence of potential microbial symbionts that fulfill that need.

This approach has already been used to successfully describe the genomic environment of one insect–microbe system: the wood-feeding termite, *Nasutitermes ephratae*, hind-gut system (Warnecke et al. 2007). In this study, DNA isolated from the wood-feeding termite hind-gut was sequenced to determine the types and origins of enzymes that allow the termites to digest wood. A large number of cellulose-degrading genes were found, and their putative origins were pinpointed, many of which are from different species of the termite-associated *Treponema* symbionts (Spiro-

chaetes: Treponamataceae). In addition to these genes, parts of the bacterial community in the termite's hind-gut were characterized based on the 16S rDNA genes sequenced. A similar approach is promising for the fungus-growing ant–microbe system, because the ants likewise process a massive amount of plant biomass. For example, both 16S/18S and functional metagenomics can be used to identify the microbial community and its role in the fungus garden. Because it seems that the cultivated fungus does not participate in cellulose degradation (Abril and Bucher 2002, 2004), it is likely that other microbial symbionts are responsible for this process; they can be identified using metagenomics.

Perhaps the most exciting application of functional metagenomics is in reconstructing the whole genome of insect symbionts to pinpoint and establish the association to specific microbes. This approach has been used in very specialized environments, where there are likely only a few predicted symbionts, thereby facilitating the assignment of functional metagenomic data to a particular genome (Piel 2002). Although this approach is still in its infancy, a recent report used stable isotope-labeled methane to tag DNA and successfully constructed the genome of a novel methylotroph from functional metagenomic data of a diverse microbial community in Lake Washington (Kalyuzhnaya et al. 2008). This approach might be particularly useful for whole genome reconstruction of insect symbionts, especially if the host provides the symbiont with nutrients. As this genome-first approach continues to develop, it will be possible to characterize microbial symbionts of insects in a purely genetic context, including those that cannot be cultured or directly observed.

Genomics of Symbiosis: Interactions at the Genetic Level. As new symbionts are identified, it is important to gain an understanding of how they interact at a genetic level. This is greatly facilitated with the advent of genome sequencing, because researchers have unprecedented access to all of the genes that define an organism's biology. Having access to the genomes of microbes associated with fungus-growing ants would be immensely valuable, allowing us to tease apart the genetic changes in both the ants and the microbes associated with the establishment of fungus farming, the genetic mechanisms of coadaptation during the long history of coevolution between the ant and the cultivar, and the genetic basis of host–symbiont recognition. These types of studies, conducted in other insect–microbe symbioses, have advanced our understanding of the genetic mechanisms of symbiosis. For example, the only complete insect–microbe genome pair is that of *Drosophila* and its endosymbiont *Wolbachia*, and it was recently reported that *Wolbachia* can integrate its entire genome into the chromosome of its *Drosophila* host (Hotopp et al. 2007). This type of cross-kingdom horizontal genome transfer has changed the way we view symbiosis and genome evolution.

Because of the prohibitive cost of sequencing their large and highly variable genome sizes, to date, only a handful of completed insect genomes are available. In fungus-growing ants, the genomes of *Atta cephalotes* (L.) and *A. colombica* are ≈300 Mb in length, whereas the genome of *Apterostigma dentigerum* is more than double the size at ≈640 Mb (Tsutsui et al. 2008). With the decreasing cost in sequencing, we expect many insect genomes to be sequenced in the near future (for a review, see Robinson et al. 2006). Sequencing the genome of the fungal symbionts is much less resource-intensive; however, as of yet, genome sequencing for these fungi has not been undertaken. Nevertheless, a genome sequencing project is underway for the first symbiont from the ant–microbe system, the actinobacterium *Pseudonocardia.* With the genome of this symbiont, it will be possible to conduct comparative genome analyses between *Pseudonocardia* and other sequenced actinobacteria not associated with fungus-growing ants, thereby providing important insights into those genomic regions directly involved in symbiosis. Moreover, the availability of a sequenced genome will help facilitate the description of genetic variation across the genome. This is powerful because it allows for the identification of genomic regions that have recently undergone selective sweeps (i.e., regions that have lost substantial genetic variation). Frequent bouts of selective sweeps are predicted to occur at regions directly involved in host–pathogen coadaptation, so identifying these regions may help pinpoint loci involved in antibiotic production and hence *Escovopsis* suppression.

Furthermore, we can also identify those genes that provide benefits to the ants that are not readily apparent. Although *Pseudonocardia* is known to produce antibiotics that inhibit *Escovopsis*, for which the genetic cluster can be readily identified from its genome (see below), there may be other genes that contribute to the ant–actinobacteria mutualism. This type of analysis has been used to study other bacterial symbionts, specifically, for the large number of endosymbionts that have been sequenced (Dale and Moran 2006). We now know that the close association of endosymbiotic bacteria with their insect host often results in severe genome reduction, with retention of mainly genes that confer a benefit to the host (Ochman and Moran 2001, Wernegreen 2002). For example, many of these endosymbionts specialize in biosynthetic pathways for the production of amino acids that are either scarce or missing from the diet of their insect host (Shigenobu et al. 2000, Nakabachi et al. 2006, McCutcheon and Moran 2007). *Pseudonocardia* will likely be the first insect exosymbiont sequenced, with a predicted genome size between 6 and 7 Mb. Far greater than the average <2 Mb of insect-endosymbiotic genomes, it may encode genetic clusters that are needed for the exosymbiotic lifestyle and possibly genes that confer benefits to the ants in addition to antibiotic production.

Many of these initial genome-level studies have focused on using comparative genomics to identify the genes involved in establishing and propagating symbiotic interactions. However, we are now realizing that the expression of genes during symbiotic inter-

actions is likely a major driving force behind their evolution. As a result, many insect–microbe symbiosis studies are beginning to use microarrays, a technology that can measure changes in the level of gene expression (Lucchini et al. 2001, Southern 2001, Ehrenreich 2006). For the ant–microbe system, this approach may be more feasible than sequencing the whole genome of an ant, because a microarray can be constructed using the ant's expressed sequence tags. Sequencing of these tags requires substantially less resources than whole genome sequencing, and a microarray for the fire ant, based on ≈12,000 expressed sequence tags, in fact already exists (Wang et al. 2007). Coupled with the genome of *Pseudonocardia*, a dual-microarray can be constructed to investigate the changes in gene expression that occur in both partners under different conditions, such as infection with *Escovopsis.* This approach has been used to evaluate changes in gene expression that occur in both partners of the *Drosophila–Wolbachia* symbiosis during *Wolbachia* colonization (Xi et al. 2008) and also in the pea aphid–*Buchnera* system during stress response to heat shock (Wilson et al. 2006). Because of the feasibility of constructing dual-microarray systems, this may be an attractive technique for many insect–microbe systems.

Symbiosis at the Molecular Level. Having access to the genome sequences of insect-microbe symbionts can also increase our understanding of symbiotic interactions at the molecular level. This is primarily because of our ability to develop genetic systems for these symbionts and study the molecular changes that occur as a result of genetic changes in the genome. For many insects, the development of RNAi to remove the expression of genes has greatly facilitated our ability to test the involvement of specific genes and proteins in symbiotic interactions (Fire 1999, Hannon 2002). Similarly, the development of genetic systems for microbial symbionts by completely disrupting transcription of specific genes can be useful for studying the symbionts role. This approach presents an exciting prospect for the fungus-growing ant–microbe system, because many of the symbiotic interactions in this system exist at multiple scales. For example, the ants engage in behavioral activities that promote symbiosis, such as the grooming and weeding behavior of *Escovopsis* from their fungal gardens. By knocking out the expression of specific genes identified to be involved in pathways such as pathogen recognition using RNAi studies, we can begin to understand the genetic and molecular basis of such behaviors.

Many of these types of analyses are already well developed for bacterial systems, and the development of a genetic system for *Pseudonocardia* would allow for the study of a number of key aspects of the ant–*Pseudonocardia* symbiosis. For example, the production of secondary metabolites by *Pseudonocardia*, such as the already identified compound (Oh et al. in prep.), can be correlated to genetic cluster(s), and a knockout can be used to definitively establish that a particular antibiotic is responsible for *Escovopsis* inhibition. Furthermore, knockout strains of *Pseudonocardia* could be tested by inoculating these strains into their original colonies to determine the set of genes that are important for successful maintenance of the symbiosis. Such knockout type experiments could be applied to all other members in the system, thereby teasing apart genes and molecules that result in the establishment, maintenance, and evolution of symbiosis.

Conclusion

Given the extraordinary amount of biodiversity held within microbes and the insects (Wilson 1992), it is somewhat surprising that few insect–microbe symbioses have been described. This is perhaps a reflection of the relatively small number of entomologists and/or microbiologists focused on these cross-kingdom associations, as well as the challenges associated with identifying and working on microbial symbionts. It is likely that insect–microbe symbioses are ubiquitous, and a future challenge for the field is describing the multitude of associations that exist. Here we have reviewed the past and present research in the fungus-growing ant model system in hopes to provide insights for describing other insect–microbe symbioses. The fungus-growing ant system is a particularly useful model system, because it contains relatively well-described symbionts ranging from mutualists to antagonists crossing multiple kingdoms. Our experience in microbial ecology and evolution, we hope, also provides useful insight into tackling complex concepts such as population and coevolutionary dynamics, as well as host-symbiont specificity, within multipartite interactions. We have also discussed future directions in insect symbioses, pointing out that the field stands to benefit greatly from recently thriving areas, such as genomics. Given the abundance of microbial symbionts, as both pathogens and producers of antibiotics, insight from insect–microbe symbioses may also have important implications for the health of all organisms, including humans.

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