Population Structure and Coevolutionary Dynamics of Fungus-Growing Ant Symbionts

By

Eric J. Caldera

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Zoology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2017

Date of final oral examination: 05/1/2017

The dissertation is approved by the following members of the Final Oral Committee:

Cameron R. Currie, Professor, Bacteriology
Carol E. Lee, Professor, Zoology
Katherine D. McMahon, Professor, Bacteriology
Tony L. Goldberg, Professor, Epidemiology
David A. Baum, Professor, Botany

Acknowledgements

This dissertation would not be possible without the incredible support of several mentors. I am forever grateful to Professor Gilliam "Mike" Burton at San Antonio College for offering me an eleventh-hour scholarship, and indeed a lifeline, that started me down this path. Dr. David Holway welcomed me into his lab at The University of California, San Diego and over a summer guided me through the process of conducting research, from collecting samples to publishing a paper. Dr. Ulrich Mueller introduced me to the fungus-growing ant system that I love and encouraged me to pursue research when I transferred to the University of Texas at Austin from community college. Dr. Daniel Bolnick introduced me to population genetic analysis and gave me many fond memories of field work on Vancouver Island. Dr. DeWayne Shoemaker provided me the opportunity to work on fire ants and graciously shared his knowledge of evolutionary genetics. Throughout my time in at the University of Wisconsin-Madison, Dr. Cameron Currie has been a wonderful graduate advisor, mentor, and friend. I'm indebted to many current and former members of the Currie Lab for valuable discussions, collaborations and field adventures: Jarrod Scott, Michael Poulson, Garret Suen, Adrian Pinto, Marc Chevrette, Kirk Grubbs, Heidi Horn, Lily Khadempour, Bradon McDonald, Alissa Hanshew, Frank Aylward. To my incredible thesis committee – Drs. Cameron R. Currie, Carol E. Lee, Katherine D. McMahon, Tony L. Goldberg, and David A. Baum. You are all my academic heroes and I thank you for your enthusiasm, encouragement, and feedback at every step.

I would also like to thank the various funding sources and organizations that have made my dissertation work possible. The Organization for Tropical Studies provided

funding and resources to conduct research in Costa Rica, and has provided invaluable training and scholarships throughout my undergraduate and graduate education. The National Science Foundation provided a predoctoral fellowship and a doctoral dissertation improvement grant. The National Institute of Health provided a microbes in health and disease fellowship. The UW-Madison graduate school provided an advanced opportunity fellowship and the Zoology department awarded funding for field work in Panama and Peru. The majority of my field work was conduced at the Smithsonian Tropical Research Institute (STRI) in Pamama. I thank STRI and the Authoridad Nacional del Ambiente (ANAM) for resources and permission to collect material in Panama.

I dedicate this dissertation to my family. To my brother and sister, who were the first in our family to graduate from university, you are both my inspiration and an incredible example for everyone. My mother's love and support is boundless – I've needed every bit of it. My father, who's often juggled multiple, blue-collar jobs just to keep food on the table, while managing to find time to be a great dad, has always been my hero and example of determination. Finally, to my beautiful wife Dr. Celeste Eno: you're my best friend, biggest cheerleader, and the hardest working person I know. I truly could not have completed this journey without your love and partnership.

Table of Contents

List of Figures and Tables

Chapter 1: Insect Symbioses: A Case Study of Past, Present, and Future Fungus-growing Ant Research

Chapter 2: Labile association between fungus-growing ant cultivars and their garden pathogens.

Chapter 3: The population structure of antibiotic-producing bacterial symbionts of *Apterostigma dentigerum* ants: Impacts of coevolution and multi-partite symbiosis.

Chapter 4: The geographic mosaic of antibiotic coevolution in a bacterial symbiont of the fungus-farming ant *Apterostigma dentigerum*: evidence for a coevolutionary hot spot on Barro Colorado Island, Panama

Chapter 5: Summary and Future Directions

Appendix:

Putative Native Source of the Invasive Fire Ant *Solenopsis invicta* in the USA.

The Genome Sequence of the Leaf-Cutter Ant *Atta cephalotes* Reveals Insights into Its Obligate Symbiotic Lifestyle

Abstract

Microbial symbionts play key roles in shaping the diversity of life, from disease causing pathogens to beneficial microbes. The fungus-growing ant (Attini, *Apterostigma dentigerum*) is ideal for exploring these dynamics, as it maintains multiple symbionts, including fungal cultivars, cultivar attacking pathogens, and mutualistic bacteria whose antibiotic secretions combat infection. Decades of Attine-symbiont research has characterized these interactions as examples of diffuse coevolution. By contrast, coevolutionary theory on a geographic scale posits that these diffuse associations may still contain tightly coevolved interactions in a mosaic pattern over space. Moreover, these mosaics are shaped by the population genetic structure of interacting species. In this dissertation, I characterize the *Apterostigma dentigerum*-symbiont dynamic from a geographic mosaic prospective. The history of attine-symbiont evolutionary ecology is reviewed in chapter 1. In chapter 2, I describe cultivar-pathogen population structure and suggest that this labile association may facilitate specific adaptation by the mutualistic bacteria, *Pseudonocardia*. In chapter 3, I describe the population genetic structure of *Pseudonocardia* and demonstrate that its fine scale phylogeograpic structure may facilitate local adaptation with a common pathogen (*Escovopsis*) morphotype. Chapter 4 uses bioassay inhibition experiments in combination with population genomic approaches to demonstrate that the *Pseudonocardia-Escovopsis* interaction is consistent with a geographic mosaic of coevolution, with a locally adapted population residing on Barro Colorado Island in Panama. Chapter 5 provides a putative framework and future directions for understanding the role biosynthetic gene clusters play in *Pseudonocardia*-

pathogen coevolution. These studies provide insights into the maintenance of antibiotic potency over evolutionary time, and microbial niche evolution.

1 Chapter 1: Insect Symbioses: A Case Study of Past, Present, and Future Fungus-growing Ant Research

This chapter was previously published in Environmental Entomology. 2009, 38(1): 78-92

Eric J. Caldera, Michael Poulsen, Garret Suen and Cameron R. Currie

All authors contributed equally to writing, and editing the paper in the following ways. EJC & CRC summarized pervious research, EJC & MP & CRC summarized contemporary research and GS, EJC, MP & CRC summarized future, genomic approaches.

1.1 Abstract and Keywords

Fungus-growing ants (Attini: Formicidae) engage in an obligate mutualism with fungi they cultivate for food. Although many biologists have been fascinated with fungus-growing ants since the resurgence of interest in 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.

Attini, *Escovopsis*, *Leucoagaricus*, *Pseudonocardia*, symbiosis

1.2 Introduction

The history of life on earth has been primarily 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 have also played key roles in biogeochemical processes, which helped make the biosphere more hospitable to other life forms. 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 of this 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 of the continuum, 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, with 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 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.

1.3 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. 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 macroorganisms, 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 leafcutters 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 by 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 when compared 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.

1.4 Contemporary Fungus-growing Ant Symbiosis Research

1.4.1 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 in association 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 found 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 anti-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 are 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. For a microbe to be a pathogen Koch's postulates require that the microorganism of interest be found in diseased organisms but not in healthy ones. The microorganism must then be isolated in pure culture and confirmed to cause disease when introduced to a healthy organism. Last, the microorganism must be re-isolated 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).

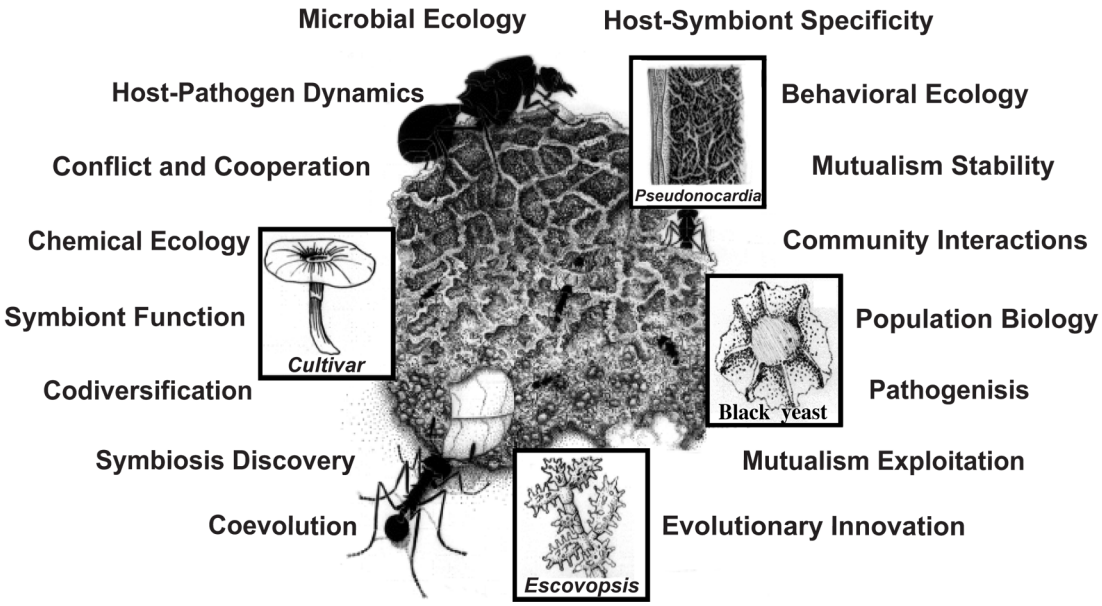


Figure 1. The fungus-growing ant symbiosis is a model system for studying the ecology and evolution of symbiotic interactions. The symbiosis currently contains five identified and characterized symbionts: Attine ants, the fungi that they cultivate for food, cultivar-attacking microfungi in the genus *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.

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 1910) has recently been characterized (Oh et al 2009). 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 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.

1.4.2 Host-symbiont Phylogenetics and Coevolution

A more complete understanding of the ant-fungus mutualism has been greatly hindered by the lack of information 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). This 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).

	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

Figure 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.

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. 2011.). 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 would typically not be appropriate for revealing patterns of codiversification at evolutionary time scales (Cohan 2006, Staley 2006; see Cafaro et al. 2011).

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.

1.4.3 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, 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, thus becoming 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, more 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.

1.5 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. 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.

1.5.1 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 Green genes

(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 (Spirochaetes: 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 appears 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.

1.5.2 Genomics of Symbiosis: Interaction 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, as it would allow 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, though 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. This should provide 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 would be powerful because it would allow 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 might 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 interactions 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.

1.5.3 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.

1.6 Conclusion

Given the extraordinary amount of biodiversity held within microbes and the insects (Wilson 1992), itis 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.

1.7 Acknowledgements

We thank J. Scott, R. Steffensen, S. Marsh, and S. Adams for comments on the manuscript and M. Cafaro for unpublished information. E.J.C. and M.P. thank U. G. Mueller and J. J. Boomsma, respectively, for teaching them about fungus-growing ants. During the writing of this review, E.J.C. was supported by an NSF Graduate Research Fellowship, M.P. by the Carlsberg Foundation, C.R.C. and G.S. by the Great Lakes Bioenergy Research Center-DOE DE-FC02- 07ER64494, and C.R.C. by the NSF (CAREER 0747002, Microbial Observatory 0702025, and Microbial Genome Sequencing 0731822).

1.8 References Cited

Aanen, D. K., P. Eggleton, C. Rouland-Lefevre, T. Guldberg, Frøslev, S. Rosendahl, and J. J. Boomsma. 2002. The evolution of fungus-growing termites and their mutualistic fungal symbionts. Proc. Natl. Acad. Sci. USA. 99: 14887- 14892.

Abril, A. B., and E. H. Bucher. 2002. Evidence that the fungus cultured by leaf-cutting ants does not metabolize cellulose. Ecol. Lett. 5: 325-328.

Abril, A. B., and E. H. Bucher. 2004. Nutritional sources of the fungus cultured by leaf-cutting ants. Appl. Soil Ecol. Agric. Ecosyst. Environ. 26: 243-247.

Abril, A. B., and E. H. Bucher. 2007. Genetic diversity of fungi occurring in nests of three *Acromyrmex* leaf-cutting ant species from Cordoba, Argentina. Microb. Ecol. 54: 417- 423.

Adams, R. M., U. G. Mueller, A. K. Holloway, A. M. Green, and J. Narozniak. 2000. Garden sharing and garden stealing in fungus-growing ants. Naturwissenschaften 87: 491- 493.

Agrios, G. N. 1988. Plant pathology. Academic, San Diego, CA. Autuori, M. 1956. La fondation des sociétés chez les fourmis champignonnistes du genre 'Atta' (Hym.

Formicidae), pp. 77-104. In M. Autuori (ed.), L'instinct dans le comportement des animaux et de l'homme. Masson et Cie, Paris, France.

Baldauf, S. L. 2003. The deep roots of eukaryotes. Science 300: 1703-1706.

Bass, M., and J. M. Cherrett. 1994. The role of leaf-cutting ant workers (Hymenoptera: Formicidae) in fungus garden maintenance. Ecol. Entomol. 19: 215-220.

Bates, H. W. 1863. The naturalist on the River Amazons, 2 vols. John Murray, London, United Kingdom.

Belt, T. 1874. The naturalist in Nicaragua. E. Bumpus, London, United Kingdom.

Borneman, J., P. W. Skroch, K. M. O'Sullivan, J. A. Palus, N. G. Rumjanek, J. L. Jansen, J. Nienhuis, and E. W. Triplett. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. Appl. Environ. Microbiol. 62: 1935-1943.

Bot, A.N.M., S. A. Rehner, and J. J. Boomsma. 2001. Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. Evolution 55: 1980 -1991.

Boucher, D. H. 1988. The biology of mutualisms. Oxford University Press, New York.

Bourtzis, K. A., and T. A. Miller. 2003. Insect symbiosis, vol. 1. CRC, Boca Raton, FL.

Bourtzis, K. A., and T. A. Miller. 2006. Insect symbiosis, vol. 2. CRC, Boca Raton, FL.

Bowers, M. A., and S. D. Porter. 1981. Effect of foraging distance on water content of substrates harvested by *Atta colombica* (Guerin). Ecology 62: 273-275.

Bronstein, J. L. 2001. The exploitation of mutualisms. Ecol. Lett. 4: 277-287.

Cafaro, M. J., and C. R. Currie. 2005. Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. Can. J. Microbiol. 51: 441- 446.

Cafaro, M. J., M. Poulsen, A. E. F. Little, S. L. Price, N. M. Gerardo, B. Wong, A. E. Stuart et al. 2011. Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society B: Biological Sciences.

Chapela, I. H., S. A. Rehner, T. R. Schultz, and U. G. Mueller. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266: 1691-1694.

Cherrett, J. M. 1968. The foraging behaviour of *Atta cephalotes* L. (Hymenoptera: Formicidae), I: foraging pattern and plant species attacked in tropical rain forest. J. Anim. Ecol. 37: 387- 403.

Cherrett, J. M. 1972. Some factors involved in the selection of vegetable substrate by *Atta* cephalotes (L.) (Hymenoptera: Formicidae) in tropical rain forest. J. Anim. Ecol. 41: 647-660.

Ciche, T. A., and S. K. Goffredi. 2007. General methods to investigate microbial synthesis, pp. 394 - 419. In C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. M. Arzluf, T. M. Schmidt, and L. R. Synder (eds.), Methods for general and molecular microbiology, 3rd ed. ASM Press, Washington, DC.

Clarkson, J. M., and A. K. Charnley. 1996. New insights into the mechanisms of fungal pathogenesis in insects. Trends Microbiol. 4: 197-203.

Cohan, F. M. 2006. Towards a conceptual and operational union of bacterial systematics, ecology, and evolution. Philo. Trans. R. Soc. B Biol. Sci. 361: 1985-1996.

Cole, J. R., B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-SyedMohideen, D. M. McGarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje. 2007. The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res. 35: D169 -D172.

Cruden, D. L., and A. J. Markovetz. 1987. Microbial ecology of the cockroach gut. Annu. Rev. Microbiol. 41: 617- 643. Currie, C. R. 2001a. Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia (Berl.) 128: 99 -106.

Currie, C. R. 2001b. A community of ants, fungi, and bacteria: amultilateral approach to studying symbiosis. Annu. Rev. Microbiol. 55: 357-380.

Currie, C. R., and A. E. Stuart. 2001. Weeding and grooming of pathogens in agriculture by ants. Proc. R. Soc. Lond. Ser. B Biol. Sci. 268: 1033-1039.

Currie, C. R., U. G. Mueller, and D. Malloch. 1999a. The agricultural pathology of ant fungus gardens. Proc. Natl. Acad. Sci. USA. 96: 7998 - 8002.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999b. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature (Lond.) 398: 701-704.

Currie, C. R., A.N.M. Bot, and J. J. Boomsma. 2003a. Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101: 91-102.

Currie, C. R., B. Wong, A. E. Stuart, T. R. Schultz, S. A. Rehner, U. G. Mueller, G. H. Sung, J. W. Spatafora, and N. A. Straus. 2003b. Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299: 386 -388.

Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311: 81- 83.

Dale, C., and N. A. Moran. 2006. Molecular interactions between bacterial symbionts and their hosts. Cell 126: 453- 465.

Dawson, S. C., and N. R. Pace. 2002. Novel kingdom-level eukaryotic diversity in anoxic environments. Proc. Natl. Acad. Sci. USA. 99: 8324 - 8329.

de Bary, A. 1879. Die Erscheinung der Symbiose. Verlag von Karl J. Trubner, Strassburg, Germany.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72: 5069 -5072.

Dillon, R. J., and V. M. Dillon. 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. 49: 71-92.

do Nascimento, R. R., E. Schoeters, E. D. Morgan, J. Billen, and D. J. Stradling. 1996. Chemistry of metapleural gland secretions of three attine ants, Atta sexdens rubropilosa, *Atta cephalotes*, and *Acromyrmex octospinosus* (Hymenoptera: Formicidae). J. Chem. Ecol. 22: 987-1000. Douglas, A. E. 2007. Symbiotic microorganisms: untapped resources for insect pest control. Trends Biotechnol. 25: 338 -342.

Ehrenreich, A. 2006. DNA microarray technology for the microbiologist: an overview. Appl. Microbiol. Biotechnol. 73: 255-273.

Ewald, P. 1994. Evolution of infectious disease. Oxford University Press, New York. Fabricius, J. C. 1804. Systema Piezatorum secundum ordines, genera, species, adjectis synonymis, locis, observationibus, descriptionibus. C. Reichard, Brunswick.

Fernández-Marín , H., J. K. Zimmerman, and W. T. Wcislo. 2004. Ecological traits and evolutionary sequence of nest establishment in fungus-growing ants (Hymenoptera, Formicidae, Attini). Biol. J. Linn. Soc. 81: 39 - 48.

Fernández-Marín , H., J. K. Zimmerman, S. A. Rehner, and W. T. Wcislo. 2006. Active use of the metaplural glands by ants in controlling fungal infection. Proc. R. Soc. London Ser. B Biol. Sci. 273: 1689 -1695.

Fire, A. 1999. RNA-triggered gene silencing. Trends Genet. 15: 358 -363.

Fisher, P. J., D. J. Stradling, B. C. Sutton, and L. E. Petrini. 1996. Microfungi in the fungus gardens of the leaf-cutting ant *Atta cephalotes*: a preliminary study. Mycol. Res. 100: 541-546. Frank, S. A. 1996. Host-symbiont conflict over the mixing of symbiotic lineages. Proc. Biol. Sci. 263: 339 -344. Frank, S. A. 2003. Perspective: repression of competition and the evolution of cooperation. Evolution 57: 693-705.

Geib, S. M., T. R. Filley, P. G. Hatcher, K. Hoover, J. E. Carlson, M. Jimenez-Gasco Mdel, A. Nakagawa-Izumi, R. L. Sleighter, and M. Tien. 2008. Lignin degradation in wood-feeding insects. Proc. Natl. Acad. Sci. USA. 105: 12932-12937.

Gerardo, N. M., and E. J. Caldera. 2007. Labile associations between fungus-growing ant cultivars and their garden pathogens. ISME J. 1: 373- 84.

Gerardo, N. M., U. G. Mueller, and C. R. Currie. 2006a. Complex host-pathogen coevolution in the Apterostigma fungus-growing ant-microbe symbiosis. BMC Evol. Biol. 6: 88.

Gerardo, N. M., S. R. Jacobs, C. R. Currie, and U. G. Mueller. 2006b. Ancient host-pathogen associations maintained by specificity of chemotaxis and antibiosis. PLoS Biol. 4: e235.

Green, A. M., U. G. Mueller, and R. M. M. Adams. 2002. Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants. Mol. Ecol. 11: 191-195.

Haine, E. R. 2008. Symbiont-mediated protection. Proc. Biol. Sci. 275: 353-361.

Hajek, A. E., and R. J. St. Leger. 1994. Interactions between fungal pathogens and insect hosts. Annu. Rev. Entomol. 39: 293-322.

Halkett, F., J. C. Simon, and F. Balloux. 2005. Tackling the population genetics of clonal and partially clonal organisms. Trends Ecol. Evol. 20: 194 -201.

Hamilton, W. D., R. Axelrod, and R. Tanese. 1990. Sexual reproduction as an adaptation to resist parasites (a review). Proc. Natl. Acad. Sci. USA. 87: 3566 -3573. Hannon, G. J. 2002. RNA interference. Nature (Lond.) 418: 244 -251.

Heath, B. D., R.D.J. Butcher, W.G.F. Whitfield, and S. F. Hubbard. 1999. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Curr. Biol. 9: 313-316.

Hervey, A., C. T. Rogerson, and I. Leong. 1977. Studies on fungi cultivated by ants. Brittonia 29: 226 -236.

Hibbett, D. S., L. B. Gilbert, and M. J. Donoghue. 2000. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. Nature (Lond.) 407: 506 -508.

Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J. H. Werren. 2008. How many species are infected with *Wolbachia?*-a statistical analysis of current data. FEMS Microbiol. Lett. 281: 215-220. Hölldobler, B., and E. O. Wilson. 1990. The ants. Harvard University Press, Cambridge, MA.

Hongoh, Y., P. Deevong, T. Inoue, S. Moriya, S. Trakulnaleamsai, M. Ohkuma, C. Vongkaluang, N. Noparatnaraporn, and T. Kudo. 2005. Intra- and interspeciFIc comparisons of bacterial diversity and community structure support coevolution of gut microbiota and termite host. Appl. Environ. Microbiol. 71: 6590 - 6599.

Hotopp, J. C., M. E. Clark, D. C. Oliveira, J. M. Foster, P. Fischer, M. C. Torres, J. D. Giebel, N. Kumar, N. Ishmael, S. Wang, J. Ingram, R. V. Nene, J. Shepard, J. Tomkins, S. Richards, D. J. Spiro, E. Ghedin, B. E. Slatko, H. Tettelin, and J. H. Werren. 2007. Widespread lateral gene transfer fromintracellular bacteria to multicellular eukaryotes. Science (NY) 317: 1753-1756.

Huigens, M. E., R. F. Luck, R. H. Klaassen, M. F. Maas, M. J. Timmermans, and R. Stouthamer. 2000. Infectious parthenogenesis. Nature (Lond.) 405: 178 -179.

Huigens, M. E., R. P. de Almeida, P. A. Boons, R. F. Luck, and R. Stouthamer. 2004. Natural interspeciFIc and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in Trichogramma wasps. Proc. Biol. Sci. 271: 509 -515.

Ihering, H. v. 1898. Die Anlange neuer Kolonien und Pilzgärten bei *Atta sexdens*. Zool. Anz. 21: 238 -245.

Jaenike, J., and S. J. Perlman. 2002. Ecology and evolution of host-parasite associations: mycophagous Drosophila and their parasitic nematodes. Am. Nat. 160(Suppl 4): S23-S39.

Janson, E. M., J. O. Stireman, M. S. Singer, and P. Abbot. 2008. Phytophagous insect-microbe mutualisms and adaptive evolutionary diversification. Evolution 62: 997- 1012.

Kaltenpoth, M., W. Gottler, G. Herzner, and E. Strohm. 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. Curr. Biol. 15: 475- 479.

Kalyuzhnaya, M. G., A. Lapidus, N. Ivanova, A. C. Copeland, A. C. McHardy, E. Szeto, A. Salamov, I. V. Grigoriev, D. Suciu, S. R. Levine, V. M. Markowitz, I. Rigoutsos, S. G. Tringe, D. C. Bruce, P. M. Richardson, M. E. Lidstrom, and L. Chistoserdova. 2008. High-resolution metagenomics targets specific functional types in complex microbial communities. Nature Biotechnol. 26: 1029-1034.

Klepzig, K. D., and D. L. Six. 2004. Bark beetle fungal symbiosis: context dependency in complex interactions. Symbiosis 37: 189 -206.

Linneaus, C. V. 1758. Systema naturae, per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Typis Ioannis Thomae, Oxford, United Kingdom.

Little, A. E., and C. R. Currie. 2007. Symbiotic complexity: discovery of a fifth symbiont in the attine ant-microbe symbiosis. Biol. Lett. 3: 501-504.

Little, A. E., and C. R. Currie. 2008. Black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants. Ecology 89: 1216 -1222.

Little, A. E., T. Murakami, U. G. Mueller, and C. R. Currie. 2006. Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biol. Lett. 2: 12-16.

Littledyke, M., and J. M. Cherrett. 1978. Defence mechanisms in young and old leaves against cutting by the leaf-cutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (Reich) (Hymenoptera: Formicidae). Bull. Entomol. Res. 68: 263-271.

Lively, C.M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. Am. Nat. 153: S34 -S47.

Lucchini, S., A. Thompson, and J. C. Hinton. 2001. Microarrays for microbiologists. Microbiology 147: 1403-1414.

Lutzoni, F., M. Pagel, and V. Reeb. 2001. Major fungal lineages are derived from lichen symbiotic ancestors. Nature (Lond.) 411: 937-940.

Maddison, D. R., D. L. Swofford, and W. P. Maddison. 1997. NEXUS: an extensible FIle format for systematic information. Syst. Biol. 46: 590 - 621.

Margulies, M., M. Egholm, W. E. Altman, S. Attiya, J. S. Bader, L. A. Bemben, J. Berka, M. S. Braverman, Y. J. Chen, Z. Chen, S. B. Dewell, L. Du, J. M. Fierro, X. V. Gomes, B. C. Godwin,W. He, S. Helgesen, C. H. Ho, G. P. Irzyk, S. C. Jando, M. L. Alenquer, T. P. Jarvie, K. B. Jirage, J. B. Kim, J. R. Knight, J. R. Lanza, J. H. Leamon, S. M. Lefkowitz, M. Lei, J. Li, K. L. Lohman, H. Lu, V. B. Makhijani, K. E. McDade, M. P. McKenna, E. W. Myers, E. Nickerson, J. R. Nobile, R. Plant, B. P. Puc,M. T. Ronan, G. T. Roth, G. J. Sarkis, J. F. Simons, J. W. Simpson, M. Srinivasan, K. R. Tartaro, A. Tomasz, K. A. Vogt, G. A. Volkmer, S. H. Wang, Y. Wang, M. P. Weiner, P. Yu, R. F. Begley, and J. M. Rothberg. 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature (Lond.) 437: 376 -380.

Margulis, L. 1970. Origin of eukaryotic cells. Yale University Press, New Haven, CT.

Margulis, L. 1981. Symbiosis in cell evolution. W.H. Freeman & Co., New York. McCutcheon, J. P., and N. A.

Moran. 2007. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc. Natl. Acad. Sci. USA. 104: 19392-19397.

Mereschkowsky, C. 1905. Über Natur und Ursprung der Chromatoporenim Pflanzenreiche. Biologisches Centralblatt 25: 593- 604.

Mikheyev, A. S., U. G. Mueller, and P. Abbot. 2006. Cryptic sex and many-to-one coevolution in the fungus-growing ant symbiosis. Proc. Natl. Acad. Sci. USA. 103: 10702- 10706.

Mikheyev, A. S., U. G. Mueller, and J. J. Boomsma. 2007. Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. Mol. Ecol. 16: 209 -216.

Mo¨ller, A. 1893. Die Pilzgärten einiger südamerikanischer Ameisen, Botanische Mitteilungen Tropen 6: 1-127.

Moran, N. A. 2006. Symbiosis. Curr. Biol. 16: R866 -R871.

Moreira, D., H. Le Guyader, and H. Philippe. 2000. The origin of red algae and the evolution of chloroplasts. Nature (Lond.) 405: 69 -72.

Muchovej, J. J., and T. M. Della Lucia. 1990. *Escovopsis*, a new genus from leaf cutting ant nests to replace Phialocladus nomem invalidum. Mycotaxon 37: 191-195.

Muchovej, J. J., T. M. Della Lucia, and R.M.C. Muchovej. 1991. *Leucoagaricus weberi sp. nov.* from a live nest of leaf-cutting ants. Mycol. Res. 95: 1308 -1311.

Mueller, U. G. 2002. Ant versus fungus versus mutualism: ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. Am. Nat. 160(Suppl): S67-S98.

Mueller, U. G., S. A. Rehner, and T. R. Schultz. 1998. The evolution of agriculture in ants. Science 281: 2034 -2038.

Mueller, U. G., T. R. Schultz, C. R. Currie, R. M. Adams, and D. Malloch. 2001. The origin of the attine ant-fungus mutualism. Q. Rev. Biol. 76: 169 -197.

Mueller, U. G., D. Dash, C. Rabeling, and A. Rodrigues. 2008. Coevolution between Attine ants and Actinomycete bacteria: a reevaluation. Evolution 62: 2894 -2912.

Munkacsi, A. B., J. J. Pan, P. Villesen, U. G. Mueller, M. Blackwell, and D. J. McLaughlin. 2004. Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants. Proc. R. Soc. Lond. Ser. B Biol. Sci. 271: 1777-1782.

Nakabachi, A., A. Yamashita, H. Toh, H. Ishikawa, H. E. Dunbar, N. A. Moran, and M. Hattori. 2006. The 160- kilobase genome of the bacterial endosymbiont *Carsonella*. Science (NY) 314: 267.

Noda, H., T. Miyoshi, Q. Zhang, K. Watanabe, K. Deng, and S. Hoshizaki. 2001. *Wolbachia* infection shared among planthoppers (Homoptera: Delphacidae) and their endoparasite (Strepsiptera: Elenchidae): a probable case of interspecies transmission. Mol. Ecol. 10: 2101-2106.

O'Neill, S. L., R. Giordano, A. M. Colbert, T. L. Karr, and H. M. Robertson. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmicincompatibilityininsects. Proc. Natl. Acad. Sci. USA. 89: 2699 -2702.

Ochman, H., and N. A. Moran. 2001. Genes lost and genes found: evolution of bacterial pathogenesis and symbiosis. Science (NY) 292: 1096 -1099.

Oh, D. C., M. Poulsen, C. R. Currie, and J. Clardy. 2009. Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nature Chemical Biology 5:391-393.

Ohkuma, M. 2003. Termite symbiotic systems: efFIcient biorecycling of lignocellulose. Appl. Microbiol. Biotechnol. 61: 1-9.

Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. Science 276: 734 -740.

Page, R.D.M., and M. A. Charleston. 1998. Trees within trees: phylogeny and historical associations. Trends Ecol. Evolut. 13: 356 -359.

Pedigo, L. P., and M. E. Rice. 2006. Entomology and pest management. Prentice Hall, Englewood Cliffs, NJ.

Piel, J. 2002. A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of Paederus beetles. Proc. Natl. Acad. Sci. USA. 99: 14002-14007.

Poulsen, M., and J. J. Boomsma. 2005. Mutualistic fungi control crop diversity in fungus-growing ants. Science 307: 741-744.

Poulsen, M., A. N. Bot, and J. J. Boomsma. 2003. The effect of metapleural gland secretion on the growth of a mutualistic bacterium on the cuticle of leaf-cutting ants. Naturwissenschaften 90: 406 - 409.

Poulsen, M., M. Cafaro, J. J. Boomsma, and C. R. Currie. 2005. Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. Mol. Ecol. 14: 3597-3604.

Poulsen, M., W.O.H. Hughes, and J. J. Boomsma. 2006. Differential resistance and the importance of antibiotic production in *Acromyrmex echinatior* leaf-cutting ant castes towards the entomopathogenic fungus Aspergillus nomius. Insectes Soc. 53: 349 -355.

Poulsen, M., D. P. Erhardt, D. J. Molinaro, T. L. Lin, and C. R. Currie. 2007. Antagonistic bacterial interactions help shape host-symbiont dynamics within the fungus-growing ant-microbe mutualism. PLoS ONE. 2: e960.

Poulsen, M., H. Fernandez-Marin, C. R. Currie, and J. J. Boomsma. Ephemeral windows of opportunity for hotizontal transmission of fungal symbionts in leaf-cutting ants. Evolution. 63: 2235 - 2247.

Price, P. W., M. Wetoby, B. Rice, P. R. Atsatt, R. S. Fritz, J. H. Thompson, and K. Mobley. 1986. Parasite mediation in ecological interactions. Annu. Rev. Ecol. Syst. 17: 487- 505.

Quinlan, R. J., and J.M. Cherrett. 1977. The role of substrate preparation in the symbiosis between the leaf cutting ant *Acromyrmex octospinosus* (Reich) and its food fungus. Ecol. Entomol. 2: 161-170.

Reynolds, H. T., and C. R. Currie. 2004. Pathogenicity of *Escovopsis weberi*: the parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. Mycologia 96: 955-959.

Richard, F.-J., M. Poulsen, A. Hefetz, C. Errard, D. Nash, and J. J. Boomsma. 2007. The origin of the chemical profiles of fungal symbionts and their significance for nest -mate recognition in *Acromyrmex* leaf-cutting ants. Behav. Ecol. Sociobiol. 61: 1637-1649.

Robinson, G. E., J. D. Evans, R. Maleszka, H. M. Robertson, D. B. Weaver, K. Worley, R. A. Gibbs, and G. M. Weinstock. 2006. Sweetness and light: illuminating the honey bee genome. Insect Molec. Biol. 15: 535-539.

Rockwood, L. L. 1975. The effects of seasonality on foraging in two species of leaf-cutting ants (Atta) in Guanacaste province, Costa Rica. Biotropica 7: 176 -193.

Rodrigues, A., M. Bacci Jr., U. G. Mueller, A. Ortiz, and F. C. Pagnocca. 2008. Microfungal “weeds” in the leafcutter ant symbiosis. Microb. Ecol. 56: 604 - 614.

Sanders, I. R. 2002. Ecology and evolution of multigenomic arbuscular mycorrhizal fungi. Am. Nat. 160(Suppl 4): S128 -S141.

Sanhudo, C.E.D., T. J. Izzo, and C.R.F. Brandao. 2008. Parabiosis between basal fungus-growing ants (Formicidae, Attini). Insectes Soc. 55: 296 -300.

Schilthuizen, M., and R. Stouthamer. 1997. Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. Proc. Biol. Sci. 264: 361-366.

Schultz, T. R., and S. G. Brady. 2008. Major evolutionary transitions in ant agriculture. Proc. Natl. Acad. Sci. USA. 105: 5435-5440.

Scott, J. J., D.-C. Oh, M. C. Yuceer, K. D. Klepzig, J. Clardy, and C. R. Currie. 2008. Bacterial protection of beetle-fungus mutualism. Science 322: 63.

Seifert, K. A., R. A. Samson, and I. H. Chapela. 1995. *Escovopsis aspergilloides*, a rediscovered hyphomycete from leaf-cutting ant nests. Mycologia 87: 407- 413.

Shah, P. A., and J. K. Pell. 2003. Entomo-pathogenic fungi as biological control agents. Appl. Microbiol. Biotechnol. 61: 413- 423.

Shigenobu, S., H. Watanabe, M. Hattori, Y. Sakaki, and H. Ishikawa. 2000. Genome sequence of the endocellular bacterial symbiont of aphids Buchnera sp. APS. Nature (Lond.) 407: 81- 86.

Six, D. L., and K. D. Klepzig. 2004. Dendroctonus bark beetles as model systems for the study of symbioses. Symbiosis 37: 207-232.

Southern, E. M. 2001. DNA arrays, pp. 1-15. In J. S. Rampal (ed.), DNA arrays: methods and protocols. Humana Press, Totowa, NJ. Staley, J. T. 2006. The bacterial species dilemma and the genomic-phylogenetic species concept. Philo. Trans. R. Soc. B Biol. Sci. 361: 1899 -1909.

Stanton, M. L. 2003. Interacting guilds: moving beyond the pairwise perspective on mutualisms. Am. Nat. 162: S10 - S23.

Strauss, S. Y., and R. E. Irwin. 2004. Ecological and evolutionary consequences of multispecies plant-animal interactions. Annu. Rev. Ecol. Evol. Syst. 35: 435- 466.

Taerum, S. J.,M. J. Cafaro, A. E. Little, T. R. Schultz, and C. R. Currie. 2007. Low host-pathogen specificity in the leaf-cutting ant-microbe symbiosis. Proc. Biol. Sci. 274: 1971-1978.

Thomas, A. M. 2008. Pest and disease challenges and insect biotechnology solutions. Entomol. Res. 38: 34 - 40.

Tomitani, A., K. Okada, H. Miyashita, H. C. Matthijs, T. Ohno, and A. Tanaka. 1999. Chlorophyll b and phycobilins in the common ancestor of cyanobacteria and chloroplasts. Nature (Lond.) 400: 159 -162.

Tsutsui, N. D., A. V. Suarez, J. C. Spagna, and J. S. Johnston. 2008. The evolution of genome size in ants. BMC Evol. Biol. 8: 64.

Vavre, F., F. Fleury, D. Lepetit, P. Fouillet, and M. Bouletreau. 1999. Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. Mol. Biol. Evol. 16: 1711-1723.

Vega, F. E., and M. Blackwell. 2005. Insect-fungal associations: ecology and evolution. Oxford University Press, New York.

Viana, A.M.M., A. Frézard, C. Malosse, T.M.C. Della Lucia, C. Errard, and A. Lenoir. 2001. Colonial recognition of fungus in the fungus-growing ant *Acromyrmex* subterraneus subterraneus (Hymenoptera: Formicidae). Chemoecology 11: 29 -36.

Wang, J., S. Jemielity, P. Uva, Y. Wurm, J. Graff, and L. Keller. 2007. An annotated cDNA library and microarray for large-scale gene-expression studies in the ant Solenopsis invicta. Genome Biol. 8: R9.

Warnecke, F., P. Luginbuhl, N. Ivanova, M. Ghassemian, T. H. Richardson, J. T. Stege, M. Cayouette, A. C. McHardy, G. Djordjevic, N. Aboushadi, R. Sorek, S. G. Tringe, M. Podar, H. G. Martin, V. Kunin, D. Dalevi, J. Madejska, E. Kirton, D. Platt, E. Szeto, A. Salamov, K. Barry, N. Mikhailova, N. C. Kyrpides, E. G. Matson, E. A. Ottesen, X. Zhang, M. Hernandez, C. Murillo, L. G. Acosta, I. Rigoutsos, G. Tamayo, B. D. Green, C. Chang, E. M. Rubin, E. J. Mathur, D. E. Robertson, P. Hugenholtz, and J. R. Leadbetter. 2007. Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. Nature (Lond.) 450: 560 -565.

Weber, N. A. 1966. Fungus-growing ants. Science 153: 587- 604.

Weber, N. A. 1972. The fungus-culturing behavior of ants. Am. Zool. 12: 577-587.

Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. Nat. Rev. Genet. 3: 850 - 861.

Werren, J. H. ,W. Zhang, and L. R. Guo. 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. Biol. Sci. 261: 55- 63.

Wetterer, J. K. 1999. The ecology and evolution of worker size distribution in leaf cutting ants (Hymenoptera: Formicidae). Sociobiology 34: 119 -144.

Wheeler, W. M. 1910. Ants: their structure, development and behavior. Columbia University Press, New York.

Whitaker, R. J., and J. F. Banfield. 2006. Population genomics in natural microbial communities. Trends Ecol. Evol. 21: 508 -516.

Wilson, A. C., H. E. Dunbar, G. K. Davis, W. B. Hunter, D. L. Stern, and N. A. Moran. 2006. A dual-genome microarray for the pea aphid, Acyrthosiphon pisum, and its obligate bacterial symbiont, Buchnera aphidicola. BMC Genomics 7: 50.

Wilson, E. O. 1980a. Caste and division of labor in leaf-cutter ants (Hymenoptera: Formicidae: *Atta*). I. The overall pattern in *Atta sexdens*. Behav. Ecol. Sociobiol. 7: 143-156.

Wilson, E. O. 1980b. Caste and division of labor in leafcutter ants (Hymenoptera: Formicidae: *Atta*). II. The ergonomic optimization of leaf cutting *Atta sexdens*. Behav. Ecol. Sociobiol. 7: 157-165.

Wilson, E. O. 1992. The diversity of life. W.W. Norton & Company, New York.

Xi, Z., L. Gavotte, Y. Xie, and S. L. Dobson. 2008. Genome-wide analysis of the interaction between the endosymbiotic bacterium *Wolbachia* and its Drosophila host. BMC Genomics 9: 1.

Zhang, M. M., M. Poulsen, and C. R. Currie. 2007. Symbiont recognition of mutualistic bacteria by *Acromyrmex* leafcutting ants. ISME J. 1: 313-320.

2 Chapter 2: Labile association between fungus-growing ant cultivars and their garden pathogens.

This chapter was previously published in The International Society for Microbial Ecology, ISME Journal. 2007, 1:373-384.

Nicole M. Gerardo and Eric J. Caldera

Author contributions: NMG & collaborators conducted the field work. NMG performed bioassay experiments and genotyping. NMG and EJC analyzed the population genetic data and wrote the paper.

2.1 Abstract

The distribution of genetic and phenotypic variation in both host and parasites over their geographic range shapes coevolutionary dynamics. Specifically, concordant host and parasite distribution facilitates localized adaptation and further specialization of parasite genotypes on particular host genotypes. We here compare genetic population structure of the cultivated fungi of the fungus-growing ant *Apterostigma dentigerum* and of the cultivar-attacking fungus, *Escovopsis,* to determine whether these microbial associations have evolved or are likely to evolve genotype-genotype specialization. Analyses based on amplified fragment length polymorphism (AFLP) genotyping of host cultivars and pathogenic *Escovopsis* from 77 *A. dentigerum* colonies reveal that populations of hosts and pathogens are not similarly diverged and that host and pathogen genetic distances are uncorrelated, indicating that genetically similar parasites are not infecting genetically similar hosts. Microbial bioassays between pathogens and cultivars of different genotypes and from different populations show little pairwise specificity; most *Escovopsis* strains tested can successfully infect all cultivar strains with which they are paired. These molecular and experimental data suggest that *Escovopsis* genotypes are not tightly tracking cultivar genotypes within the *A. dentigerum* system. The diffuse nature of this host–pathogen association has implications for evolutionary and ecological disease dynamics of the fungus-growing ant–microbe symbiosis.

2.2 Introduction

Parasites specialize on hosts at numerous ecological scales. At the broad level, most parasites attack only particular taxonomic host groups (e.g. birds, annual plants, bacteria); at the finest level, some pathogen genotypes are specialized at attacking only a narrow range of host genotypes (Carius et al, 2001; Schulenburg and Ewbank, 2004). Specialization is a consequence of reciprocal adaptation by sympatric hosts and parasites. As predicted by the Red Queen hypothesis, this can lead to increased ability of parasites to infect and utilize local hosts, i.e. local adaptation (Parker, 1985; Lively, 1989; Gandon et al, 1996; Kaltz and Shykoff, 1998; Lively and Dybdahl, 2000; Dybdahl and Storfer, 2003).

Whether specialization arises is contingent on a variety of complex ecological and evolutionary factors. In particular, gene flow across host and parasite metapopulations is expected to play an important role in the evolution of localized adaptation within populations (Price, 1980; Kaltz and Shykoff, 1998; Lively, 1999; Thompson, 1999; Gandon and Michalakis, 2002; Thompson and Cunningham, 2002; Criscione et al., 2005). In general, host and parasite migration among populations can provide novel genetic variation that natural selection can subsequently act on, creating the potential for local adaptation, but it can also counteract adaptation by introducing locally maladapted alleles (Slatkin, 1985). More specifically, theory predicts that it is the relative gene flow of hosts and parasites that is important in defining local adaptive processes. First, when both hosts and their parasite have low gene flow, and particularly when their patterns of gene flow are similar, local parasite adaptation is likely to arise (Price, 1980; Kirkpatrick and Barton, 1997; Gandon, 1998). When parasites migrate more freely than their hosts (that is,

parasite populations are less genetically structured than host populations), parasites are also expected to be locally adapted to hosts (Kaltz and Shykoff, 1998), but when hosts experience higher gene flow than parasites, however, parasites may be better suited to attack allopatric hosts over sympatric ones, thus appearing locally maladapted (Gandon et al., 1996).

Therefore, given that gene flow plays a critical role in coevolutionary dynamics, to understand the evolution of host–parasite interactions, it is important to have information on the relative population structure of the two players within a system. To date, only a handful of studies have characterized the population genetic structure of natural host and parasite metapopulations across their geographic range (Mulvey et al., 1991; Dybdahl and Lively, 1996; Davies et al., 1999; Delmotte et al., 1999; Martinez et al., 1999; Jobet et al., 2000; Sire et al., 2001; Jerome and Ford, 2002; Johannesen and Seitz, 2003), a very small fraction of which involve microbial pathogens (Delmotte et al., 1999). Results of these studies have been varied. Jobet et al (2000), for example, found similar genetic differentiation between populations of the urban cockroach (*Blatella germanica*) and its nematode parasite (*Blatticola blattae*). Many other studies, however, have found asymmetrical patterns of genetic structuring in hosts and parasites. Delmotte et al (1999), for instance, found that populations of the fungal pathogen *Microbotryum violaceum* were much more strongly differentiated than were populations of its host plant, *Silene latifolia*, while Dybdahl and Lively (1996) found that populations of trematode parasites (*Microphallus* sp.) were much less differentiated than those of their host snails (*Potamopyrgus antipodarum*).

The fungus-growing ant–microbe symbiosis provides a unique opportunity to study microbial host– pathogen population structure, parasite specificity and disease dynamics. In this insect agricultural system, more than 200 ant species in the Tribe Attini are known to cultivate fungus as their primary food source. When new colonies are formed, founding queens take a piece of fungus from their mother's colony to start new colonies. Each colony cultivates a single fungal strain (Poulsen and Boomsma, 2005; Gerardo, personal observation; Scott et al., manuscript in preparation), either in the family Lepiotaceae or Pterulaceae (Basidiomycota, Agaricales) (Chapela et al., 1994; Munkacsi et al., 2004). These cultivars are attacked by *Escovopsis*, a genus of microfungi (Ascomycota, Hypocreales) thus far only found in association with fungus-growing ant colonies (Currie et al., 1999; Reynolds and Currie, 2004). Though only two species of *Escovopsis*, *E. weberi* and *E. aspergilliodes*, are currently formally described (Seifert et al., 1995), morphological and genetic diversity of *Escovopsis* strains suggests that the genus is comprised of many species (Gerardo et al., 2006a). Analyses at the broad, interspecific level indicate that the diverse *Escovopsis* types, as defined by morphology and genetics, are specialized: particular *Escovopsis* clades only attack specific clades of fungal cultivars (Currie et al., 2003; Gerardo et al., 2004, 2006a). This specificity is not maintained by transmission along with the host cultivar, as infection patterns suggest that *Escovopsis spp*. are horizontally transmitted via a yet unknown mechanism (Currie et al., 1999; Currie, 2001; Gerardo et al., 2004).

The outcome of cultivar–*Escovopsis* encounters, as well as other host–parasite interactions, is ultimately predicated upon adaptations on part of the host to resist parasite establishment and adaptations on part of the parasite to overcome host defenses and

establish infection (Combes, 2004). In many systems, adaptive mechanisms on the part of the host and parasite are difficult to elucidate. In the fungus-growing ant–microbe symbiosis, however, recent studies of the microbes associated with the ant genus *Apterostigma* suggest mechanisms by which host cultivars can prevent some infections and mechanisms by which pathogenic *Escovopsis* can efficiently infect some hosts. Experimental bioassays interacting *Escovopsis* with cultivars raised by a range ant species indicate that these pathogens are attracted to chemical signals produced by their host cultivars and by closely-related fungi (Gerardo et al., 2006b). This attraction to cultivar signals may increase the efficiency by which *Escovopsis* establishes infection. Conversely, the same *Escovopsis* strains are not attracted to, but are inhibited by, distantly-related cultivars raised by other fungus-growing ants. These phenotypes, *Escovopsis* host-seeking and cultivar resistance, may limit this parasite to successfully infecting only the cultivars raised by a subset of fungus-growing ant species (Gerardo et al., 2006b). It is unknown whether these adaptive mechanisms could function to maintain tight intraspecific specificity of *Escovopsis* strains on strains of its natural host.

To date, we know little about intraspecific host–pathogen population dynamics in the fungus-growing ant symbiosis, as the few fine-scale genetic analyses of fungus-growing ant-associated microbes have focused only on the host cultivars in a single geographical region (in and around the Panama Canal zone) (Bot et al., 2001; Green et al., 2002; Poulsen et al., 2005; Mikheyev et al., 2007), rather than on both host and parasites across populations. Here, we use amplified fragment length polymorphism (AFLP) genotyping to characterize the population genetic structure of cultivars and *Escovopsis* pathogens isolated in tandem from the colonies of *Apterostigma dentigerum* over a large

geographic scale spanning Central and South America. We use these population genetic data to compare patterns of host and parasite genetic structure. We also examine the distribution of host and parasite genotypes to look for signatures of fine-scale parasite specialization. These molecular analyses are coupled with experimental bioassays pairing genetically diverse parasite and cultivar strains in an attempt to identify signatures of local adaptation and mechanisms controlling parasite infectivity. By assessing both patterns of host and parasite distribution over large geographic scales and specificity of host–parasites interactions at finer scales, we gain insight into the evolutionary and ecological dynamics of the *Apterostigma* ant–microbe symbiosis.

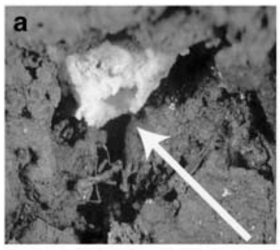
2.3 Materials and Methods

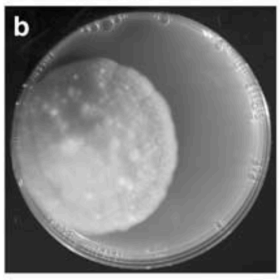
2.3.1 Study System and Collecting.

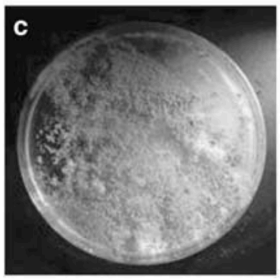
Colonies of *A. dentigerum* are common along stream banks and under logs throughout much of Central and South America (Gerardo pers. obs.). Unlike subterranean ant colonies, *A. dentigerum* colonies can be easily detected and identified because of a conspicuous white veil of fungus that protects their internal fungal garden (Villesen et al., 2004; Fig. 1a), facilitating collection of both garden material and ants from many colonies across populations. All *A. dentigerum* ants raise cultivars in the family Pterulaceae (Basidiomycota, Agaricales).

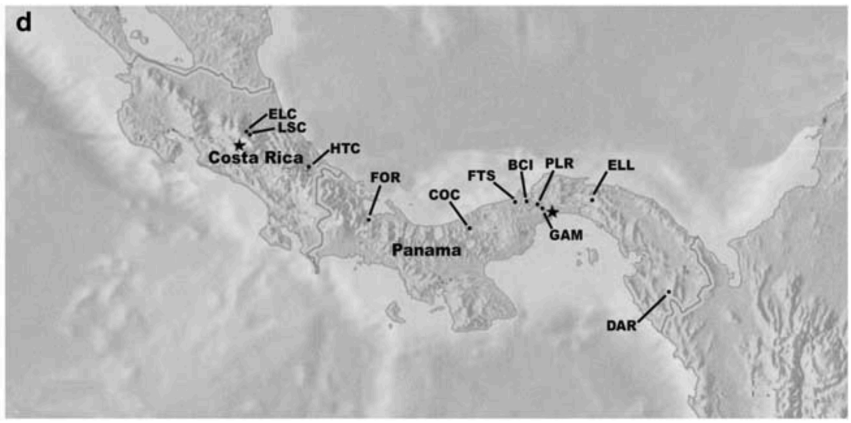
Based on DNA sequence analysis of *Apterostigma* cultivars, all *A. dentigerum* appear to grow genetically similar cultivar strains of the G2 clade (Villesen et al, 2004). Within the context of a larger set of *Apterostigma* cultivar sequences, *A. dentigerum* cultivars are monophyletic at two genes (25S rDNA and COI) but not at ITS internal

transcription spacer, a hypervariable marker. Although species boundaries have not been subscribed to fungal cultivars, the G2, G3, and G4 groupings are considered well supported monophyletic clades, and genetic differences within these clades are referred to as "cultivar strains" or "cultivar types" (Villesen et al, 2004). These host cultivars are attacked frequently by three genetically and morphologically distinct parasite types: brown-spored, yellow-spored and white-spored *Escovopsis* (Gerardo et al, 2006a). All three *Escovopsis* types form monophyletic clades with little sequence divergence and are specific to attacking *Apterostigma* spp. cultivars (Gerardo et al, 2006a). Based on the morphological and sequence similarity within each clade, we refer to each *Escovopsis* type as a "species".









Country	Locality	# Colonies
Costa Rica	El Ceibo Biological Station (ELC)	11
	La Selva Biological Station (LSC)	9
	Hitoy Cerere Biological Reserve (HTC)	7
Panama	approximately 25km north of Fortuna Biological Station (FOR)	5
	near Coclecito (COC)	3
	Fort Sherman (FTS)	8
	Barro Colorado Island (BCI)	10
	Pipeline Road (PLR)	12
	Gamboa (GAM)	7
	El Llano - Cartí Suitupo Road (ELL)	1
	Rancho Frío in Darien Province (DAR)	2
Ecuador	La Selva Lodge and Biological Station (not shown)	1
Argentina	Parque Pical (not shown)	1

Figure 1. Sampling of *Apterostigma dentigerum* colonies. (a) *A. dentigerum* colonies are easily located in the field because of the conspicuous, white fungal veil (see arrow) that protects their garden. After collection for this study, gardens were sampled to obtain pure isolates of the ant's cultivated fungi (b) and the parasitic fungus *Escovopsis* (c). (d) Collecting sites and sample sizes. Cultivar and *Escovopsis* were collected from 77 colonies throughout Costa Rica, Panama, Ecuador and Argentina (the latter two are not shown). Stars mark the country capitals.

Garden material from 472 *A. dentigerum* colonies was collected in 2001–2003 in Central and South America at thirteen localities (Fig. 1). At least ten garden pieces (~8mm^3) from each colony were grown on potato dextrose agar (PDA; Difco, Detroit, MI) with bacteria-inhibiting antibiotics (50mg/L each of penicillin and streptomycin), and pieces identified as either cultivar or as *Escovopsis* were subsequently subcultured to obtain axenic (pure) cultures before storage at -80^{O}C.

From 77 of the 472 colonies collected, axenic cultures of both cultivar (Fig. 1b) and brown-morphotype *Escovopsis* (Fig. 1c) were isolated and stored successfully. Only samples from these colonies were included here, because use of host–parasite pairs isolated in tandem (i.e. cultivar and *Escovopsis* from the same colony) assured that both hosts and pathogens were sampled similarly across space and time. Sample sizes for each population are listed in Fig. 1. *Escovopsis* isolates of the yellow-spored and white-spored morphotypes were not included in the study, because Ef-1 alpha sequences indicate that they are genetically distinct species from the brown-morphotype *Escovopsis*, and, furthermore, these pathogens are less common than the brown-morphotype, limiting sample size (Gerardo et al., 2006a).

2.3.2 AFLP Genotyping.

To examine the distribution of host and pathogen genotypes both across populations and relative to one another, we used amplified fragment length polymorphisms, AFLPs, to profile cultivars and *Escovopsis* isolated from the same 77 colonies. Preceding amplification, DNA from a single cultivar and a single *Escovopsis* isolate from each colony was extracted according a CTAB protocol modified from

Bender et al (1983). Cultivar and parasite isolates from two randomly selected colonies were extracted twice and genotyped separately to detect the amount of noise (unreliable marker differences) generated during the amplification and scoring process. These duplicates were not included in graphical or statistical analyses. For all samples, reactions followed the AFLP protocol for small plant genomes (www.appliedbiosystems.com; protocol 4303146) with the following modifications found to increase reliability of AFLP reactions: 1) prior to pre-selective amplification, 120µl of $TE_{0.1}$ was added to the restriction-ligation product instead of the recommended 189µl; 2) the pre-selective product was not diluted for selective amplification; 3) for both preselective and selective amplifications, 25 thermocycler cycles were used instead of the recommended 20 cycles; 4) Three microliters of each selective product was mixed with 0.4µl of GeneScan 500 Rox and 6.6µl of Hi-Di Formamide prior to generation of AFLP markers on an ABI Prism 3100 Genetic Analyzer. For selective amplifications, six combinations of AFLP-primer extensions were chosen because they generated high levels of polymorphic markers that could be scored reliably: AC/CAA, AC/CTG, AC/CTC, TG/CAG, TG/CTC and TC/CAG. Markers were scored blindly in Genotyper 2.5 by simultaneously comparing all fragments of a given length across all 77 *Escovopsis* isolates and, in a separate analysis, across all 77 cultivar isolates. Only markers that could be scored as unambiguously present/absent across all pathogen or host samples were used in analyses. At all stages, all cultivar and *Escovopsis* samples were run at the same time in 96-well plates and using the same reagents to minimize experimental artifacts.

2.3.3 Host and Parasite Population Structures.

For analysis of population structure, we performed parallel analyses of the cultivar (host) and *Escovopsis* (parasite) datasets and then compared results between the two. We conducted two Analyses of Molecular Variance (AMOVA) in Arlequin (Schneider et al, 2000) to partition genetic variation both among host and among parasite isolates within and between localities. The AMOVA module in Arlequin generates Φ statistics, equivalent to Weir and Cockerham's (1984) θ, which is a molecular analog to Fisher's F_{st} (Excoffier, 2001). Population pairwise Φ_{st} values were also generated to determine the proportion of differences between hosts, and separately between parasites, associated with each locality. Levels of significance were determined through 100 000 random permutation replicates. Additional analogs of overall F_{st} (Θ^B and $G_{st}{}^B$, an estimate of Nei's $G_{st,}$ (Nei, 1977) for di-allelic loci) were calculated using Bayesian methods implemented in the program Hickory v1.0.4 (Holsinger et al, 2002). We ran all possible models available in Hickory, with default parameters recommended by the authors (Holsinger et al, 2002), but there were no substantial differences, so we report values obtained using the full model. For all population analyses, we excluded three localities at which only a single sample was collected (LSE, ARG, ELL), because no within-locality variation could be determined.

For hosts, and separately for parasites, we conducted Mantel tests in ZT (Bonnet and Van de Peer, 2002) to determine correspondence between each pairwise Φ_{st} (genetic distance) matrix and a pairwise geographical distance matrix. A significant, positive correlation would indicate effects of isolation by distance. All Mantel tests mentioned hereafter were also conducted using ZT and were performed with 10 000 permutations.

Pairwise, linear geographical distances between localities were calculated using the program Range (Luetgert). We also plotted the relationship between pairwise Φ_{st} and geographical distance for all pairs of host populations and all pairs of parasite populations.

To determine whether host and parasite populations exhibited a similar spatial pattern of divergence, we used ARLEQUIN to construct two matrices: 1) the Nei's corrected average pairwise cultivar population differences; and 2) the Nei's corrected average pairwise *Escovopsis* population differences (Nei and Li, 1979). We then used a Mantel test to assess correspondence between the matrix of cultivar pairwise Φ_{st} values and parasite pairwise Φ_{st} values. A significant correlation would indicate that parasite populations show similar relative divergence to the host populations that they are attacking. We also visually compared cultivar population structure against parasite population structure using Structure v2.1 (Pritchard et al, 2000). Structure uses multilocus genotypic data to evaluate models of different numbers of clusters (K), with each individual probabilistically assigned to a reconstructed genetic cluster based on its multilocus genotype and the allele frequencies estimated for each cluster. Each genotype was treated as effectively haploid, where each locus contained one of two alleles determined by the presence or absence of a band. We applied a model of no-admixture and allele frequencies were assumed to be correlated within populations. Simulations were run from $K = 1$ to $K = 9$ with each K iterated 5 times, and we did not include any priors regarding sample location. To select the appropriate K, we calculated a ΔK statistic according to Evanno et al (2005). Structure was also used to calculate F_k values, interpreted as F_{st} analogs between each population and a single hypothetical common ancestral population (Pritchard et al, 2000; Falush et al, 2003). F_k values were obtained

by running the no-admixture model with K = 10, utilizing locality as the population origin. All Structure simulations began with a burn-in of 100 000 and a runtime of 300 000.

2.3.4 Association of host and parasite genotypes.

Correlation between host and parasite population pairwise differences would indicate that more genetically similar populations of hosts and more genetically similar populations of parasites are associated, but this would not reveal parasite specialization at finer levels (i.e. whether each parasite genotype within a population is attacking a narrow range of host genotypes within a population). To determine whether genetically similar parasites are attacking genetically similar hosts, we first visualized the relationships between the 77 cultivar isolates using mean character distances (i.e. the sum of marker differences between two samples / total no. of markers), generated in PAUP* (Swofford, 2002), to construct a non-metric multidimensional scaling (NMDS) plot using NCSS (ver. 2000, Hintze 2001). NMDS is an ordination technique that detects nonhierarchical structure by reducing the multidimensional relationship between entities to a smaller number of dimensions. A similar plot was created for the pathogen isolates. Mean character distances were used for these and all subsequent analyses, because though Nei-Li restriction distances (Nei and Li, 1979) are often selected for AFLP data analysis, many *Escovopsis* pair distances were undefined using this method. The Nei-Li and mean character distances for the cultivars were highly correlated (Mantel test, $r = 0.78$, $p < 0.0001$), and the results of no analysis were changed if the cultivar Nei-Li distances were used in place of the mean character differences. To visually verify the clustering produced through NMDS, we also used PAUP* to construct UPGMA dendrograms.

In addition to visual inspection, we conducted two separate statistical analyses. First, we used a Mantel test to assess correlation between the host and parasite mean character difference matrices. Significance would indicate that more genetically similar parasite isolates (those with smaller mean character differences) attack more genetically similar host isolates, both between and within populations. Second, in both the NMDS plot and the UPGMA dendrogram, cultivar isolates fell into the same six visually distinct clusters. To verify the genetic distinctiveness of these clusters, we assigned each cultivar isolate to a cluster (cluster 1–6) and then used AMOVA to determine the proportion of the genetic variation among cultivar isolates. Pairwise comparisons among clusters were also conducted. Then, to determine whether the host-cluster with which an *Escovopsis* isolate is associated could explain genetic variation among parasite isolates, we assigned each *Escovopsis* isolate to the cluster in which its host belonged and used these groups as a basis for AMOVA. A significant overall Φ_{st} would indicate the proportion of *Escovopsis* variation attributable to their association with genotypically distinct host clusters. We also conducted pairwise comparisons to determine which parasite groups, as defined based on host genotype cluster, were genetically differentiable. Significant pairwise differences would indicate cases in which genotypically differentiable parasite groups are attacking genotypically differentiable hosts.

2.3.5 Experimental Test for Specificity

We conducted microbial bioassays, following procedures in Gerardo et al (2006b), to see if there is variation in the outcome of interactions between a set of genetically and geographically diverse *A. dentigerum*-associated *Escovopsis* strains and a set of equally diverse *A. dentigerum*-associated cultivar strains. For each bioassay, we

placed a single isolate of cultivar near the edge of a 9 cm Petri dish with PDA + antibiotics. After one week, we inoculated the center of each plate with a single *Escovopsis* isolate. Each of nine cultivar isolates was interacted with each of the same nine *Escovopsis* isolates for a total of 81 bioassays. Experimental strains were chosen to maximize geographic and genetic diversity. Bioassays were monitored for up to two months. Interactions were scored for presence/absence of inhibition by the cultivar on *Escovopsis* growth and presence/absence of attraction by *Escovopsis* to the cultivar (Fig. 5).

To determine whether the variation that we observed in interactions between *Escovopsis* and cultivar isolates was associated with either genetic differences or geographic distances, we conducted a series of Mantel tests looking for the correlation between various distance matrices. Correlations with geographic distances would be consistent with local adaptation, while correlations with genetic distance would indicate specialization on particular genotypes. Four interaction distance matrices were constructed. The first matrix consisted of the inhibition distances between each pair of the nine cultivar strains, where each inhibition distance ranged from 0 to 1 and increased 0.11 for each case in which the two cultivar strains had a different inhibition result with the same *Escovopsis* strain (i.e., one cultivar inhibited the *Escovopsis* strain while the other did not). A second matrix consisted of *Escovopsis* inhibition distances; each inhibition distance ranged from 0 to 1 and increased 0.11 for each case in which the two *Escovopsis* strains had a different inhibition result with the same cultivar strain (i.e. one *Escovopsis* strain was inhibited while the other was not). The third and fourth matrices were

comprised of cultivar and *Escovopsis* attraction distances, which were determined similarly to the inhibition distances.

With the AFLP data, we constructed two genetic distances matrices using PAUP*: a mean character difference distance matrix for the nine experimental cultivar strains and a mean character difference distance matrix for the nine experimental *Escovopsis* strains. We also constructed two geographic distance matrices: a matrix of distances between collection sites of the cultivar strains and a matrix of distances between collection sites of the *Escovopsis* strains. We then used ZT to conduct Mantel tests to examine the correlation between matrices of: 1) cultivar inhibition distances and cultivar genetic distances, 2) cultivar inhibition distances and cultivar geographic distances, 3) cultivar attraction distances and cultivar genetic distances, 4) cultivar attraction distances and cultivar geographic distances, 5) *Escovopsis* inhibition distances and *Escovopsis* genetic distances, 6) *Escovopsis* inhibition distances and *Escovopsis* geographic distances, 7) *Escovopsis* attraction distances and *Escovopsis* genetic distances, and 8) *Escovopsis* attraction distances and *Escovopsis* geographic distances To account for multiple tests, we applied a Bonferroni correction on all mantel tests.

Table 1. AMOVA Φ_{st} values and pairwise comparisons for host and parasite localities.

AMOVA results	**Cultivar (host)** *(a)*			***Escovopsis* (parasite)** *(b)*		
Population differences	variance	d.f.	% total	variance	d.f.	% total
Between populations	7.3	9	11.1	4.8	9	7.4
Within populations	58.9	64	88.9	60.5	64	92.6
	overall Φ_{st} = 0.11, p < 0.01			**overall Φ_{st} = 0.07, p < 0.01**		

between-population pairwise Φ_{st} values
Cultivar (below diagonal) & *Escovopsis* (above diagonal)

	ELC	LSC	HIT	FOR	COC	FTS	BCI	PLR	GAM	DAR
ELC		0.01	0.06	0.04	0.05	**0.06**	**0.28**	**0.13**	0.06	0.06
LSC	**0.13**		0.03	0	0	0.01	**0.2**	0.05	0.01	0.01
HIT	**0.32**	0.02		0.01	0.04	0.05	**0.31**	**0.11**	0.06	**0.23**
FOR	**0.26**	0.002	0		0	0	**0.24**	0	0	**0.30**
COC	0.11	0.09	**0.24**	0.16		0.06	0.12	0.04	0.14	0.01
FTS	0.06	0	0.12	0.10	0		0.02	0	0	0.02
BCI	**0.10**	**0.17**	**0.27**	**0.15**	0.07	**0.13**		0.03	**0.11**	**0.30**
PLR	**0.09**	**0.08**	**0.15**	**0.14**	0	0	0		0.01	0
GAM	**0.12**	**0.21**	**0.32**	**0.29**	0	0.06	0	0		0.01
DAR	0.02	0.17	**0.36**	0.15	0.03	0.10	0.02	0.15	0.08	

Overall Φ_{st} values indicate the proportion of variation attributable to host *(a)* and parasite *(b)* genotype differences between populations. Pairwise comparisons are between populations, with pairwise Φ_{st} values for cultivar below and for parasite above the diagonal. All p-values were derived by permuting genotypes among samples (100 000 permutations). Signficant pairwise Φ_{st} values ($p < 0.05$) are in bold.

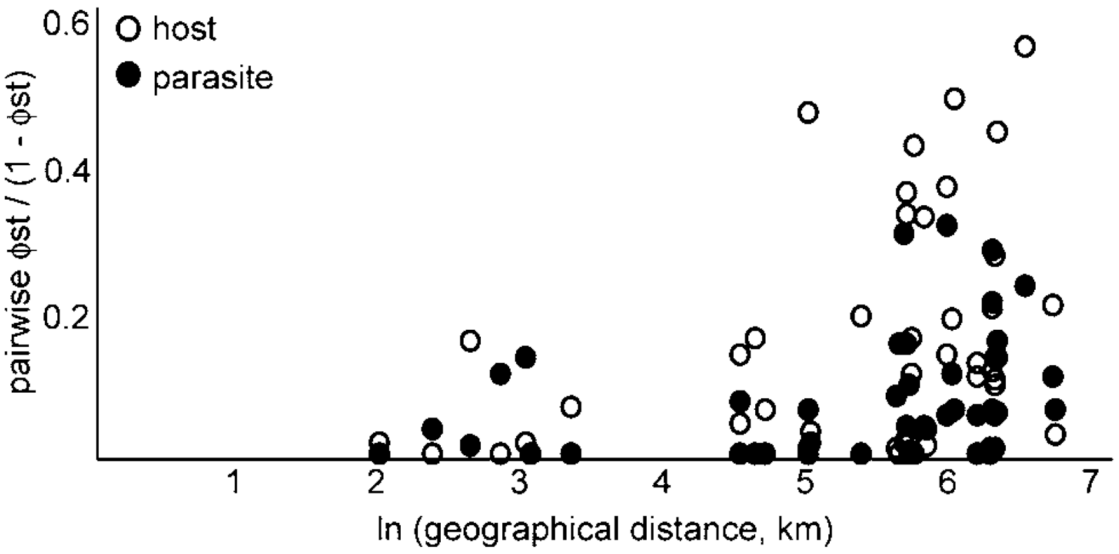


Figure 2. Isolation by distance. Plot of pairwise Φst / (1 – Φst) against pairwise geographical distance between each of 10 populations of hosts (open circles) and parasites (filled circles). The relationship between genetic and spatial distances was assessed using Mantel tests and is significant for both cultivars (r = 0.34, p = 0.04) and *Escovopsis* (r = 0.38, p = 0.03).

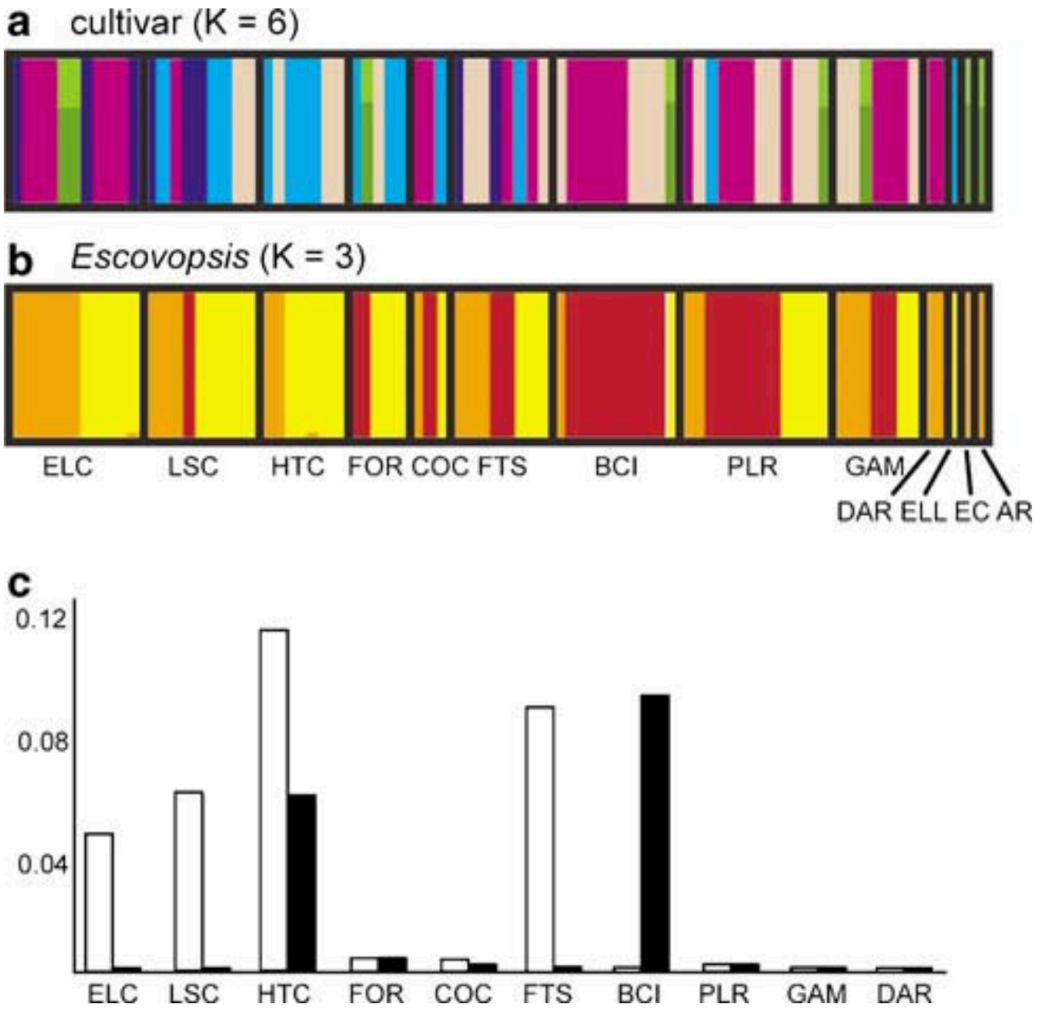


Figure 3. Host and parasite population differentiation. *(a)* Genetic clusters for cultivars (K = 6) and *(b)* for *Escovopsis* (K = 3), determined using Bayesian methods in Structure and a ΔK statistic of Evanno et al. 2005. Each vertical line represents a single individual and each color corresponds to the genetic cluster to which an individual is probabilistically assigned. *(c)* F_K values (an F_{st} analog between each population and a hypothetical common ancestor of all populations) for cultivars (white) and *Escovopsis* (black). Populations are arranged in order from northern Costa Rica to eastern Panama.

2.4 Results

2.4.1 AFLP Diversity.

For the host cultivars, a total of 804 AFLP markers were identified using the six primer systems; all are polymorphic and 208 (26%) are autapomorphic. For pathogenic *Escovopsis*, a total of 933 AFLP markers were identified; all are polymorphic and 334 (36%) are autapomorphic. Both cultivars and *Escovopsis* samples are diverse; mean character differences between cultivar isolates range from 0.02 to 0.29 (mean = 0.16, s.d. = 0.05), and mean character differences between *Escovopsis* isolates range from 0.04 to 0.22 (mean = 0.14, s.d. = 0.04). Of the four duplicated samples, the mean character difference between duplicates is low, ranging from 0.02 to 0.07 (mean = 0.04, s.d. = 0.02), and the difference between cultivar duplicates and between parasite duplicates is similar.

2.4.2 Host and Parasite Population Structures.

While both cultivar and *Escovopsis* populations exhibit little genetic structure along geographically determined boundaries, cultivar populations are relatively more structured than those of their parasite. Eleven percent of the variation among cultivar isolates is attributable to between population differences (AMOVA overall Φ_{st} = 0.11, Table 1a; Θ^{B} = 0.11, 95% C.I. 0.10 - 0.12), while six to seven percent of the variation among *Escovopsis* isolates is attributable to population differences (AMOVA overall Φ_{st} = 0.07, Table 1a; Θ^{B} = 0.06, 95% C.I. 0.10 - 0.12). Estimates of Gst^{B} are very similar for cultivar (Gst^{B} = 0.10, 95% C.I. 0.09 - 0.11) and *Escovopsis* (Gst^{B} = 0.05, 95% C.I. 0.04 - 0.06).

Cultivar and *Escovopsis* population structures are not significantly correlated to one another. A Mantel test of the correspondence between cultivar pairwise Φ_{st} values and pairwise geographic distances confirms the effect of geographic isolation by distance (r = 0.34, p = 0.04). *Escovopsis* exhibits similar correlation between population genetic and geographic distances (r = 0.38, p = 0.03). This positive relationship between population genetic and geographic distances is represented in Fig. 2. There is not significant correspondence between cultivar population pairwise Φ_{st} values and *Escovopsis* population pairwise Φ_{st} values (r = 0.22. p = 0.139). This absence of corresponding population structure between host and parasite can be visualized by comparing the genetic clusters of each symbiont (Fig. 3a & 3b). Consistent with the pattern of *Escovopsis* being slightly less structured than its host, as determined by the F_{st} analogs above, the genetic structure of *Escovopsis* conforms best to a model of $K = 3$ genetic clusters, while the cultivar host is best explained by $K = 6$. Though discrepancy in the values of K between the cultivar and *Escovopsis* does not necessarily translate to incongruent population genetic structure, as it is possible for finer scale cultivar genetic structure to be nested within the three clusters of *Escovopsis*, we did not observe such a clustering pattern. For the cultivar, F_K values (Fig. 3c) are greatest in the Costa Rican population HCE (F_K = 0.12), followed by FTS in Panama (F_K = 0.10), while the most divergent *Escovopsis* population occurs in BCI (F_K = 0.10), the only region with a substantial F_K for the pathogen. This implies that the most divergent host populations sampled do not correspond to the most divergent parasite population sampled, but it is important to note that these values, in general, are relatively low.

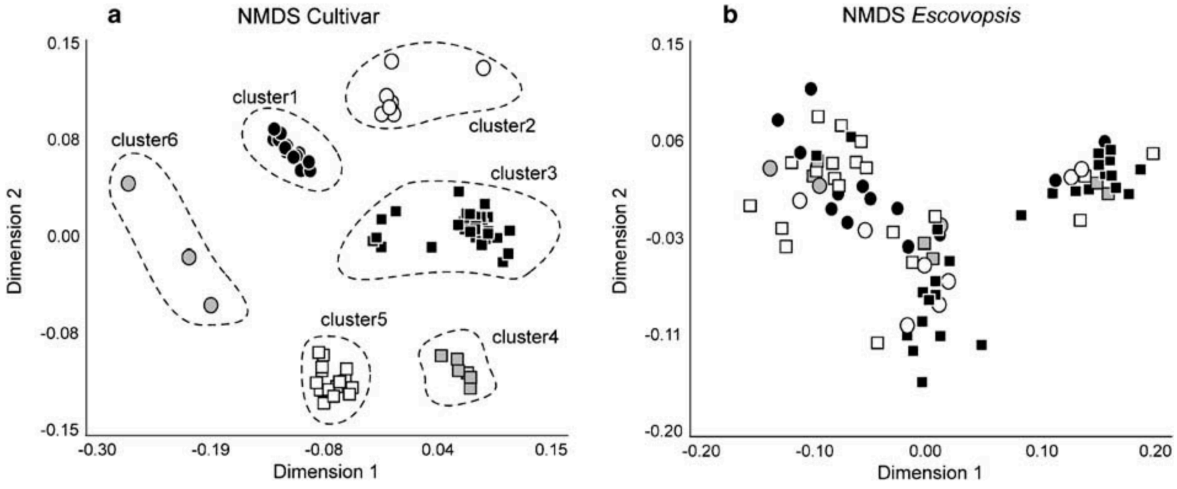


Figure 4. Clustering of all host and parasite isolates. *(a)* NMDS solution for all cultivar isolates. Dashed lines demarcate six main host genotype clusters. Pairwise Φst comparisons indicate that all clusters are genetically differentiable (Table 2). *(b)* NMDS solution for all *Escovopsis* isolates. Isolates are coded by the cluster (1 - 6) of their associated host.

Table 2. AMOVA Φ_{st} values and pairwise comparisons for host genotype clusters and their associated parasites.

AMOVA results **Host Clusters**	**Cultivar (host)** *(a)* variance	d.f.	% total	***Escovopsis* (parasite)** *(b)* variance	d.f.	% total
Between host clusters	40.4	5	54.1	1.64	5	2.53
Within host clusters	34.3	71	45.9	63.13	71	97.47
	overall Φ_{st} = 0.54, p < 0.001			**overall Φ_{st} = 0.025, p = 0.048**		

between-host cluster pairwise Φ_{st} values
Cultivar (below diagonal) & *Escovopsis* (above diagonal)

	cluster1	cluster 2	cluster 3	cluster 4	cluster 5	cluster 6
cluster1		0.04	**0.08**	0	0	0
cluster2	**0.66**		0.02	0	0.01	0.06
cluster3	**0.54**	**0.46**		0	0.03	0.08
cluster4	**0.77**	**0.71**	**0.42**		0	0
cluster5	**0.61**	**0.59**	**0.50**	**0.52**		0
cluster6	**0.60**	**0.49**	**0.43**	**0.61**	**0.49**	

Overall Φ_{st} values indicate the proportion of cultivar genotypic variation that is captured by a posteriori assignment of each host to a genotype cluster *(a)* and the proportion of *Escovopsis* genotypic variation that is captured by assignment of parasites to their respective host clusters *(b)*. Pairwise comparisons below the diagonal are between each cultivar cluster, and pairwise comparisons above the diagonal are between groups of *Escovopsis* isolates assigned to their hosts' clusters. All p-values were calculated by permuting genotypes among samples (100 000 permutations). Significant pairwise Φ_{st} values ($p < 0.05$) are in bold.

2.4.3 Association of Host and Parasite Genotypes.

Cultivars exhibit substantial genetic structure, as seen through the clustering of isolates in both the NMDS plot (Fig. 4a) and UPGMA dendrogram (not shown) of all cultivar samples. Both clustering algorithms group cultivar isolates into six main clusters, and all isolates fall into the same cluster in both analyses. As can be seen in the Structure analysis (Fig. 3b), these clusters do not correspond to geographically isolated populations. For the NMDS plot, dimension one captures 29% of the cultivar variation and dimension two captures an additional 22%. Upon a posteriori assignment of each of the cultivars to one of the six genotypic clusters, the resulting clusters account for 54% of the variation among isolates (Table 2a, AMOVA, overall $\Phi_{st} = 0.54$), substantially more than when the cultivars are assigned to populations (Table 1a, AMOVA, overall $\Phi_{st} = 0.11$). All six genotype clusters are significantly genetically distinct from one another (Table 2, pairwise differences). As can be seen in the Structure analysis (Figure 3b), these clusters do not correspond to geographically isolated populations.

Escovopsis exhibits less genetic structure, with little clustering in the NMDS plot (Fig. 4b) or UPGMA dendrogram (not shown). For the NMDS plot, dimension one captures 37% of the isolate variation, and dimension two captures an additional 15%. Lesser parasite clustering in Fig. 4 as compared to host clustering suggests that there may be little concordance between host genetic similarity and parasite genetic similarity, and absence of tight parasite tracking of hosts genotypes is confirmed by a lack of significant correspondence between mean host differences and their respective mean parasite differences ($r = 0.04$, $p = 0.065$).

When *Escovopsis* isolates were assigned to groups based on the genotypic cluster with which their host cultivar was associated, this clustering explains a small, though significant portion of the variation among parasite isolates (Table 2b, overall $\Phi_{st} = 0.025$). Pairwise comparisons between groups of *Escovopsis* isolates that attack the different host genotype clusters indicate only one significant difference: parasites attacking cultivars in cluster one are only slightly, though significantly, genotypically distinct from parasites attacking cultivars in cluster three (Table 2, pairwise differences).

2.4.4 Experimental Test for Specificity.

In the vast majority of bioassays, the *Escovopsis* isolate was attracted to the cultivar isolate, and the cultivar isolate was not able to inhibit the *Escovopsis* isolate (Fig. 5). Of the eight mantel tests looking for a correlation between an interaction distance matrix and either a genetic or geographic distance matrix, only one test had a p-value less than 0.1; there is a negative correlation between cultivar attraction distance and cultivar geographic distance (simple mantel test: $r = - 0.3$, $p = 0.04$), likely driven by the fact that one cultivar isolate from PLR inhibited an *Escovopsis* isolate not inhibited by the other two PLR cultivar strains. This weak correlation is not significant when a Bonferroni correction is applied.

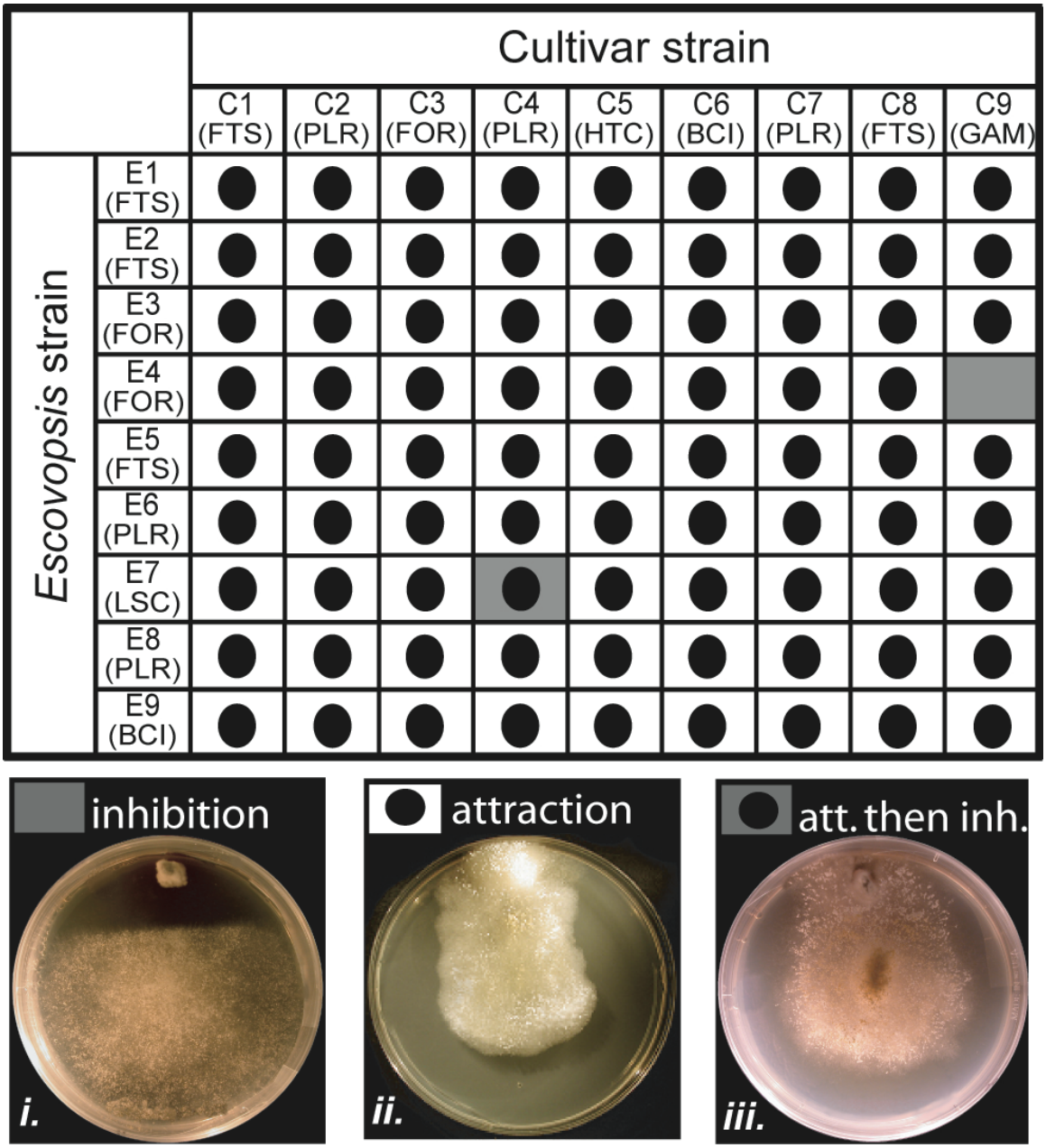


Figure 5. Microbial Bioassays. Each cell represents the outcome of the interaction between one cultivar and one *Escovopsis* isolate. Gray indicates inhibition (*i.*), a dot indicates attraction and subsequent infection (*ii*.), and gray with a dot indicates attraction followed by inhibition (*iii*.). The collection locality of each fungal strain is indicated in parentheses under its sample name.

2.5 Discussion

The distribution of parasite genotypes relative to their hosts, when analyzed over large geographic scales, gives insight into both local and global disease dynamics. Here, at a broad geographic scale, we see that host and parasite populations are not similarly differentiated. First, there is no correspondence between host and pathogen population pairwise Φ_{st} values, suggesting that hosts and pathogen populations are not diverging in tandem. Second, the most divergent host populations (indicated by Fk) are not the same as the most divergent pathogen populations (Fig. 3c). At a finer scale, we see no evidence of specialized pairings of host and parasite genotypes. Genetic analyses of neutral markers indicate that there is no correspondence between a matrix of host mean character differences and a matrix of pathogen mean character differences, and, whereas cluster analyses suggest several genetically distinct host clusters, there is no corresponding divergence in pathogen isolates (Figs 3 & 4). Supporting this lack of genotype-genotype specificity, experimental bioassays indicate that genetically distinct *Escovopsis* isolates are able to successfully infect a range of genetically diverse cultivar isolates in microbial bioassays (Fig. 5), and there is no correspondence between genetic similarity of hosts and/or of parasites and infection success.

Many life-history traits of hosts and pathogens critically shape disease processes and likely lead to the patterns we see in the cultivar-*Escovopsis* system. Specifically, different modes of transmission increase the likelihood that parasite and host genotypes do not stay coupled across space and time (Huyse et al. 2005). Previous epidemiological studies have suggested that, while cultivars are vertically transmitted by the ants from a mother's colony to new colonies, *Escovopsis* is independently horizontally transmitted

(Currie et al, 1999). It has been hypothesized that *Escovopsis* could be vectored between colonies by microarthropods, or acquired by foraging ants either from the environment or via ant-ant contact. (Currie, 2001; Gerardo et al, 2004). These mechanisms of disease spread likely have different barriers to gene flow than vertical transmission of the cultivar by the ants. Our results corroborate the existence of different barriers to gene flow for the host and for the parasite. Specifically, based on F_K values (Fig. 3c), the most divergent host population (HCE), in Costa Rica's Talamanca mountains, is not the most divergent parasite population (BCI), an island in the Panama Canal (Fig. 1). While water would provide a barrier to *Escovopsis* migration to BCI by any of the above-mentioned horizontal transmission mechanisms, it would not prevent dispersal of the host cultivar by winged, migrating ants, which could easily fly the short distance from BCI to the mainland. On the other hand, cultivar-dispersing ants may have difficulty migrating to and from HCE, because of their sensitivity to cooler temperatures in such higher-elevation regions. If different barriers to gene flow exist, then parasite genotypes would be less able to tightly track host genotypes.

Even without coupled dispersal, tight congruence of host-parasite population structures can be maintained by parasite-specificity, such that parasites are only able to establish in a population if the appropriate host genotype is there. For example, the global population structure of *Mycobaterium tuberculosis*, which causes tuberculosis in humans, is defined by six lineages each associated with different human populations. In urban areas where different human populations have recently become sympatric, the *M. tuberculosis* strains historically associated with these human populations are present. This matching of human and pathogen distributions is shaped in part by tandem host-pathogen

dispersal but also by coevolutionary adaptation. In a diverse urban host population, *M. tuberculosis* strains are much more likely to be infective in humans from populations with which they coevolved than with humans from other lineages (Gagneux et al, 2006). It is likely that specific host and parasite traits, namely host immune-related phenotypes and parasite virulence genes, maintain this specificity. We hypothesized that specific cultivar and *Escovopsis* traits would also maintain tight specificity of *Escovopsis* strains on cultivar strains. Host cultivars are known to be able to produce antibiotics that inhibit the growth of some *Escovopsis* spp., and *Escovopsis* spp. are known to efficiently recognize and establish infection on some hosts species (Gerardo et al, 2006b). Results here suggest that these mechanisms are not facilitating genotype-genotype specificity or local adaptation at a population-level, as a range of genetically and geographically diverse *Escovopis* strains were able to successfully infect a range of genetically and geographically diverse cultivar strains. Thus, these previously identified adaptive mechanisms (Gerardo et al, 2006b) may prevent a given *Escovopsis* from switching between host species but do not necessarily prevent *Escovopsis* from switching between different host strains.

The patterns of host and parasite association that we see here, driven by relatively independent host-parasite dispersal and a lack of genotype-genotype specificity, have implications for the coevolutionary dynamics of the cultivar-*Escovopsis* association. First, focusing on the likelihood of *Escovopsis* to become adapted to local cultivar strains, theory suggests higher gene flow between parasite populations than between cultivar populations, particularly when coupled with substantial genetic structuring across host populations, will facilitate local adaptation of parasites on hosts (Gandon et al, 1996;

Gandon and Michalakis, 2002). Though *Escovopsis* does exhibit slightly lower genetic structuring than cultivars, this discrepancy is likely not sufficient to support local adaptation, as the host cultivars exhibit little genetic population structure. As a comparison, in systems where population genetic analyses detected more extensive parasite than host migration and experimental work confirmed that parasites perform better on local than on novel host genotypes, the relative difference between parasite and host migration rates was much greater than is seen in this study. For example, Dybdahl and Lively (1996) found much higher levels of gene flow in trematode parasites relative to their snail hosts, and it has been experimentally demonstrated that these parasites are locally adapted to common host genotypes (Lively, 1989; Lively and Dybdahl, 2000). Similarly, Mutikainen and Koskela (2002) found higher parasite gene flow in parasitic plants than their perennial hosts, and these parasites had been previously reported to be locally adapted to their hosts (Koskela et al, 2000) In these cases, host populations were respectively 10 and 3 times more differentiated than their parasites, whereas here, with cultivar and *Escovopsis*, overall Φ_{st} values of host and parasite populations are less than twofold different. Moreover, results of Mantel tests between genetic and geographic distance indicate that the effects of isolation by distance in cultivars and *Escovopsis* are similar (Fig. 3), and suggest that though there may be less parasite population differentiation at distances greater than 150km (i.e. when the natural log of geographic distance is greater than 5, Fig. 3). At smaller distances, there is equal mixing between host populations as between parasite populations. Therefore, extensive local adaptation may be less likely in the cultivar-*Escovopsis* system than in other host-parasite associations where the difference between parasite and host genetic structure is greater.

Overall, it seems that local adaptation and strain-specific specialization is not likely to arise in the *A. dentigerum* cultivar-*Escovopsis* system. This is in contrast to the specificity that we see at broader scales, where *Escovopsis* strains are constrained to attacking particular host species or groups of closely related species. This pattern of fine-scale lability underlying broad-scale specificity is consistent with other coevolutionary interactions. For example, a recent study of the host-range of tropical plant pathogens found fungal pathogens were likely to be able to infect a range of closely-related hosts, but were less likely to infect plants distantly related to their host. In other words, the likelihood of infection establishment decreased with increasing phylogenetic distance between the source host plant and the target plant species, with the steepest decline occurring in the most closely-related plant pairs (Gilbert and Webb, 2007). Another example is the mutualistic association of sepiolid squid and their bioluminescent bacteria of the genus *Vibrio*. In this mutualism, phylogenetic congruence and co-adaptation have been observed at a broad scale (Nishiguchi et al, 1998), but finer scale genetic analyses show incongruent and low levels of genetic structuring across a geographic scale (Jones et al, 2006). The question remains as to how species-specificity arises given the lability of associations at finer scales. Resolving this question will require studies coupling multiple-scale specificity (among strains, species, etc.) with studies of the genetics underlying host-parasite adaptations.

2.6 Acknowledgements

We are especially grateful to S. Xhao for her help with laboratory work, B. Wee for help with analysis and interpretation, and C. Currie and U. Mueller for teaching us about the attine system. Research and export permits were granted by the Autoridad Nacional del Ambiente (Panama), Smithsonian Tropical Research Institute (Panama), Ministerio del Ambiente (Ecuador), Museo de Ciencias Naturales (Ecuador), Administración de Parques Nacionales (Argentina), Dirección de Conservación y Manejo (Argentina), Organization for Tropical Studies (Costa Rica) and Ministerio del Ambiente y Energia (Costa Rica). Critical logistical support was provided by M. Leone (STRI), O. Arosemena (STRI) and S. Villamarin (Ecuador). Collections were made by C. Currie, A. Himler, L. Laudermilk, A. Mikehev, S. Price, J. Scott, A. Smith and U. Mueller. Helpful discussion and comments were provided by C. Currie, U. Mueller, M. Singer, B. Wee and three anonymous reviewers. This work was supported by NSF Doctoral Dissertation Improvement Grant DEB-0308757 and NSF IRCEB Grant DEB-0110073. N. Gerardo was supported by fellowships from the University of Texas at Austin Graduate School and the U.T. Department of Integrative Biology, and E. Caldera is supported by an NSF Graduate Research Fellowship.

2.7 References

Bender, W., Spierer, P., and Hogness, D.S. (1983) Chromosomal walking and jumping to isolate DNA from the Ace and Rosy loci and the bithorax complex in *Drosophila melanogaster*. *J. Mol. Biol.* 168: 17-33.

Bonnet, E., and Van de Peer, Y. (2002) ZT: a software tool for simple and partial Mantel tests. *Journal of Statistical Software* 7: 1-12.

Bot, A.N.M., Rehner, S.A., and Boomsma, J.J. (2001) Partial incompatibility between ants and symbiotic fungi in two sympatric species of *Acromyrmex* leaf-cutting ants. *Evolution* 55: 1980-1991.

Carius, H.J., Little, T.J., and Ebert, D. (2001) Genetic variation in a host-parasite association: potential for coevolution and frequency-dependent selection. *Evolution* 55: 1136-1145.

Chapela, I.H., Rehner, S.A., Schultz, T.R., and Mueller, U.G. (1994) Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* 266: 1691-1694.

Combes, C. (2004) *Parasitism: The Ecology and Evolution of Intimate Interactions*. Chicago: University of Chicago Press.

Criscione, C.D., Poulin, R., and Blouin, M.S. (2005) Molecular ecology of parasites: elucidating ecological and microevolutionary processes. *Mol. Ecol.* 14: 2247-2257.

Currie, C.R. (2001) Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia* 128: 99-106.

Currie, C.R., Mueller, U.G., and Malloch, D. (1999) The agricultural pathology of ant fungus gardens. *Proc. Natl. Acad. Sci. U. S. A.* 96: 7998-8002.

Currie, C.R., Wong, B., Stuart, A.E., Schultz, T.R., Rehner, S.A., Mueller, U.G. et al. (2003) Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science* 299: 386-388.

Davies, C.M., Webster, J.P., Kruger, O., Munatsi, A., Ndamba, J., and Woolhouse, M.E.J. (1999) Host-parasite population genetics: a cross-sectional comparison of *Bulinus globosus* and *Schistosoma haematobium*. *Parasitology* 119: 295-302.

Delmotte, F., Bucheli, E., and Shykoff, J.A. (1999) Host and parasite population structure in a natural plant-pathogen system. *Heredity* 82: 300-308.

Dybdahl, M.F., and Lively, C.M. (1996) The geography of coevolution: Comparative population structures for a snail and its trematode parasite. *Evolution* 50: 2264-2275.

Dybdahl, M.F., and Storfer, A. (2003) Parasite local adaptation: Red Queen vs. Suicide King. *Trends Ecol. Evol.* 18: 523-530.

Evanno, G., Regnaut, S., and Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* 14: 2611-2620.

Excoffier, L. (2001) Analysis of population subdivision. In *Handbook of Statistical Genetics*. Balding, A.R., Bishop, M., and Cannings, C. (eds). New York: John Wiley & Sons, pp. 271-307.

Falush, D., Stephens, M., and Pritchard, J.K. (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. *Genetics* 164: 1567-1587.

Gagneux, S., DeRiemer, K., Van, T., Kato-Maeda, M., de Jong, B.C., Narayanan, S. et al. (2006) Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. *Proc. Natl. Acad. Sci. U. S. A.* 103: 2869-2873.

Gandon, S. (1998) Local adaptation and host-parasite interactions. *Trends in Ecology & Evolution* 13: 214-216.

Gandon, S., and Michalakis, Y. (2002) Local adaptation, evolutionary potential and host-parasite coevolution: interactions between migration, mutation, population size and generation time. *Journal of Evolutionary Biology* 15: 451-462.

Gandon, S., Capowiez, Y., Dubois, Y., Michalakis, Y., and Olivieri, I. (1996) Local adaptation and gene-for-gene coevolution in a metapopulation model. *Proceedings of the Royal Society of London Series B-Biological Sciences* 263: 1003-1009.

Gerardo, N.M., Mueller, U.G., and Currie, C.R. (2006a) Complex host-pathogen coevolution in the *Apterostigma* fungus-growing ant-microbe symbiosis. *BMC Evol. Biol.* 6.

Gerardo, N.M., Mueller, U.G., Price, S.L., and Currie, C.R. (2004) Exploiting a mutualism: parasite specialization on cultivars within the fungus-growing ant symbiosis. *Proceedings of the Royal Society of London Series B-Biological Sciences* 271: 1791-1798.

Gerardo, N.M., Jacobs, S.R., Currie, C.R., and Mueller, U.G. (2006b) Ancient host-pathogen associations maintained by specificity of chemotaxis and antibiosis. *Public Library of Science - Biology* 4: 1358-1363.

Gilbert, G.S., and Webb, C.O. (2007) Phylogenetic signal in plant pathogen-host range. *Proc. Natl. Acad. Sci. U. S. A.* 104: 4979-4983.

Green, A.M., Mueller, U.G., and Adams, R.M.M. (2002) Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants. *Mol. Ecol.* 11: 191-195.

Holsinger, K.E., Lewis, P.O., and Dey, D.K. (2002) A Bayesian approach to inferring population structure from dominant markers. *Mol. Ecol.* 11: 1157-1164.

Jerome, C.A., and Ford, B.A. (2002) Comparative population structure and genetic diversity of *Arceuthobium americanum* (Viscaceae) and its *Pinus* host species: insight into host-parasite evolution in parasitic angiosperms. *Mol. Ecol.* 11: 407-420.

Jobet, E., Durand, P., Langand, J., Muller-Graf, C.D.M., Hugot, J.P., Bougnoux, M.E. et al. (2000) Comparative genetic diversity of parasites and their hosts: population structure of an urban cockroach and its haplo-diploid parasite (oxyuroid nematode). *Mol. Ecol.* 9: 481-486.

Johannesen, J., and Seitz, A. (2003) Comparative population genetic structures of the fruit fly *Urophora cardui* and its primary parasitoid *Eurytoma robusta*. *Entomologia Experimentalis Et Applicata* 108: 149-157.

Jones, B.W., Lopez, J.E., Huttenburg, J., and Nishiguchi, M.K. (2006) Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol. Ecol.* 15: 4317-4329.

Kaltz, O., and Shykoff, J.A. (1998) Local adaptation in host-parasite systems. *Heredity* 81: 361-370.

Kirkpatrick, M., and Barton, N.H. (1997) Evolution of a species' range. *American Naturalist* 150: 1-23.

Koskela, T., Salonen, V., and Mutikainen, P. (2000) Local adaptation of a holoparasitic plant, *Cuscuta europaea*: variation among populations. *Journal of Evolutionary Biology* 13: 749-755.

Lively, C.M. (1989) Adaptation by a parasitic trematode to local-populations of its snail host. *Evolution* 43: 1663-1671.

Lively, C.M. (1999) Migration, virulence, and the geographic mosaic of adaptations by parasites. *Am. Nat.* 153: S34-S47.

Lively, C.M., and Dybdahl, M.F. (2000) Parasite adaptation to locally common host genotypes. *Nature* 405: 679-681.

Luetgert, J. Range. United States Geological Service.

Martinez, J.G., Soler, J.J., Soler, M., Moller, A.P., and Burke, T. (1999) Comparative population structure and gene flow of a brood parasite, the great spotted cuckoo (*Clamator glandarius*), and its primary host, the magpie (*Pica pica*). *Evolution* 53: 269-278.

Mikheyev, A.S., Mueller, U.G., and Boomsma, J.J. (2007) Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. *Mol. Ecol.* 16: 209-216.

Mulvey, M., Aho, J.M., Lydeard, C., Leberg, P.L., and Smith, M.H. (1991) Comparative population genetic structure of a parasite (*Fascioloides magna*) and its definitive host. *Evolution* 45: 1628-1640.

Munkacsi, A.B., Pan, J.J., Villesen, P., Mueller, U.G., Blackwell, M., and McLaughlin, D.J. (2004) Convergent coevolution in the domestication of coral mushrooms by fungus-growing ants. *Proceedings of the Royal Society of London Series B-Biological Sciences* 271: 1777-1782.

Mutikainen, P., and Koskela, T. (2002) Population structure of a parasitic plant and its perennial host. *Heredity* 89: 318-324.

Nei, M. (1977) F-statistics and analysis of gene diversity in subdivided populations. *Ann. Hum. Genet.* 41: 225-233.

Nei, M., and Li, W.H. (1979) Mathematical-model for studying genetic-variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. U. S. A.* 76: 5269-5273.

Nishiguchi, M.K., Ruby, E.G., and McFall-Ngai, M.J. (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-vibrio symbioses. *Appl. Environ. Microbiol.* 64: 3209-3213.

Parker, M.A. (1985) Local-population differentiation for compatibility in an annual legume and its host-specific fungal pathogen. *Evolution* 39: 713-723.

Poulsen, M., and Boomsma, J.J. (2005) Mutualistic fungi control crop diversity in fungus-growing ants. *Science* 307: 741-744.

Poulsen, M., Cafaro, M., Boomsma, J.J., and Currie, C.R. (2005) Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. *Mol. Ecol.* 14: 3597-3604.

Price, P.W. (1980) *Evolutionary biology of parasites*. Princeton: Princeton University Press.

Pritchard, J.K., Stephens, M., and Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics* 155: 945-959.

Reynolds, H.T., and Currie, C.R. (2004) Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia* 96: 955-959.

Schneider, S., Roessli, D., and Excoffier, L. (2000) ARLEQUIN: A Software for Population Genetics Data Analysis. Genetics and Biometry Laboratory, University of Geneva.

Schulenburg, H., and Ewbank, J.J. (2004) Diversity and specificity in the interaction between *Caenorhabditis elegans* and the pathogen *Serratia marcescens*. *BMC Evol. Biol.* 4.

Seifert, K.A., Samson, R.A., and Chapela, I.H. (1995) *Escovopsis aspergilloides*, a rediscovered hyphomycete from leaf-cutting ant nests. *Mycologia* 87: 407-413.

Sire, C., Langand, J., Barral, V., and Theron, A. (2001) Parasite (*Schistosoma mansoni*) and host (*Biomphalaria glabrata*) genetic diversity: population structure in a fragmented landscape. *Parasitology* 122: 545-554.

Slatkin, M. (1985) Gene flow in natural-populations. *Annual Review of Ecology and Systematics* 16: 393-430.

Swofford, D.L. (2002) PAUP*: Phylogenetic analysis using parsimony (*: and other methods). Sinauer Associates
.

Thompson, J.N. (1999) Specific hypotheses on the geographic mosaic of coevolution. *American Naturalist* 153: S1-S14.

Thompson, J.N., and Cunningham, B.M. (2002) Geographic structure and dynamics of coevolutionary selection. *Nature* 417: 735-738.

Villesen, P., Mueller, U.G., Schultz, T.R., Adams, R.M.M., and Bouck, A.C. (2004) Evolution of ant-cultivar specialization and cultivar switching in *Apterostigma* fungus-growing ants. *Evolution* 58: 2252-2265.

3 Chapter 3: The population structure of antibiotic-producing bacterial symbionts of *Apterostigma dentigerum* ants: Impacts of coevolution and multi-partite symbiosis.

This chapter was previously published in The American Naturalist. 2012. 180(5): 604-617.

Eric J. Caldera[1,2,] and Cameron R. Currie [1,2,3]

Author contributions: EJC conducted the field work, culture work, genotyping and analysis. EJC & CRC wrote the paper.

3.1 Abstract

Fungus-growing ants (Attini) are part of a complex symbiosis with Basidiomycetous fungi that they cultivate for food, Ascomycetous fungal pathogens (*Escovopsis*) that parasitize cultivars, and Actinobacteria that produce antibiotic compounds that suppress pathogen growth. Previous studies characterizing the association between attines and their bacterial symbionts have employed broad phylogenetic approaches, with conclusions ranging from a diffuse coevolved mutualism to no specificity being reported. However, the geographic mosaic theory of coevolution, proposes that coevolved interactions likely occur at a level above local populations, but within species. Moreover, the scale of population subdivision is likely to have impacts on coevolutionary dynamics. Here, we describe the population structure of bacteria associated with the attine *Apterostigma dentigerum* across Central America using multi-locus sequence typing (MLST) of six housekeeping genes. The majority (90%) of bacteria isolated grouped into a single clade within the genus *Pseudonocardia*. In contrast to studies suggesting that *Pseudonocardia* dispersal is high and therefore unconstrained by ant associations, we found highly structured (F_{ST}=0.39) and dispersal-limited (i.e. significant isolation by distance r=0.517, P=0.05) populations over even a relatively small scale (e.g. within the Panama Canal Zone). Estimates of recombination versus mutation were uncharacteristically low compared to free-living Actinobacteria (e.g ρ/θ=0.028 in La Selva, Costa Rica), suggesting recombination is constrained by association with ant hosts. Further, *Pseudonocardia* population structure was correlated with that of *Escovopsis* (r=0.67, P=0.02), supporting the bacteria's role in disease suppression. Overall, the population dynamics of symbiotic *Pseudonocardia* are more consistent with a specialized

mutualistic association than recently proposed models of low specificity and frequent horizontal acquisition.

3.2 Introduction

Discerning how individuals within a species are structured over geographic space is essential to understanding how populations evolve. For example, allopatric speciation (divergence due to geographic isolation) is typically regarded as the most common mechanism of speciation (Mayr 1963; Lande 1980; Coyne and Orr 2004), and migration can either facilitate or hinder local adaptation (Lenormand 2002; Kawecki and Ebert 2004). Further, the scale of population subdivision plays an important role in shaping coevolutionary dynamics (Lively 1999; Thompson and Cunningham 2002; Thompson 2005, 2009). Within host-symbiont systems, symbiont populations can be structured by hosts (resulting in congruent population structure), although such congruence is more likely when specificity is maintained either through vertical transmission or adaptive mechanisms that determine symbiont selection (Wirth et al. 2005; Vollmer et al. 2010; Zanatta and Wilson 2011). In contrast, horizontal transmission and diffuse associations can decouple host-symbiont populations and constrain opportunities for pairwise coevolution.

The attine (Attini, Formicidae) ant-microbe system represents a complex community of well-characterized symbionts, providing an opportunity to study the impact of multi-partite symbiont associations on population structure. In addition to the ants, this symbiosis also includes mutualistic Basidiomycetous fungi, parasitic Ascomycetous fungi, and antibiotic-producing Actinobacteria (reviewed in Caldera et al. 2009). Attine ants are a monophyletic tribe of approximately 230 ant species, all of which

cultivate fungi as their primary food source. The association between attine ants and their fungal cultivars is a textbook example of both obligate mutualism and agriculture. Like many agricultural crops, attine cultivars are susceptible to pathogens, the most common being *Escovopsis* (Ascomycota, Hypocreales) microfungi that persist as chronic infections within the fungal gardens (Currie et al. 1999 a, Currie et al. 2003 a). If *Escovopsis* infection is not maintained at a low level, the pathogen can rapidly consume the cultivar, thus destroying the entire ant colony (Currie et al. 1999 a, Currie et al. 2001). To combat *Escovopsis* infection, most attine species rely on antibiotic compounds produced by symbiotic *Pseudonocardia* (Actinobacteria, Actinomycetales) that grow on specialized regions of the ant cuticle and suppress *Escovopsis* growth (Currie et al. 1999 b, Currie et al. 2003 a, Currie et al. 2006, Oh et al. 2009, Cafaro et al. 2011).

Our current understanding of how symbiotic associations might shape population structure within the attine system begins with the ant-cultivar association. Before departing on nuptial flights, virgin queens carry in their mouth an inoculum of a fungal cultivar from the mother colony, which they use to start the fungal garden after mating (Hölldobler and Wilson 1990). This vertical transmission of cultivar clones from mother to daughter is believed to have contributed to the broad patterns of phylogenetic congruence between attine ants and their cultivars over evolutionary time (Chapela et al. 1994, Mikheyev et al. 2010). Despite the coupling of cultivar transmission with the reproductive lifecycle of attine queens, horizontal exchange among closely related ant species through cultivar sharing and/or stealing appears rampant (Adams et al. 2000, Green et al. 2002, Mikheyev et al. 2006, Mikheyev et al. 2007, Poulsen et al. 2009), and some basal attine species may be capable of utilizing free-living fungi (Mueller et al.

1998, Vo et al. 2009). Horizontal acquisition of cultivars occurs early during colony founding (Poulsen et al. 2009) and mature colonies appear to propagate only one cultivar clone despite maintaining multiple garden chambers (Mueller et al. 2010 a). To date, the geographic population genetic structure of an ant and its cultivar has been described in only one attine, *Trachymyrmex septentrionalis* (Mikheyev et al. 2008). Although both ants and cultivars exhibited significant isolation by distance (IBD) across the eastern United States (r=0.23, P=0.034 and r=0.38, P=0.003 respectively), there was no correlation between the ant's genetic and the fungal community similarity matrix, suggesting that cultivar population structure is not shaped by transmission with its ant host.

Whereas opportunities for both vertical and horizontal transmission exist for cultivars, *Escovopsis* pathogens are horizontally acquired, likely from invertebrates that associate with attine ant colonies, and are absent in the early stages of colony founding (Currie et al. 1999 a). Although *Escovopsis* is acquired horizontally, broad phylogenetic congruence exists between fungal cultivars and *Escovopsis*, suggesting that additional mechanisms maintain pathogen specificity over evolutionary time scales (Currie et al. 2003 b). The adaptive mechanisms governing cultivar-*Escovopsis* specificity are best understood in the basal attine genus *Apterostigma*. Among *Apterostigma* species, phylotype-specific patterns of *Escovopsis* attraction to cultivars through chemotaxis, and cultivar resistance to *Escovopsis* reflect patterns of phylogenetic congruence (Gerardo et al. 2006 a and b). A population-level study of *Escovopsis*-cultivar dynamics in the ant species *Apterostigma dentigerum* across Central America, however, found that cultivar resistance is ineffective against the most common pathogen phylotype (brown

Escovopsis) of *A. dentigerum*, regardless of genotype or location (Gerardo and Caldera 2007). Moreover, while both cultivars and brown *Escovopsis* showed significant IBD (r=0.34, P=0.04 and r=0.38, P=0.03 respectively), their population genetic structure was not congruent, indicating a lack of pairwise specificity at the population level. It remains possible that the seemingly labile association between *A. dentigerum* cultivars and brown *Escovopsis* persists because the role of specialization against *Escovopsis* is maintained by *Pseudonocardia*.

Less is known about the transmission and population dynamics of attine-associated *Pseudonocardia* than cultivars or *Escovopsis*. Like cultivars, a clear mechanism for vertical transmission of *Pseudonocardia* exists, but the frequency of horizontal acquisition or recombination among strains remains ambiguous. Virgin queens harbor a visible inoculum of *Pseudonocardia* on specialized cuticular structures prior to departing on mating flights (Currie et al. 2006); therefore, it is not necessary to acquire novel strains during colony founding. Doing so would also require the new bacteria to out compete the original strain. Studies of *Acromyrmex*-associated *Pseudonocardia* have recovered only one genotype per colony (Poulsen et al. 2005), demonstrated sophisticated strain-level recognition by the ants (Zhang et al. 2007), and shown that acquisition from non-maternal colonies results in less abundant bacterial cover (Armitage et al. 2011). This evidence together suggests that the ants transmit and maintain the same clone present during colony founding. Phylogenetic studies have shown a degree of congruence between attines and *Pseudonocardia* indicative of vertical transmission, but incongruences also suggested that horizontal switches are perhaps more frequent than cultivar switching over evolutionary time (Cafaro and Currie 2005, Cafaro et al. 2011).

Opportunity for horizontal acquisition could exist through contact with males during mating; however, *Pseudonocardia* are often absent or in low abundance on males, and males are not present during colony founding, so they are not likely to be the primary vector. Contact with the soil, foraging material, and other foraging fungus-growing ants may also provide opportunities for horizontal acquisition. Recently, a new model of attine-*Pseudonocardia* association (termed the acquisition model) has emerged, which argues that the ant-*Pseudonocardia* symbiosis is substantially less specialized than previously thought (Kost et al. 2007, Haeder et al. 2009; Sen et al. 2009), and may not be a mutualistic association, but rather encompasses a range of associations including parasitism (Mueller et al. 2008; Mueller et al. 2010 b). More specifically, the acquisition model emphasizes limited vertical transmission of *Pseudonocardia* across many ant generations and low potential (or absence) of *Pseudonocardia-Escovopsis* coevolution and specificity.

Studies both in support of and in conflict with the acquisition model have drawn heavily on phylogenetic and/or across-species approaches among many ant species (Cafaro and Currie 2005, Currie et al. 2006, Mueller et al. 2008, Mueller et al. 2010 b, Sen et al. 2009, Cafaro et al. 2011). The geographic mosaic concept of coevolution, however, posits that coevolved interactions likely occur at a scale above local populations, but below species (i.e. at the geographic population scale; Thompson 1994). Consequently, studies at this scale are likely to yield important insights into attine-microbe coevolution. To date, the geographic population structure of attine-associated *Pseudonocardia* has only been described in *Trachymyrmex serptentrionalis* (Mikheyev et al. 2008). Across the eastern United States, *Pseudonocardia* had little population

structure at the *EF-Tu* locus and genetic distances were not correlated with the ant host, which did show substantial geographic structure. This led the authors to conclude that the bacteria's dispersal capacity is high and not constrained by association with the ants, although bacterial population structure might be shaped by environmental factors and/or interactions with *Escovopsis*. Currently, most genetic based studies of symbiotic *Pseudonocardia* (Cafaro and Currie 2005; Poulsen et al. 2005, Zhang et al. 2007, Mikheyev et al. 2008; Mueller et al. 2008; Sen et al. 2009; Mueller et al. 2010 b), including the only description of *Pseudonocardia* population structure, have been limited by the use of a single, well conserved, housekeeping gene. Studies of microbial geographic distributions and niche evolution, however, strongly caution against interpretations drawn from a single locus, as evidence for finer geographic population structure tends to emerge as additional loci and molecular markers are added (Cho and Tiedje 2000, Whitaker et al. 2003; Hedlund and Staley 2004). Therefore, it remains possible that multi-locus analysis of ant-associated *Pseudonocardia* may expose a finer degree of geographic structure yet unseen.

Here, we describe the population genetic structure of *A. dentigerum*-associated *Pseudonocardia* in Panama and Costa Rica using loci from six different housekeeping genes (multi locus sequence typing MLST). We use both phylogenetic and population genetic analyses to answer the following questions: 1) Are populations geographically structured within Central America? 2) Do populations conform to isolation by distance, and at what scale? 3) Are *Pseudonocardia* populations recombining, and to what extent? 4) Is *Pseudonocardia* population structure correlated with the population structure of the

brown *Escovopsis* phylotype and/or cultivars? Finally, we discuss our findings in the context of differing models of attine-*Pseudonocardia* association.

3.3 Materials and Methods

3.3.1 Collection of *Apterostigma dentigerum*

We collected colonies of *A. dentigerum* from La Selva Biological Station, Costa Rica, during October 2006 and July 2007, and from seven locations in Panama during June 2008 (fig. 1a & b). Within Panama, sampling was greatest around the Panama Canal Zone, including the following locations: Gamboa Forest (GAM), Pipeline Road (PLR), and Barro Colorado Island (BCI). Collections on BCI were divided roughly into the northern and southern regions of the island, and collections along PLR were divided into colonies collected along the banks of rivers intersecting the northern part of the road (rivers Agua Salud, Guacharo, Pelon, and Macho) and the southern part (rivers Limbo, Seda, Frijoles, and Frijolito). We collected additional ant colonies on the peninsulas surrounding BCI and in Bocas del Toro province (BT), near the Costa Rican border.

3.3.2 Symbiont Isolation

To obtain pure cultures of symbiotic Actinobacteria, we followed the methods of Poulsen et al. (2005) for *Acromyrmex* ants, but with the following modifications specific to *Apterostigma dentigerum*. Before scraping bacteria off the ant cuticle, we first removed the head and forelegs from workers, to expose the mesosternal lobe where the bacteria are localized in *A. dentigerum* (Currie et al. 2006). Depending on the number of workers in a given nest, we scraped the mesosternal lobes of one to four ants. An additional 16

bacterial isolates collected during 2001-2003 by researchers from the University of Texas at Austin and the University of Kansas were included. These additional isolates were stored in 2 ml tubes at -80°C and contained either frozen cells or extracted DNA.

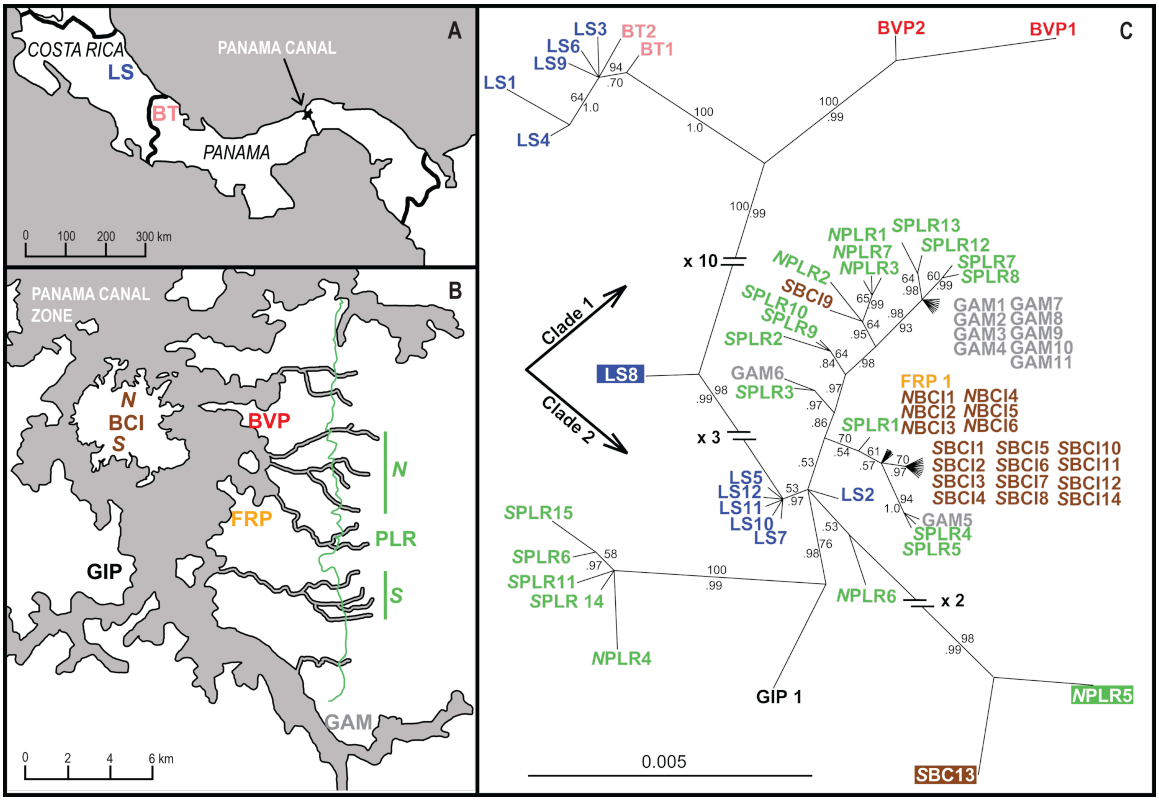


Figure 1. Collection locations (A & B) and phylogenetic construction (C) of symbiotic *Pseudonocardia*. The phylogeny was based on a six-locus concatenated alignment and utilized Bayesian methods. The scale bar corresponds to 0.005 substitutions per site. Maximum likelihood methods yielded trees with similar topologies. Bayesian posterior probabilities are reported below branches – bootstrap support values from maximum likelihood are above. Highlighted isolates are recombinant.

3.3.3 DNA Extraction and Sequencing

DNA was extracted from pure cultures following Poulsen et al. (2005). To amplify the *16S* rDNA gene, we used the polymerase chain reaction (PCR) primer sets 243F and 1378R (Heuer et al. 1997). MacVector 10.6 was used to design PCR primers for an additional five loci comprised of partial sequences from the following housekeeping genes: *atpD, dnaA, EF-Tu, gyrB, rpoB* (table 2). DNA sequences for these five additional genes were extracted from a *Pseudonocardia* draft genome that was isolated from *A. dentigerum* (Currie unpublished data). Amplifications were conducted using standard PCR methods and positive amplifications were sequenced at the University of Wisconsin-Madison Biotechnology Center (biotech.wisc.edu) using Sanger sequencing technology. DNA sequence chromatograms were edited using Sequencher 4.6 (Gene Codes Corporation, An Arbor, MI, USA). Because the *16S* rDNA PCR primers are specific to Actinobacteria (Heuer et al. 1997), whereas primers for the additional five loci are potentially specific to a *Pseudonocardia* strain associated with *A. dentigerum*, isolates were first sequenced at the*16S* locus. Samples that were named *Pseudonocardia* species by BLAST searches (blast.ncbi.nlm.nih.gov) were subsequently genotyped for the remaining five housekeeping genes. Of the isolates named *Pseudonocardia* in BLAST searches (>98% sequence identity) 71 isolates were sequenced for all six loci (table 1). MLST sequences have been deposited in Genbank under the following accession numbers (HQ540730-HQ541155).

Table 1. Location and number of *Pseudonocardia* symbionts isolated from the mesosternal lobes of *Apterostigma dentigerum* and successfully PCR-amplified at six loci.

Country	Location	Abbr.	Dist.(km)*	*N*
Costa Rica	La Selva Biological Station	LS	482	12
Panama	Bocas del Toro province	BT	273	2
Panama	Gigante Peninsula	GIP	6.3	1
Panama	Frijoles Peninsula	FRP	6.8	1
Panama	Buena Visa Peninsula	BVP	4.7	2
Panama	*N*. Barro Colorado Island	*N*BCI	0	6
Panama	*S*. Barro Colorado Island	*S*BCI	3.5	14
Panama	Gamboa Forest	GAM	17.9	11
Panama	*N*. Pipeline Road	*N*PLR	7	7
Panama	*S*. Pipeline Road	*S*PLR	21.9	15
			Total:	71

*Distance from *N*. BCI

Table 2. Housekeeping genes used in multi-locus genotyping, including PCR primer pairs, dN/dS ratios, and Tajima's D.

Target	Forward primer	Reverse primer	Product size (bp)	dN/dS	Tajima's D
16S rRNA					
(Heuer et al. 1997)	GGATGAGCCCGCGGCCTA	CGGTGTGTACAAGGCCCGGGAACG	1175		
atpD	AGGAGATGATCCAGCGTGTC	GTTCTTCTCCAGGTCGTCCA	890	0.031	-0.483
dnaA	GTCGACGACATCCAGTTCCT	CTCGTTGCGGATCTTCTTGT	645	0.000	1.038
EF-Tu	CACGACAAGTACCCGAACCT	AGTTGTTGAAGAACGGGGTG	880	0.196	0.090
gyrB	AGCTACACGCTGGAGACGA	GGTGATGATCGACTGGACCT	999	0.192	0.480
rpoB	AACAAGAAGCTCGGTCTGGA	CATGACGGTGATGTAGTCGG	1038	0.127	0.275

3.3.4 Phylogenetic Analysis

Nucleotide sequences were aligned using CLUSTAL X 2.0.11 (Larkin et al. 2007) and models of nucleotide substitution were determined using jMODELTEST (Posada 2008; Guindon and Gascuel 2003). We constructed phylogenetic trees independently for each of six genes, and also using a concatenation of all genes. Single-locus phylogenetic relationships were determined using the maximum-likelihood (ML) criterion, and the concatenated alignment utilized both ML and Bayesian methods implemented in the programs GARLI 0.951(Zwickl 2006; www.bio.utexas.edu/faculty/antisense/garli/Garli.html) and MrBayes 3 (Ronquist and Huelsenbeck 2003). To ensure that parameter space was thoroughly explored, MrBayes was run four separate times. Each run utilized four heated chains and each chain featured four million generations with sampling every 100th generation, following a burn-in period of 100,000 generations. GARLI was run 10 times, using default parameters. 100 bootstrap replicates were generated with the generation threshold for topology termination (*genthreshfortopoterm*) parameter set to 5,000, as recommended by the GARLI manual. Finally, to confirm that phylogenetic inferences were not impacted by recombination among isolates, we constructed additional phylograms in ClonalFrame (Didelot and Falush 2007), which removes recombination, and we visualized multiple phylograms using SplitsTree (Huson and Bryant 2006).

3.3.5 Population Genetic Analyses

To test whether the housekeeping genes are under selection, we compared synonymous and nonsynonymous substitutions by calculating overall dN/dS ratios, and Tajima's D for each translated locus using MEGA (Kumar et al. 2008). Significance was assessed with a

Z test utilizing 500 bootstraps and the Nei-Gojobori method. To test for significant population structure among geographically defined regions, we calculated the overall fixation index, F_{ST} (Weir and Cockerham 1984) using ARLEQUIN 3.11 (Excoffier et al. 2005). Genetic distances among populations were calculated using F_{ST} and Nei's net D_A (Nei et al. 1983). Genetic diversity within populations was assessed using π (mean number of nucleotide differences; Tajima 1993). *P*-values represent the proportion of permutations leading to a value larger or equal to the observed one.

Our tests for isolation by distance (IBD), involved exploring correlation between matrices of genetic distance (F_{ST} /(1- F_{ST}) and ln(geographic distance), and were conducted using a Mantel test with a Pearson's product moment correlation coefficient (Slatkin 1993). Geographic distances were determined using Mapsource® 6. Due to low sampling, the following locations were excluded from IBD and F_{ST} analyses: BT, BVP, and FRP.

Finally, to examine whether *Pseudonocardia* population genetic structure is geographically congruent with *A. dentigerum* fungal symbionts, we obtained pairwise Φ_{ST} (an analog of F_{ST}, Excoffier 2001) values for cultivars and brown *Escovopsis* among the following populations (with corresponding sample sizes): BCI=10, GAM=7, PLR=12, and LS=9. Fungal isolates were genotyped by N. Gerardo using AFLP (amplified fragment length polymorphism) genotyping in a prior study of cultivar-*Escovopsis* interactions in *A. dentigerum* (Gerardo and Caldera 2007). Isolation of fungal symbionts was conducted prior to this study so fungal and bacterial isolates derive from different individual ant colonies, although they are from the same geographic locations

and host ant species. Correlation between the pairwise matrices of *Pseudonocardia* F_{ST} values and Φ_{ST} in fungal symbionts was also assessed with a Mantel test.

3.3.6 Tests for Recombination

To assess whether *Pseudonocardia* populations are clonal or recombining, we began with the *a priori* assumption that populations are clonal and subsequently tested for evidence of recombination events. Recombination was initially examined by looking for phylogenetic incongruence among six loci, in addition to tests for recombination using two methods implemented in the Recombination Detection Program (RDP) 3.44 software package (Heath et al. 2006): RDP (Martin and Rybicki 2000) and GENECONV (Padidam et al. 1999). Estimates of the population recombination parameter $\rho=2N_e r$ and the population mutation rate Waterson's $\theta=2N_e\mu$ were determined using the coalescent method LDhat (McVean et al. 2002). LDhat simulations utilized 1,000,000 Marcov chain Monte Carlo (MCMC) iterations following a burn-in of 100,000. As recommended by the RDP documentation, we conducted an initial estimate of ρ and used the output as the starting ρ for subsequent simulations. Employing a range of block penalties (5-50) had little impact on ρ and θ estimates. ρ and θ were calculated within geographic regions and incorporating all isolates.

3.4 Results

3.4.1 Phylogenetic Inferences

A concatenated phylogeny across six loci revealed multiple, well-supported clades of *A. dentigerum*-associated *Pseudonocardia* that corresponded to geographic locations (fig. 1). ClonalFrame analyses confirmed that topologies resulting from phylogenetic

reconstructions were not impacted by recombination (fig. S1 and S2). Bayesian and ML approaches yielded virtually identical topologies. Isolates grouped with into two major clades (Clades 1 & 2). Clade 1 consisted of two sub-clades, the first of which contained five isolates from LS, Costa Rica and the only two isolates from BT, Panama, near the border of Costa Rica. Clade 1 also contained a sub-clade comprised of the only two isolates from BVP in the Panama Canal Zone. With the exception of the two isolates from BVP, most isolates collected in the Panama Canal Zone were contained in Clade 2. While approximately half of the Costa Rican isolates (LS) were also found in Clade 2, the majority of these isolates formed a single group. One isolate (LS 8), fell between Clade 1 and Clade 2 because of a recombination event among isolates from the two clades (see below). Within the Panama Canal Zone, the majority of isolates from SBCI, NBCI, and GAM formed distinct clades. In contrast, isolates collected along PLR formed multiple sub-clades throughout Clade 1, and thus appeared less geographically distinct, although many of the sub-clades did correspond to the northern or southern regions of PLR. All six locus-specific phylogenies recovered Clade 1 and Clade 2 (fig. 2), but individually lacked variation to capture many of the geographically distinct sub-clades of the concatenation. The BVP clade was recovered by four loci and a PLR specific clade was recovered in five loci.

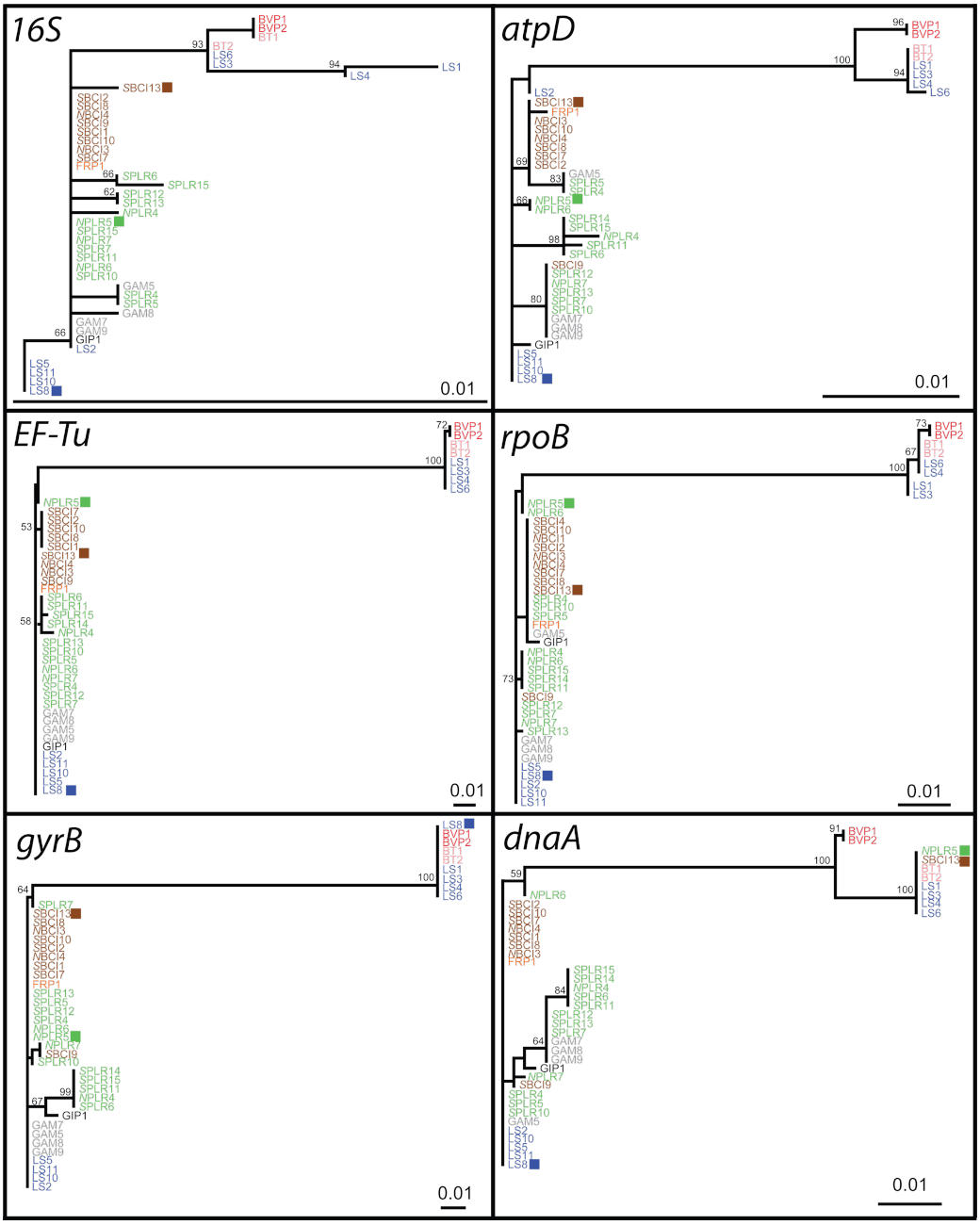


Figure 2. Phylogenetic analysis (maximum likelihood) of partial sequences from each of six house-keeping genes. The scale bar corresponds to 0.01 substitutions per site, and values at nodes report % bootstrap support. Topologies were largely congruent with the exception of three isolates showing incongruence across Clade1 and Clade 2, suggesting they are recombinant. Note that 29 isolates, none of which showed incongruence, were removed for visual purposes. LS8 shows incongruence within *gyrB*, and isolates *N*PLR5 and *S*BCI13 show incongruence in *dnaA*. These isolates were also identified as recombinant using RDP and GENECONV (see methods); however, the overall recombination to mutation rate was marginal (ρ/θ=0.09).

3.4.2 Population Genetic Structure

All translated housekeeping genes showed dN/dS ratios below 1 (average 0.109, table 2), and thus are not under positive selection. Consistent with conserved housekeeping genes, Z-tests were highly significant for purifying selection (dN>dS). Tajima's D values were relatively low, ranging from -0.48 to 1.04, further showing that positive selection is not occurring on these loci. Because of the high divergence between Clade 1 and Clade 2, it is possible that these groups may reflect species level differences, so population genetic analyses were restricted to Clade 2. The overall F_{ST}=0.390 (P<0.0001) revealed significant structure among the populations LS, BCI, GAM, PLR (P<0.0001). A second F_{ST} further dividing BCI and PLR into northern and southern sampling sites returned a similar level of structure (F_{ST}=0.391, P<0.0001) across all populations; however, pairwise F_{ST} between northern and southern BCI revealed significant structure (Table 3, NBCI-SBCI F_{ST}=0.367, P<0.05). In contrast, PLR was not structured between north-south sampling sites (Table 3, NPLR-SPLR F_{ST}=0.0297, P>0.05). Population specific genetic diversity (π) and pairwise Nei's genetic distances D_A are also presented in table 3. Mantel tests showed significant isolation by distance (IBD) across Costa Rica and Panama (r=0.4711, P=0.03); however, because LS is the only-well sampled population outside of the Panama Canal Zone, the sampling scheme does not thoroughly address IBD across the two countries. Thus, we conducted a subsequent test for IBD at a smaller scale (i.e. within the Panama Canal Zone), which also showed a significant correlation between genetic and geographic distances among populations (r=0.517, P=0.05). Fig. 3 presents the relationship between genetic divergence (F_{ST}) and geographic distance (km) in the Panama Canal Zone.

Table 3. Pairwise population genetic distances.

	NBCI	SBCI	GAM	NPLR	SPLR	CR
NBCI	0.000	**0.846**	**7.036**	**6.881**	**3.417**	**6.667**
SBCI	**0.367**	1.692	**6.931**	**6.617**	**3.562**	**6.897**
GAM	**0.698**	**0.713**	4.109	**2.502**	1.281*	**6.612**
NPLR	**0.329**	**0.472**	**0.221**	21.095	0.456	**3.833**
SPLR	0.204*	**0.344**	**0.132**	0.030	15.611	**4.083**
LS	**0.947**	**0.831**	**0.686**	**0.232**	**0.269**	0.667

Note: Pairwise F_{ST} is presented below the diagonal, D_A is above the diagonal, and mean pairwise genetic differences (π) are in the diagonal. Bold denotes pairwise distances with $P<0.05$ and * denotes $0.05<P<0.1$

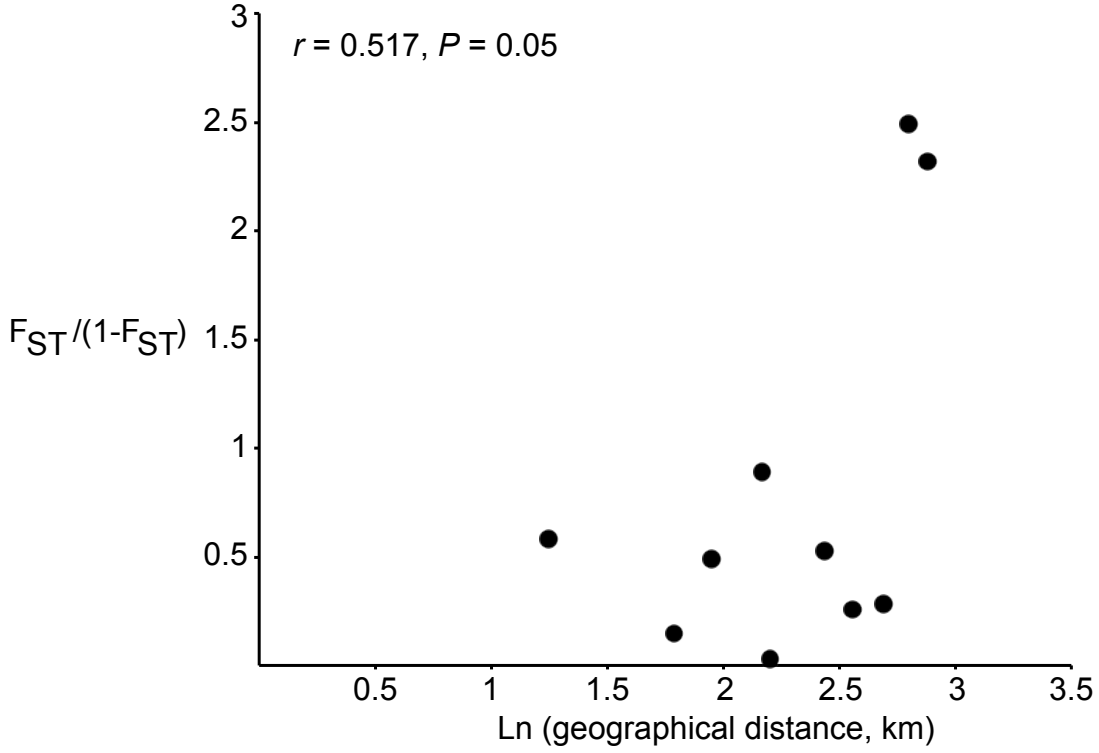


Figure 3. Isolation by distance in symbiotic *Pseudonocardia* in the Panama Canal Zone. Correlation between genetic and geographic distance was assessed using a Mantel test.

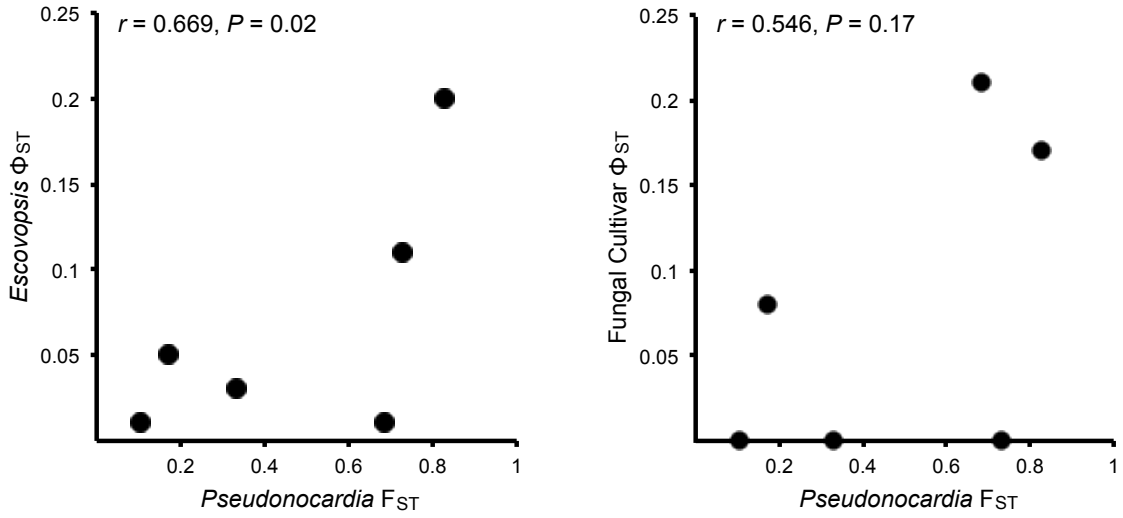


Figure 4. Association between the population genetic structure of symbiotic, antibiotic-producing *Pseudonocardia* associated with *Apterostigma dentigerum* in Central America (LS-CR, BCI-PA, PLR-PA, GAM-PA) and two fungal symbionts (*Escovopsis*-pathogen, cultivar-mutualist). Population structure was congruent between *Pseudonocardia* and the *Escovopsis* pathogens they inhibit (A). No significant association was detected between *Pseudonocardia* and the cultivars that serve as the ants food source. Significance was assessed using a Mantel test. *Escovopsis* and cultivar Φ_{ST} values were taken from Gerardo and Caldera (2007).

Pseudonocardia population genetic structure was congruent with the genetic structure of brown *Escovopsis* (fig. 4a; $r = 0.669$, $P = 0.02$). In contrast, there was no significant association between fungal cultivars and *Pseudonocardia* (fig. 4b $r = 0.546$, $P = 0.17$).

3.4.3 Recombination

The topologies of the phylogenies constructed for each of the six loci were largely congruent, suggesting that recombination is relatively infrequent among *Pseudonocardia* isolates (fig. 2). Three isolates out of 71 (LS8, NPLR5, SBCI13), however, showed incongruence between Clade 1 and Clade 2. LS8 grouped with Clade 2 across five loci but fell within Clade 1 for the *gyrB* locus. Similarly, NPLR5 and SBCI13 clustered with Cade 2 across five loci but associated with Clade 1 within the *dnaA* phylogeny, indicating that some recombination events have taken place. The same three recombination events were identified using the RDP and GENECONV methods (P-values $< 1x10^{-22}$).

The population recombination rate ($\rho=2N_e r$) across all isolates was estimated as 5.069 (95% upper and lower bounds: 3.953-6.273), with a per site estimate of $9.886x10^{-4}$ ($7.712x10^{-4}$ –$1.224x10^{-3}$). The mutation rate, Watterson's $\theta=2N_e\mu$ was 56.45, with a per-site rate of $1.1x10^{-2}$. Region specific estimates of ρ and θ are presented in table 3. We do not report estimates for BCI because the 95% confidence intervals spanned five orders of magnitude.

Table 4. Population scaled recombination rates, mutation rates, and r/u ratios.

Region	ρ	95% bounds	Θ	ρ/Θ
all isolates	5.069	3.953-6.273	56.45	0.0898
LS	2.096	2.018-2.295	74.435	0.028
GAM	2.079	0.213-4.921	9.273	0.224
PLR	8.369	3.971-16.918	22.48	0.372

3.5 Discussion

In contrast to previous studies suggesting that *Pseudonocardia* dispersal is far greater than its ant host (Mikheyev et al. 2008, Mueller et al. 2008, Mueller et al. 2010) we found highly structured and dispersal-limited populations across Central America and within the Panama Canal Zone. Estimates of recombination versus mutation were uncharacteristically low compared to free-living Actinobacteria (Pérez-Losada et al. 2006, Deletoile et al. 2010, Doroghazi and Buckley 2010), suggesting recombination is constrained by association with its ant host. Further, *Pseudonocardia* population structure was correlated with *Escovopsis*, but not cultivars across Central America, supporting the bacteria's role in disease suppression. These findings may reflect the geographic mosaic coevolutionary concept that coevolved dynamics occur at a geographic scale above local populations, but below the species level (Thompson 1994).

3.5.1 Geographic Structure

Despite the extraordinary dispersal capacity of many bacterial species, *A. dentigerum*-associated *Pseudonocardia* populations are both highly structured and dispersal-limited over even a relatively small geographic space. Multi-locus phylogenetic constructions (fig. 1) revealed several clades that correspond to specific locations (e.g. Gamboa forest and Barro Colorado Island in the Panama Canal Zone), and the overall F_{ST}=0.39 showed substantial structure. In addition, we found a significant correlation between genetic distance and geographic distance over even the relatively small geographic scale of the Panama Canal Zone (r=0.517, P=0.05).

Given the substantial amount of structure in *Pseudonocardia*, it seems likely that host associations play a role in shaping the bacteria's populations. A previous description of *Pseudonocardia* geographic structure in the attine *Trachymyrmex septentrionalis* ruled out vertical transmission with ant hosts as a likely force structuring populations, but hypothesized that interactions with *Escovopsis* might (Mikheyev et al. 2008). A single locus (*EF-Tu*) analysis revealed little geographic structure and slight IBD in *Pseudonocardia* across the eastern United States (whereas the ants showed a clear east-west divide), leading the authors to conclude that the bacteria are likely capable of existence outside the symbiosis, or are better dispersers than the ants. That finding was later cited in support of the recruitment hypothesis (Mueller et al. 2008) as evidence that bacterial symbionts are not closely tied to their ant hosts.

While it does appear that *Escovopsis* interactions do shape *Pseudonocardia* genetic structure (fig. 4, and below), our findings suggest that the lack of geographic structure in *T. septentrionalis* was due to low variation in the *EF-Tu* locus and not of greater dispersal by the bacteria. Our *EF-Tu* locus alone also recovered little phylogeographic structure (as did each individual locus, fig. 2); however, the concatenation of all six loci revealed substantial geographic structure and significant IBD (r=0.471, P=0.03). Thus, it is likely that additional loci will reveal structure yet unseen in *T.septentrionalis*-associated *Pseudonocardia*. Consequently, the assumption that *Pseudonocardia* dispersal is not tied to ant hosts should be revisited – future work should incorporate the population structure of *A. dentigerum* to address this further. We now know that the scale of *Pseudonocardia* population subdivision is finer than *Escovopsis* and cultivars, and thus perhaps more closely mirrors that of *A. dentigerum*, whose

dispersal capacity is likely under 500 m per year, based on flight distances of the much larger and more powerful leaf-cutter species *Acromyrmex octospinosus* (Mikheyev 2008).

3.5.2 *Pseudonocardia* and Fungal Symbionts

It appears that interactions with *Escovopsis* play a role in shaping *Pseudonocardia* population structure – there was a significant correlation between the population structures of *Pseudonocardia* and brown *Escovopsis* ($r = 0.669$, $P = 0.02$; fig. 4). While congruence between *Pseudonocardia* and cultivars was similarly correlated ($r = 0.546$, $P = 0.17$) the correlation lacked significance, so it remains possible that the lack of difference is the result of a type II error. A previous study of cultivar-*Escovopsis* (brown) population dynamics in *A. dentigerum* found no congruence between the population structures of the two fungal symbionts, and bioassay experiments demonstrated that the secondary metabolites of fungal cultivars are not effective in combating brown *Escovopsis*, regardless of genotype, or geographic location (Gerardo and Caldera 2007). This finding was interesting given that cultivar secondary metabolites are known to inhibit other *Escovopsis* phylotypes that associate with different, sympatric *Apterostigma* species (Gerardo et al. 2006). Together, these findings illustrate two important points about attine-microbe interactions: I) The "indirect" association between *Pseudonocardia* and *Escovopsis* is more specialized than the "direct" cultivar-*Escovopsis* interaction at the population level, and II) this pairwise specialization arose within the seemingly diffuse associations attines have with multiple cultivar and *Escovopsis* phylotypes (Gerardo et al. 2006 b, Mikheyev et al. 2007, Taerum et al. 2007). Although this seems counter intuitive, the emergence of specialized interactions within seemingly diffuse associations is

consistent with coevolutionary theory (Thompson 1994), and we can imagine how these dynamics occur in nature. Attine colonies encounter several *Escovopsis* phylotypes. The cultivar is capable of suppressing many of these phylotypes; however, in the case of *A. dentigerum* cultivars, they do not suppress brown *Escovopsis* (Gerardo et al. 2006). Under this scenario, there would be strong selection for colonies whose Actinobacteria inhibit the brown phylotype. This selection for *Escovopsis* resistance could then be a force structuring bacterial populations (e.g. Buckling and Rainey 2002). Indeed, the correlated population structure between the fungal pathogens and bacterial symbionts implies that such dynamics are occurring between *Pseudonocardia* and *Escovopsis*. To better understand these interactions, future work should include bioassay experiments to detect co-adaptation by *Pseudonocardia* to *Escovopsis*, perhaps through local adaptation experiments across the geographically structured populations recovered here.

3.5.3 Recombination

Recombination rates in bacterial populations can range from highly clonal to reaching linkage equilibrium (Spratt et al. 1999 and 2001). If *Pseudonocardia* dispersal is restricted by vertical transmission with ant hosts, one predicts restricted opportunities for recombination with other strains. This restriction would be consistent with mutualism evolutionary theory, which predicts that hosts are selected to prevent mixing of genetically different symbionts when competition among symbionts negatively impacts the mutualism (Hoekstra 1987, Frank 1996, Herre et al. 1999, Poulsen and Boomsma 2005). Indeed, such antagonistic interactions between *Pseudonocardia* lineages have been demonstrated in leaf-cutter ants (Poulsen et al. 2007), and would likely have similar

impacts in *Apterostigma* colonies. Vertical transmission also aligns host-symbiont interests because both partners benefit from successful host transmission, in addition to ensuring that offspring initially acquire a single symbiont genotype (Ewald 1987, Bull and Rice 1991, Frank 1996 and 2003, Herre et al. 1999). Consistent with these predictions, *A. dentigerum* associated *Pseudonocardia* showed restricted recombination. Only three recombination events were detected among 71 isolates and across six loci (fig. 2 and RDP/GENCOVE results). The low ratio of estimated recombination ρ=5.065 to mutation θ=56.43 (ρ/θ=0.0898) across all isolates also indicates mutation occurs more frequently than recombination. Within regions, LS provided the most robust estimates of recombination (due to higher sequence variation at that site) and returned an even lower ρ/θ ratio (0.028). These ρ/θ estimates are also very low in comparison to other bacterial species. Among 19 non-Actinobacteria species for which we could obtain estimates of ρ/θ, only one species had lower recombination ρ/θ (Pérez-Losada et al. 2006; de Bakker et al. 2008, Yan et al. 2008, Deletoile et al. 2010, Doroghazi and Buckley 2010). Unfortunately, recent surveys of recombination rates in bacteria have included little or no Actinobacteria, so direct comparisons within this phylum are limited (Vos and Didelot 2009, Pérez-Losada et al. 2006). Nonetheless, among Actinobacteria species in the genera *Bifidobacterium* and *Streptomyces*, ρ/θ values were either one, but in most cases two, orders of magnitude higher than *Pseudonocardia* (Deletoile et al. 2010, Doroghazi and Buckley 2010). Overall, *Pseudonocardia* recombination is substantially restricted compared with other bacterial species.

At this time, we can only conjecture that this restriction is due to association with ant host because we do not have estimates of recombination in free-living

Pseudonocardia. We can, however, draw useful comparisons with free-living *Streptomyces* Actinobacteria because ρ/θ calculations utilized the same six loci as our study (Doroghazi and Buckley 2010). Moreover, this genus has been suggested as an additional associate of attines under the acquisition model (Kost et al. 2007, Haeder et al. 2009). The acquisition model posits that *Streptomyces* bacteria could be acquired regularly from the environment (as would other groups, such as *Pseudonocardia*), and thus recombination rates in ant-associated isolates would mirror that of free-living populations. Interestingly, the spectrum of recombination *Streptomyces* and *A. dentigerum*-associated *Pseudonocardia* are on opposite ends – free-living *Streptomyces* recombination reaches linkage equilibrium. To explain this difference under the acquisition model, free-living *Pseudonocardia* populations are predicted to have a similarly low ρ/θ ratio. Alternatively, it is also possible that *Streptomyces*-attine associations are transient, whereas *Pseudonocardia* associations are specialized. We could not describe the population genetic structure of *A. dentigerum* associated *Streptomyces* here because we only obtained one isolate, further suggesting that attine-*Streptomyces* associations are only transient.

Finally, the rarity of recombination could be driven by a lack of genotype diversity within individual ant nests, thus masking the detection of recombination. The dominance of particular *Pseudonocardia* genotypes recovered here in *Apterostigma*, and a previous study in *Acromyrmex* leaf-cutter ants that found only one genotype per ant nest lends credence to this idea (Poulsen et al. 2005). However, if the low rates of recombination were indeed caused by low detection due to low sequence diversity, that

bottleneck in diversity would arise due to restricted transmission imposed by association with hosts and would remain inconsistent with acquisition model.

3.5.4 Reexamining Horizontal Aquistion: Methodical Considerations

Support for the acquisition model stems, in large part, from the observation that ant-associated *16S* rDNA *Pseudonocardia* genotypes are often identical to cosmopolitan isolates, implying they are horizontally acquired from the environment and not limited to dispersal by their ant hosts. For example, Mueller et al. (2008) and Mueller et al. (2010) showed that ant-associated genotypes have close affinities with free-living *Pseudonocardia*, including isolates from the soil, plants, geographically distant locations, and disparate environments, such as marine sediment near china, industrial sludge from France, Panamanian attine ants, and Argentinean attine ants. Given the lack of sequence variation in *16S* recovered here (fig. 2), and what is known about the level of variation in *16S* (i.e. that it should not be used for identifications below the genus level Cohan, 2006, Staley 2006), it is not surprising that ant-associated genotypes cluster with environmental isolates. These affinities, however, may not reflect exchange with the environment, as additional genomic analyses could reveal higher specificity, just as our multi-locus analysis revealed geographic structure not detectable with a single locus. A recent two-locus (*16S* and *EF-Tu*) phylogenetic study of attine associated *Pseudonocardia* (Cafaro et al. 2011) has revealed higher specificity to attine lineages than the single locus studies in support of the recruitment hypothesis (Mueller et al. 2008 & 2010). Overall, these differences illustrate the importance of genome wide analyses for both phylogenetic and population genetic analyses.

Just as single versus multi-locus analyses have lead to different conclusions about attine-*Psuedoncoardia* specificity, so have different microbial isolation techniques. Studies detecting diverse Actinobacteria genera (e.g. *Streptomyces*) in addition to *Pseudonocardia* have argued that the presence of these additional bacteria negates specificity in the ant-*Pseudonocardia* association (Kost et al. 2007, Mueller et al. 2008). 90% of Actinobacteria isolated from the mesosternal lobes of *A. dentigerum* were *Pseudonocardia* and we take this strong bias as evidence that attines maintain a specialized association with this lineage. Attines house their bacterial symbionts on specialized, genus-specific, locations on the cuticle, and these sites often contain crypts and glands that facilitate bacterial growth (Currie et al. 2006). Actinobacteria are highly concentrated at these cuticular sites such that they are visible to the naked eye, and these high concentrations likely facilitate accumulation of antibiotic compounds. Here, we isolated from the mesosternal lobe because it is the site of visibly high concentrations of Actinobacteria in *Apterostigma*. Studies that did not recover a similar bias towards *Pseudonocardia*, however, utilized less targeted approaches. For example, the original study proposing the acquisition model isolated Actinobacteria bacteria by smearing the entire ventral side of *Acromyrmex* ants across soy agar plates (Kost et al. 2007), although the specialized crypt and gland cells are concentrated on the propleural plate in *Acromyrmex* species (Currie et al. 2006). A subsequent phylogenetic study in support of the acquisition model did not isolate Actinobacteria from the ant's cuticle, but instead used the infrabuccal pellets and fungus gardens of *Atta texana* (Mueller et al. 2008). Because of the different isolation techniques among studies, one should consider whether

isolates obtained from areas other than the specialized cuticular locations represent transient associations.

An additional argument against the specificity of the Attine-*Pseudonocardia* association includes the potential for 'lab specific biases' in the isolation of particular *Pseudonocardia* lineages, which have created false phylogenetic clusters that imply specificity (Mueller et al. 2008; Mueller et al. 2010). In consideration of these potential biases, we incorporated *Pseudonocardia* isolates that were: 1) cultured by different individuals, 2) cultured at different locations, and 3) PCR amplified and sequenced at different facilities. None of these factors determined the phylogenetic or population genetic placement of isolates, thus lab specific biases have not impacted this study.

3.6 Conclusions

Since the discovery of attine-associated bacteria over a decade ago, several studies have examined the coevolutionary dynamics between symbiotic *Pseudonocardia* and their ant host, and over the past five years support for a model of association lacking pairwise specificity has grown. Thus far, these conclusions have been drawn with little to no information about the population level dynamics of *Pseudonocardia*. Our multi-locus population genetic analysis of *A. dentigerum* associated *Pseudonocardia* shows that the geographic scale of population subdivision is smaller than fungal symbionts and overturns previous ideas that *Pseudonocardia* dispersal is greater than its ant host. Uncharacteristically low recombination rates suggest that horizontal interactions are restricted by a symbiotic lifestyle, which is consistent with coevolutionary theory for mutualistic associations. Finally, correlated population structure with the *Escovopsis*

phylotype found most commonly in *Apterostigma dentigerum* gardens suggests that *Pseudonocardia* population structure is shaped, in part, by its role in disease suppression. Our findings support the geographic mosaic of coevolution concept that coevolved dynamics operate within species, but across local populations. Our findings have helped identify local populations and define the geographic scale at which attine-micobe dynamics occur. Future studies of attine-microbe coevolution should seek to operate at this scale.

3.7 Acknowledgements

We are grateful to J.J. Scott for valuable assistance with field collection, G. Suen for identifying housekeeping loci, G. Emmerich for help with DNA sequencing, M. Poulsen for insights into *Pseudonocardia* isolation, members of the Currie Lab for support and discussion, Carol Lee for comments on the manuscript, the Smithsonian Tropical Research Institute (STRI) for providing logistical support in Panama and facilities to work in Gamboa, Autoridad Nacional del Ambiente y el Mar (ANAM) for collection and export permits in Panama, Organization for Tropical Studies (OTS) for logistical support and facilities at La Selva Biological Station, Ministerio del Ambiente y Energia for collection and export permits in Costa Rica. This research was supported by a National Science Foundation (NSF) Career Award (DEB74702) and an NSF Microbial Genome Sequencing grant (MCB0731822) to C.R. Currie. Support to E.J. Caldera included the following: NSF Graduate Research Fellowship, National Institute of Health (NIH) Microbes in Health and Disease Fellowship (NIH National Research Service Award AI55397), OTS Christiane and Christopher Tyson Award, UW-Madison Department of Zoology Graduate Research Grant.

3.8 Supplemental Figures

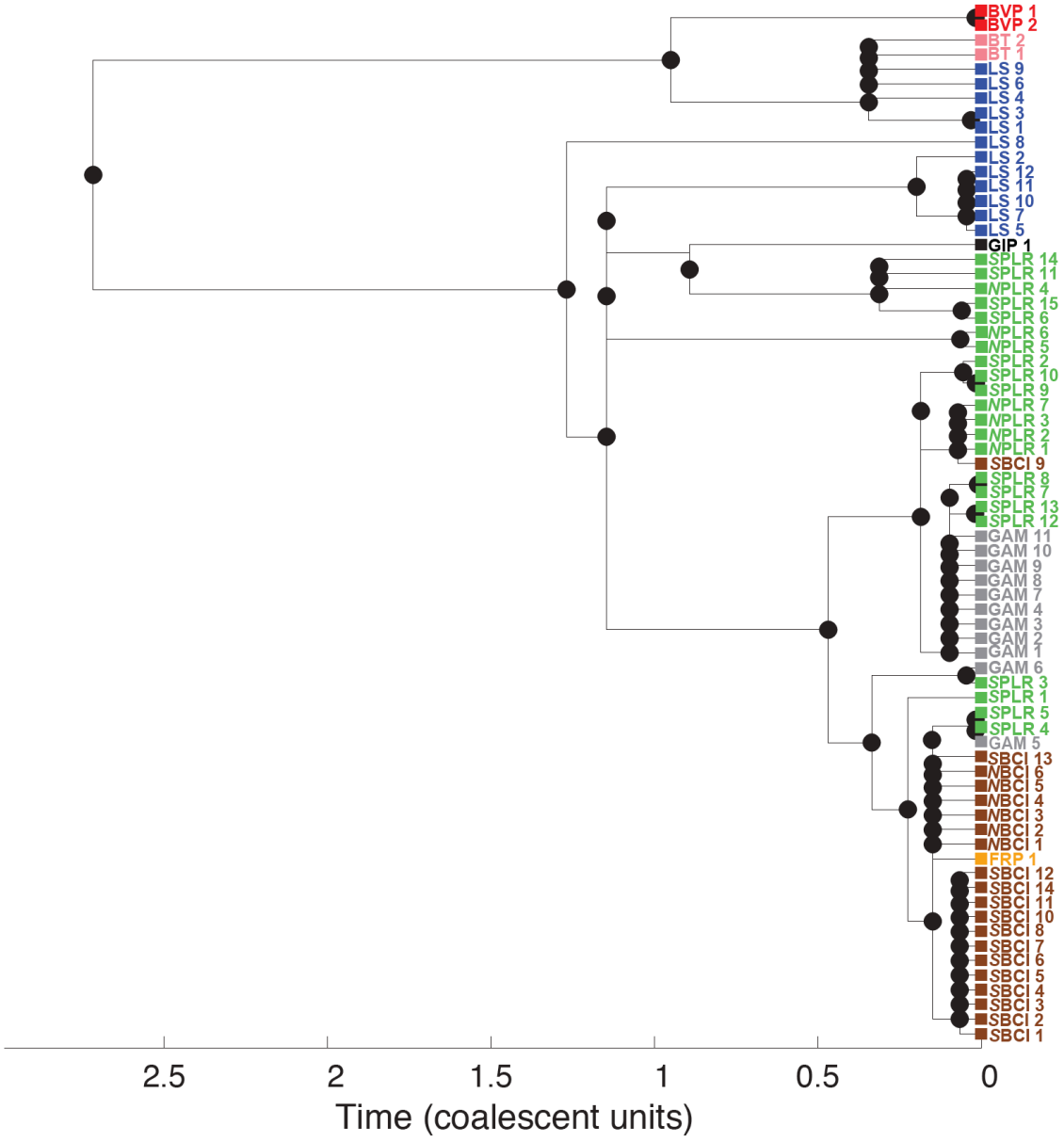


Figure S1. 75% ClonalFrame phylogram of symbiotic *Pseudonocardia*. Because ClonalFrame removes recombination, congruence between the ClonalFrame topology and the Bayesian phylogenetic reconstruction confirms that the phylogenetic reconstruction is accurate.

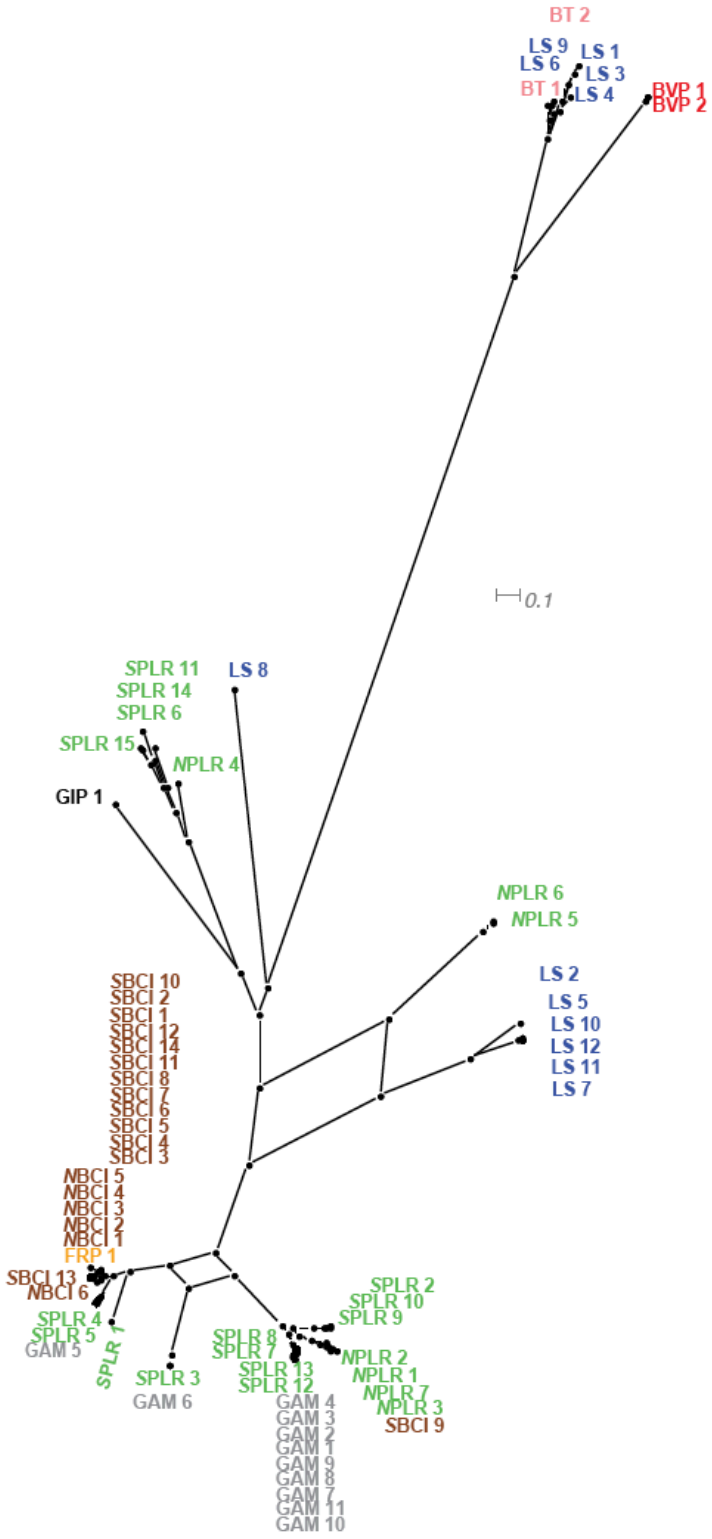


Figure S2. SplitsTree network of Clonal Frame output (1000 phylograms). Branches linking clades were drawn at a 25% threshold.

3.9 References

Adams, R. M. M., U. G. Mueller, A. K. Holloway, A. M. Green, and J. Narozniak. 2000. Garden sharing and garden stealing in fungus-growing ants. Naturwissenschaften 87:491-493.

Armitage, S. A. O., J. F. Broch, H. F. Marin, D. R. Nash, and J. J. Boomsma. 2011. Immune defense in leaf-cutting ants: a cross-fostering approach. Evolution 65:1791-1799.

Buckling, A., and P. B. Rainey. 2002. The role of parasites in sympatric and allopatric host diversification. Nature 420:496-499.

Bull, J. J., and W. R. Rice. 1991. Distinguishing mechanisms for the evolution of cooperation. Journal of Theoretical Biology 149:63-74.

Cafaro, M. J., and C. R. Currie. 2005. Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. Canadian Journal of Microbiology 51:441-446.

Cafaro, M. J., M. Poulsen, A. E. F. Little, S. L. Price, N. M. Gerardo, B. Wong, A. E. Stuart et al. 2011. Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society B: Biological Sciences 278:1814-22.

Caldera, E. J., M. Poulsen, G. Suen, and C. R. Currie. 2009. Insect Symbioses: A Case Study of Past, Present, and Future Fungus-growing Ant Research. Environmental Entomology 38:78-92.

Chapela, I. H., S. A. Rehner, T. R. Schultz, and U. G. Mueller. 1994. Evolutionary history of the symbiosis between fungus-growing ants and their fungi. Science 266:1691-1694.

Cho, J. C., and J. M. Tiedje. 2000. Biogeography and degree of endemicity of fluorescent *Pseudomonas* strains in soil. Applied and Environmental Microbiology 66:5448-5456.

Cohan, F. M. 2006. Towards a conceptual and operational union of bacterial systematics, ecology, and evolution. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1985-1996.

Coyne, J. A., and A. H. Orr. 2004, Speciation. Sunderland, Massachusetts, Sinauer Associates.

Currie, C. R. 2001. Prevalence and impact of a virulent parasite on a tripartite mutualism. Oecologia 128:99-106.

Currie, C. R., A. N. M. Bot, and J. J. Boomsma. 2003a. Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101:91-102.

Currie, C. R., U. G. Mueller, and D. Malloch. 1999a. The agricultural pathology of ant fungus gardens. Proceedings of the National Academy of Sciences of the United States of America 96:7998-8002.

Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81-83.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999b. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701-704.

Currie, C. R., B. Wong, A. E. Stuart, T. R. Schultz, S. A. Rehner, U. G. Mueller, G. H. Sung et al. 2003b. Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299:386-388.

Didelot, X., and D. Falush. 2007. Inference of bacterial microevolution using multilocus sequence data. Genetics. 175:1251-1266.

Deletoile, A., V. Passet, J. Aires, I. Chambaud, M. J. Butel, T. Smokvina, and S. Brisse. 2010. Species delineation and clonal diversity in four *Bifidobacterium* species as revealed by multilocus sequencing. Research in Microbiology 161:82-90.

den Bakker, H. C., X. Didelot, E. D. Fortes, K. K. Nightingale, and M. Wiedmann. 2008. Lineage specific recombination rates and microevolution in *Listeria monocytogenes*. Bmc Evolutionary Biology 8:13.

Doroghazi, J. R., and D. H. Buckley. 2010. Widespread homologous recombination within and between *Streptomyces* species. Isme Journal 4:1136-1143.

Ewald, P. W. 1987. Transmission modes and evolution of the parasitism-mutualism continuum. Annals of the New York Academy of Sciences 503:295-306.

Excoffier, L. 2001. Analysis of population subdivision, Pages 271-307 *in* A. R. Balding, M. Bishiop, and C. Cannings, eds. Handbook of statistical genetics. New York, John Wiley and Sons.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evolutionary Bioinformatics 1:47-50.

Frank, S. A. 1996. Host-symbiont conflict over the mixing of symbiotic lineages. Proceedings of the Royal Society of London Series B-Biological Sciences 263:339-344.

—. 2003. Perspective: Repression of competition and the evolution of cooperation. Evolution 57:693-705.

Gerardo, N. M., and E. J. Caldera. 2007. Labile associations between fungus-growing ant cultivars and their garden pathogens. Isme Journal 1:373-384.

Gerardo, N. M., S. R. Jacobs, C. R. Currie, and U. G. Mueller. 2006a. Ancient host-pathogen associations maintained by specificity of chemotaxis and antibiosis. Plos Biology 4:1358-1363.

Gerardo, N. M., U. G. Mueller, and C. R. Currie. 2006b. Complex host-pathogen coevolution in the Apterostigma fungus-growing ant-microbe symbiosis. Bmc Evolutionary Biology 6.

Green, A. M., U. G. Mueller, and R. M. M. Adams. 2002. Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants. Molecular Ecology 11:191-195.

Guindon, S., and O. Gascuel. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52:696-704.

Haeder, S., R. Wirth, H. Herz, and D. Spiteller. 2009. Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. Proceedings of the National Academy of Sciences of the United States of America 106:4742-4746.

Heath, L., E. van der Walt, A. Varsani, and D. P. Martin. 2006. Recombination patterns in aphthoviruses mirror those found in other picornaviruses. Journal of Virology 80:11827-11832.

Hedlund, B. P., and J. T. Staley. 2004. Microbial Endemism and Biogeography *in* A. T. Bull, ed. Microbial diversity and bioprospecting. Washington, ASM.

Herre, E. A., N. Knowlton, U. G. Mueller, and S. A. Rehner. 1999. The evolution of mutualisms: exploring the paths between conflict and cooperation. Trends in Ecology & Evolution 14:49-53.

Heuer, H., M. Krsek, P. Baker, K. Smalla, and E. M. H. Wellington. 1997. Analysis of Actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Applied and Environmental Microbiology 63:3233-3241.

Hoekstra, R. F. 1987. The evolution of sexes, Pages 59-91 *in* S. C. Stearns, ed. Evolution of sex and its consequences Basel, Birkhäuser.

Hölldobler, B., and E. O. Wilson. 1990, The Ants. Cambridge, MA, Harvard University Press.

Huson, D. H., and D. Bryant. 2006. Application of phylogentic networks in evolutionary studies. Molecular Biology and Evolution 23:254-267.

Kawecki, T. J., and D. Ebert. 2004. Conceptual issues in local adaptation. Ecology Letters 7:1225-1241.

Kost, C., T. Lakatos, I. Bottcher, W. R. Arendholz, M. Redenbach, and R. Wirth. 2007. Non-specific association between filamentous bacteria and fungus-growing ants. Naturwissenschaften 94:821-828.

Kumar, S., M. Nei, J. Dudley, and K. Tamura. 2008. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics 9:299-306.

Lande, R. 1980. Genetic variation and phenotypic evolution during allopatric speciation. American Naturalist 116:463-479.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin et al. 2007. Clustal W and clustal X version 2.0. Bioinformatics 23:2947-2948.

Lenormand, T. 2002. Gene flow and the limits to natural selection. Trends in Ecology & Evolution 17:183-189.

Lively, C. M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. American Naturalist 153:S34-S47.

Martin, D., and E. Rybicki. 2000. RDP: detection of recombination amongst aligned sequences. Bioinformatics 16:562-563.

Mayr, E. 1963, Animal species and evolution. Cambridge, MA, Harvard University Press.

McVean, G., P. Awadalla, and P. Fearnhead. 2002. A coalescent-based method for detecting and estimating recombination from gene sequences. Genetics 160:1231-1241.

Mikheyev, A. S. 2008. History, genetics and pathology of a leaf-cutting ant introduction: a case study of the Guadeloupe invasion. Biological Invasions 10:467-473.

Mikheyev, A. S., U. G. Mueller, and P. Abbot. 2006. Cryptic sex and many-to-one colevolution in the fungus-growing ant symbiosis. Proceedings of the National Academy of Sciences of the United States of America 103:10702-10706.

—. 2010. Comparative Dating of Attine Ant and Lepiotaceous Cultivar Phylogenies Reveals Coevolutionary Synchrony and Discord. American Naturalist 175:E126-E133.

Mikheyev, A. S., U. G. Mueller, and J. J. Boomsma. 2007. Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. Molecular Ecology 16:209-216.

Mikheyev, A. S., T. Vo, and U. G. Mueller. 2008. Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists. Molecular Ecology 17:4480-4488.

Mueller, U. G., D. Dash, C. Rabeling, and A. Rodrigues. 2008. Coevolution between attine ants and Actinomycete bacteria: a reevauation. Evolution 62:2894-2912.

Mueller, U. G., H. Ishak, J. C. Lee, R. Sen, and R. R. Gutell. 2010b. Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (Pseudonocardiaceae, Actinomycetales): a test of two symbiont-association models. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 98:195-212.

Mueller, U. G., S. A. Rehner, and T. R. Schultz. 1998. The evolution of agriculture in ants. Science 281:2034-2038.

Mueller, U. G., J. J. Scott, H. D. Ishak, M. Cooper, and A. Rodrigues. 2010a. Monoculture of Leafcutter Ant Gardens. Plos One 5:7.

Nei, M., F. Tajima, and Y. Tateno. 1983. Accuracy of estimated phylogenetic trees from molecular data . 2. gene-frequency data. Journal of Molecular Evolution 19:153-170.

Oh, D. C., M. Poulsen, C. R. Currie, and J. Clardy. 2009. Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nature Chemical Biology 5:391-393.

Padidam, M., S. Sawyer, and C. M. Fauquet. 1999. Possible emergence of new geminiviruses by frequent recombination. Virology 265:218-225.

Perez-Losada, M., E. B. Browne, A. Madsen, T. Wirth, R. P. Viscidi, and K. A. Crandall. 2006. Population genetics of microbial pathogens estimated from multilocus sequence typing (MLST) data. Infection Genetics and Evolution 6:97-112.

Posada, D. 2008. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution 25:1253-1256.

Poulsen, M., and J. J. Boomsma. 2005. Mutualistic fungi control crop diversity in fungus-growing ants. Science 307:741-744.

Poulsen, M., M. Cafaro, J. J. Boomsma, and C. R. Currie. 2005. Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of Acromyrmex leaf-cutting ants. Molecular Ecology 14:3597-3604.

Poulsen, M., D. P. Erhardt, D. J. Molinaro, T. L. Lin, and C. R. Currie. 2007. Antagonistic Bacterial Interactions Help Shape Host-Symbiont Dynamics within the Fungus-Growing Ant-Microbe Mutualism. Plos One 2.

Poulsen, M., H. Fernandez-Marin, C. R. Currie, and J. J. Boomsma. 2009. Ephemeral windows of opportunity for horizontal transmission of fungal symbionts in leaf-cutting ants. Evolution 63:2235-2247.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572-1574.

Sen, R., H. D. Ishak, D. Estrada, S. E. Dowd, E. K. Hong, and U. G. Mueller. 2009. Generalized antifungal activity and 454-screening of *Pseudonocardia* and Amycolatopsis bacteria in nests of fungus-growing ants. Proceedings of the National Academy of Sciences of the United States of America 106:17805-17810.

Slatkin, M. 1993. Isolation by distance in equilibrium and nonequilibrium populations. Evolution 47:264-279.

Spratt, B. G., W. P. Hanage, and E. J. Feil. 2001. The relative contributions of recombination and point mutation to the diversification of bacterial clones. Current Opinion in Microbiology 4:602-606.

Spratt, B. G., and M. C. J. Maiden. 1999. Bacterial population genetics, evolution and epidemiology. Philosophical Transactions of the Royal Society B: Biological Sciences 354:701-710.

Staley, J. T. 2006. The bacterial species dilemma and the genomic-phylogenetic species concept. Philosophical Transactions of the Royal Society B-Biological Sciences 361:1899-1909.

Taerum, S. J., M. J. Cafaro, A. E. F. Little, T. R. Schultz, and C. R. Currie. 2007. Low host-pathogen specificity in the leaf-cutting ant-microbe symbiosis. Proceedings of the Royal Society B-Biological Sciences 274:1971-1978.

Thompson, J. N. 1994, The coevolutionary process. Chicago, University of Chicago Press.

—. 2005, The geographic mosaic of coevolution. Chicago, University of Chicago Press.

—. 2009. The Coevolving Web of Life. American Naturalist 173:125-140.

Thompson, J. N., and B. M. Cunningham. 2002. Geographic structure and dynamics of coevolutionary selection. Nature 417:735-738.

Vo, T. L., U. G. Mueller, and A. S. Mikheyev. 2009. Free-living fungal symbionts (Lepiotaceae) of fungus-growing ants (Attini: Formicidae). Mycologia 101:206-210.

Vollmer, S. A., A. Bormane, R. E. Dinnis, F. Seeling, A. D. M. Dobson, D. M. Aanensen, M. C. James et al. 2010. Host migration impacts on the phylogeography of Lyme *Borreliosis spirochaete* species in Europe. Environmental Microbiology.

Vos, M., and X. Didelot. 2009. A comparison of homologous recombination rates in bacteria and archaea. Isme Journal 3:199-208.

Weir, B. S., and C. C. Cockerham. 1984. Estimating F-statistics for the analysis of population structure. Evolution 38:1358-1370.

Whitaker, R. J., D. W. Grogan, and J. W. Taylor. 2003. Geographic barriers isolate endemic populations of hyperthermophilic archaea. Science 301:976-978.

Wirth, T., A. Meyer, and M. Achtman. 2005. Deciphering host migrations and origins by means of their microbes. Molecular Ecology 14:3289-3306.

Yan, S. C., H. J. Liu, T. J. Mohr, J. Jenrette, R. Chiodini, M. Zaccardelli, J. C. Setubal et al. 2008. Role of recombination in the evolution of the model plant pathogen Pseudomonas syringae pv. tomato DC3000, a very atypical tomato strain. Applied and Environmental Microbiology 74:3171-3181.

Zanatta, D. T., and C. C. Wilson. 2011. Testing congruency of geographic and genetic population structure for a freshwater mussel (Bivalvia: Unionoida) and its host fish. Biological Journal of the Linnean Society 102:669-685.

Zhang, M. M., M. Poulsen, and C. R. Currie. 2007. Symbiont recognition of mutualistic bacteria by Acromyrmex leaf-cutting ants. Isme Journal 1:313-320.

Zwickl, D. J. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequences datasets under the maximum likelihood criterion, University of Texas at Austin.

4 Chapter 4: The geographic mosaic of antibiotic coevolution in a bacterial symbiont of the fungus-farming ant *Apterostigma dentigerum*: evidence for a coevolutionary hot spot on Barro Colorado Island, Panama

Eric J. Caldera, Marc G. Chevrette, Bradon R. McDonald & Cameron R. Currie

Author contributions: EJC conducted the field work, culture work, multi-locus genotyping and analysis, bioassay experiments, and wrote the paper. MGC facilitated genome sequencing/assembly, characterized biosynthetic gene clusters and edited the paper. BRM facilitated whole genome sequencing/assembly, performed genome wide SNP analysis and edited the paper. CRC facilitated the study and helped write the paper.

4.1 Abstract

Fungus-farming ants use antibiotic-producing bacterial symbionts (genus *Pseudonocardia*) to control specialized ascomycetous pathogens (genus *Escovopsis*) of their basidiomycetous cultivars. The ant-*Pseudonocardia* association is ancient, suggesting that these ants have been using symbiont-derived antibiotics for millions of years. Here, we use bioassay experiments, population genetic analyses, and whole genome sequencing to examine whether symbiont interactions are characterized by a coevolutionary framework. On average *Pseudonocardia* inhibition of rare pathogen morphotypes was significantly lower ($p<0.001$) than common morphotypes across Central America, suggesting pathogen rare-morph advantage. In Petri plate bioassay experiments, *Pseudonocardia* strains from Barro Colorado Island (BCI) in the canal-zone of Panama had higher zones of inhibition (ZIO) to a common *Escovopsis* morphotype from BCI (ZOI=2.65cm) in comparison to mainland parasite populations on pipeline road (PLR, ZOI=1.93, p-value<0.001) and Gamboa forest (ZOI=2.19, p-value-0.007 GAM). *Pseudonocardia*-GAM *Escovopsis* inhibition (ZOI=2.75) was higher only in comparison to GAM *Pseudonocardia*-PLR *Escovopsis* (ZOI=2.23, p-value=0.003) and there was no significant difference in PLR *Pseudonocardia* inhibition regardless of parasite population. BCI population structure showed elevated Fk in comparison to all other populations. Genome sequencing identified the chemical biosynthetic potential from 30 families of secondary metabolite, biosynthetic gene clusters (BGCs), six of which were unique to BCI. We found no significant correlation between BGC inhibition and parasite location. We detected significantly elevated Fst in a bactriocin BGC on BCI, suggesting a selective sweep. Our results indicate that *Pseudonocardia-Escovopsis* dynamics conform

to a theoretical framework called the geographic mosaic of coevolution with a “hot spot” of local adaptation on BCI. Further, this study implies that the long-term maintenance of antibiotic potency against parasites relies on evolutionary genetic changes within specific BGCs and not simply the acquisition of novel genetic elements.

4.2 Introduction

Coevolution shapes the diversity of life, as the multitude of species on Earth engage in complex webs of antagonistic and mutualistic interactions that undergo reciprocal selection. Most studies of coevolutionary dynamics have focused on interactions at the extremes of this spectrum. Within-population studies described pairwise interactions between a single population of one species and a single population of another species (e.g., Thompson 1994, Carius et al. 2001; Brodie et al. 2005). Coevolutionary studies at a deeper resolution have explored patterns of phylogenetic congruence among interacting species (e.g., Hafner & Ladner 1988; Chapela 1994; Moran et al. 1994; Currie et al. 2003). In contrast, the geographic mosaic theory of coevolution (GMC) posits that what we perceive at the phylogenetic scale is the sum of local coevolutionary processes that vary over geographic space (Thompson 2005). An important utility of the GMC is that it provides a framework that incorporates both components of population biology and species level interactions. Central to the GMC is the assumption that populations are the basic units of coevolutionary dynamics, and the genetic differentiation of host and symbiont populations over space has impacts on the emergence of geographic selection mosaics (Thompson & Cunningham 2002).

The attine ant fungus-farming ant symbiosis provides a unique opportunity to test components of the GMC in a multi-partite system where our understanding of the

underlying population genetic structure of multiple microbial symbionts continues to take shape. Ants of the monophyletic tribe Attini cultivate basidiomycetous fungi in an agricultural system that is analogous to human farming (Mueller et al. 1998). Like nearly all human crops, these fungal cultivars have low genetic diversity (Mueller et at. 2010a) and face higher incidence of infection (Currie et al. 1999a). Specialized ascomycetous pathogens in the genus *Escovopsis* form persistent and chronic infection throughout most of the lifecycle of a given ant colony (Currie et al. 1999a; Currie 2001). *Escovopsis* is capable of rapidly overwhelming and consuming the entire fungal garden if infection is not maintained at a low level. Thus, the *Escovopsis* parasite imposes a persistent and strong selection pressure. To combat *Escovopsis* infection, the ant hosts perform generalized defense behaviors, such as garden weeding, to physically remove infected portions of the cultivar and/or *Escovopsis* spores (Currie and Stuart 2001). These behaviors mediate pathogen infection, but are not sufficient for maintaining healthy fungus gardens. As such, the ants also chemically defend against *Escovopsis* through a symbiotic association with antibiotic-producing Actinobacteria (Currie et al. 1999b; Currie et al. 2003; Oh et al. 2009; Poulsen et al. 2010). Although fungal cultivars are the primary target of *Escovopsis*, an indirect and perhaps equally strong, selection pressure is also imposed on the (secondary) ant hosts, who cannot survive without the cultivar, and the (tertiary) bacterial symbionts, which reside within specialized structures on the ant exoskeleton (Currie et al. 2006). Interactions are summarized in table 1.

Table 1. Summary of microbial symbiont interactions associated with the fungus-farming ant *Apterostigma dentigerum.*

Mutualists	Parasite Target	Parasite Interaction
Fungal Cultivar	Primary	Labile
Ant Host	Secondary	General
Pseudonocardia	Tertiary	Specific

Symbionts of the phylogenetically basal attine ant genus *Apterostigma* are particularly poised for examining coevolutionary dynamics, as much is known about interactions at both the phylogenetic and population levels. Pair-wise inhibition assay experiments challenging different fungal cultivars with different *Escovopsis* strains across this genus found that fungal cultivars appear relatively poor at inhibiting *Escovopsis* morphotypes from their native host species but are able to inhibit non-native strains that occur sympatrically in other *Apterostigma* species (Gerardo et al. 2006a). These interspecific interactions provide a mechanism by which specificity and congruence among symbionts is maintained at a phylogenetic scale. A subsequent study of *Apterostigma dentigerum*-associated cultivars and a single *Escovopsis* morphotype (henceforth referred to as 'brown') also examined these interactions, but conducted parasite-cultivar switches among geographic locations to elucidate coevolutionary dynamics at the population level (Gerardo and Caldera 2007). There was a lack of congruence in the symbiont population genetic structures. Bioassay interactions only yielded on example of inhibition among 36 (i.e., neither genotype nor geography had any bearing on this interaction at the population level). The labile association between *A. dentigerum* cultivars and brown *Escovopsis* is not likely to foster coevolution.

If cultivars, the primary target of *Escovopsis*, are seemingly ill-equipped to combat their most common pathogen, are the symbiotic, antibiotic-producing, Actinobacteria (*Pseudonocardia*) instead capable of local adaptation to populations of brown *Escovopsis*? Our finding of congruent *Pseudonocardia-Escovopsis* population genetic structure (Caldera & Currie 2012) may indeed facilitate local adaptation (Lively et al. 1999; Lively & Dybdahl 2000), but see (Mikheyev 2007, Mueller et al. 2008, Mueller et

al. 2010b). Here, we use bioassay experiments to examine whether the population level *A. dentigerum* - associated *Pseudonocardia-Escovopsis* interaction conforms to a GMC. First, we establish whether *Pseudonocardia* - *Escovopsis* interactions are fixed across the canal-zone of Panama (compared to cultivar - *Escovopsis* interactions), and whether the scale of symbiont selection is occurring within ant colonies. Second, we test for parasite rare morph advantage in *Pseudonocardia* inhibition against *Escovopsis* morphotypes. Third, we describe the broad geographic differences in *Pseudonocardia* antibiotic inhibition across Central America while also testing for coevolutionary hot spots of local adaptation predicted by the GMC. Finally, to begin understanding the specific secondary metabolite and genetic mechanisms conferring antibiotic inhibition, we sequenced the genome of a subset (30) of *Pseudonocardia* symbionts and discuss the role biosynthetic gene clusters play in *Pseudonocardia-Escovopsis* coevolution.

4.3 Materials and Methods

4.3.1 Symbiont collection and isolation

We sampled *Pseudonocardia* and *Escovopsis* symbionts associated with the fungus–growing ant *Apterostigma dentigerum* throughout Central America, including La Selva Biological Station in Costa Rica (LS), and populations in the canal–zone of Panama: Barro Colorado Island (BCI), Pipeline Road (PLR), Gamboa Forest (GAM), Frijoles Peninsula (FRP), and Buena Vista Peninsula (BVP), from 2006 through 2010. Ant colonies were collected aseptically in the field, using flame sterilized forceps and spoons to reduce the risk of lateral transfer of symbionts between nests. John T. Longino's Key to Costa Rican *Apterostigma* was used to identify *A. dentigerum* species. *Pseudonocardia*

isolations were conducted by first removing the ants head and forelegs to expose the mesosternal lobes where the bacteria are concentrated. We then used fine, sterilized probes to scrape small tufts of bacteria onto chitin media. For details on *Pseudonocardia* isolation from *A. dentigerum* see Caldera & Currie (2012). To isolate parasites, we plated pieces of fungal cultivar onto PDA media and sub–cultured *Escovopsis* spores as they emerged, following Gerardo and Caldera 2007. *Escovopsis* were sampled from the same geographic range as *Pseudonocardia* in the Panama canal–zone but with additional *Escovopsis* sampling of *Apterostigma sp*. from the Los Amigos Station in Peru (PU). In total, we accumulated 50 *Pseudonocardia* strains and 55 *Escovopsis* strains. BCI, PLR, and GAM were used for within-population versus across-population tests for local adaptation, as they were the most thoroughly sampled.

4.3.2 Bioassay Analysis

To quantify *Escovopsis* inhibition by *Pseudonocardia* we performed bioassay experiments pairing each of the two symbionts and measured the zone of inhibition (ZOI). The *in vitro* ZOI corresponds to fungus garden loss in higher attines, and thus provides a valuable proxy for the impact of *Escovopsis in vivo* (Poulson et al. 2010). Petri plate bioassay experiments and statistical comparisons for local adaptation followed Cafaro et al. 2011, as summarized here: *Pseudonocardia* was point inoculated in the center of a YMEA plate and allowed to grow for three weeks, after which *Escovopsis* was inoculated at the edge of the plate. Plates were inspected bi–weekly and when a clear zone of inhibition had formed in a given pairing (typically within two to three weeks after fungus inoculation), the minimum zone of inhibition (ZOI) was measured. We used two–

tailed t–tests in pairwise comparisons of within–population, and cross population *Escovopsis–Pseudonocardia* pairings. P–values were adjusted using Bonferroni correction to reduce type I error in multiple comparisons. F–tests were performed to establish whether individual t–tests should be performed with equal or unequal variances. To examine whether colony–level symbiont selection is occurring, in 2008 we performed a bioassay utilizing eight ant colonies from the canal–zone of Panama for which we isolated both *Pseudonocardia* and *Escovopsis* Here we compared within-colony and between-colony inhibition. In 2009, we performed a bioassay pairing *Pseudonocardia* from across Costa Rica and Panama against three *Escovopsis* test strains representing each of three *Escovopsis* morphotypes (brown, yellow and fuzzy brown). These *Escovopsis* morphotypes represent distinct phylogenetic clades and are taken to represent species (Gerardo et al. 2006 BMC). Here we make the first reference to the "fuzzy brown" morphotype, although it has been noted by attine researchers since the late 1990s. Finally, in 2010 we performed a bioassay pairing all *Pseudonocardia* and *Escovopsis* morphotypes available in actively growing cultures.

4.3.3 Population Genomics

To describe the population genetic structure of *Pseudonocardia* we used the Bayesian program STRUCTURE (Pritchard et al. 2000; Falush et al. 2003), which assigns genetic populations (K) to strains without prior information about sampling location. *Pseudonocardia* strains were previously genotyped at six housekeeping genes (Caldera & Currie 2012). We treated each polymorphic site (355 total) as an individual locus and simulations were run with both no–admixture and admixture/linkage models. The

admixture model assumes that each individual derives ancestry from only one population and is most appropriate for discrete populations. For the no–admixture model, we assumed a constant λ and correlated allele frequencies, which improves clustering for closely related populations. The linkage model allows for individuals to have mixed ancestry (i.e. admixture), but also accounts for linkage among loci. This is important because STRUCTURE utilizes linkage disequilibrium to define population structure. Following Falush et al. (2003) the linkage model assumed correlated allele frequencies, estimated λ and treated polymorphic sites within genes as linked, with linkage being proportional to genetic distance in base pairs. The appropriate K was selected by observing the K that generated the highest likelihood score, and by calculating the ΔK (Evanno et al. 2005). All simulations were run for K =1 to K =20 with 20,000 iterations following a burn–in period of 10,000. Each run was repeated five times to assess consistency and facilitate ΔK calculations.

To begin understanding the genetic mechanisms of antibiotic resistance to *Escovopsis*, we sequenced a subset (30) of the *Pseudonocardia* strains used in bioassay experiments and characterized the presence of conserved biosynthetic gene clusters. Sequencing of *Pseudonocardia* strains was performed by Duke University or Washington University in St. Louis. Pacific Biosciences (EC080625–04, Duke) assemblies utilize HGAP 1.4 (Chin et al., 2013); whereas, Illumina (Wash. U.) genomes were assembled using Velvet (Zerbino and Birney, 2008). Prodigal v2.60 (Hyatt et al., 2010) was used for protein prediction; whereas, RFAM (Nawrocki et al., 2015) hidden markov models and Infernal 1.1.1 (Nawrocki and Eddy, 2013) were used for ribosomal RNA prediction. Annotation of protein coding genes was through TIGRFam v15 (Haft et al., 2013),

PFAM v29, KEGG, and actNOG (Powell et al., 2012) hidden markov models and HMMer 3.1 (Eddy, 2011). In each genome, secondary metabolite clusters were identified by antiSMASHv3 (Blin et al., 2013) with manual curation of cluster boundaries and grouped into families by 80% nucleotide identity via nucmer alignment and 50% coverage for each segment of a cluster that matched another. For additional detiail on the above methods see McDonald et al. 2016 (dissertation). To assess extent to which the presence/absence of specific secondary metabolite clusters contributes local adaptation we calculated Pearson correlation of BCG with ZOI by location.

For whole genome analysis, all Illumina genome assemblies were aligned to the PacBio EC080625–04 assembly using nucmer (Kurtz et al. 2004). Reference SNP positions covered by a contig for every genome were used for fineSTRUCTURE analysis to avoid missing data for any SNP positions. Multiple runs with varying values of c and estimated population size had little effect on overall strain clustering, except for very high values of c merging neighboring clusters together.

4.4 Results

In our eight by eight bioassay experiment pairing strains of *Pseudonocardia* and brown *Escovopsis* across the canal-zone of Panama, we found that all bacterial strains exhibited the ability to inhibit strains of the garden pathogen and that there is variation in the degree of inhibition (fig. 1b). This contrasts with pairings of the fungal cultivar and brown *Escovopsis*, where in 35 of 36 combinations no sign of pathogen inhibition was observed (fig. 1a). In the *Pseudonocardia-Escovopsis* pairings, there was no significant difference (p-value = 0.8533) in zone of inhibition distance (or variance p-value = 0.393)

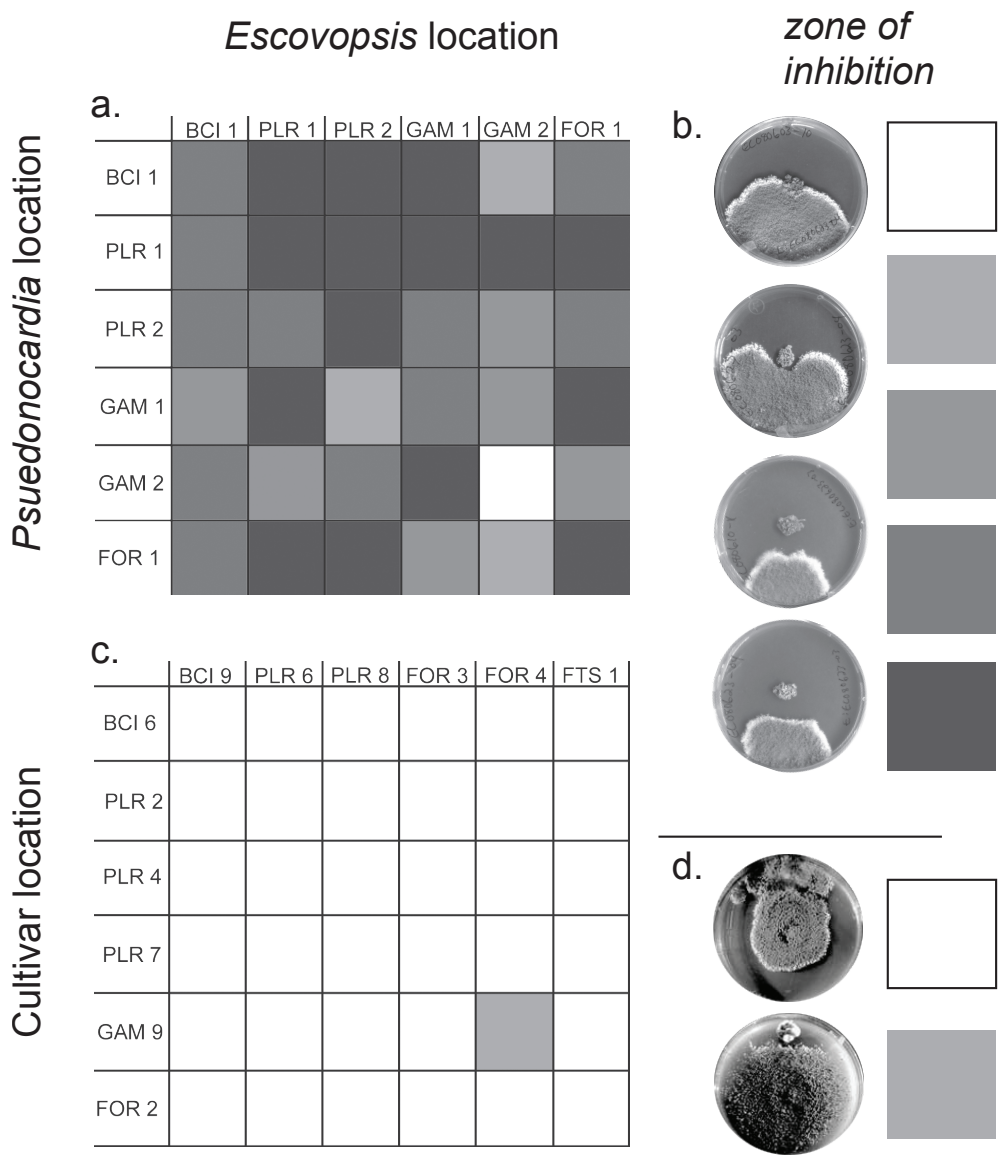


Figure 1. Trait mismatches in *Pseudonocardia-Escovopsis* inhibition across Panama. (a). A lack of differentiation between within-colony (i.e. along the diagonal of panel a) and among-colony interactions refutes colony-level symbiont selection. In contrast, cultivars have little antibiotic inhibition towards brown *Escovopsis* (b. adapted from Gerardo and Caldera, 2007). Darker shades indicate a greater zone of inhibition (ZOI) against *Escovopsis* in Petri plate bioassays. The horizontal line separates *Pseudonocardia* interactions (b. above) and cultivar interactions (d. below).

between within-colony parings (mean 0.882, SE 0.209) and among-colony parings (mean 0.847, SE 0.074).

A test strain bioassay pairing each of three *Escovopsis* morphotypes (brown, yellow, and fuzzy brown) against *Pseudonocardia* from across Panama and Costa Rica revealed

significant hierarchical inhibition in paired t-tests: brown was inhibited more than yellow, and yellow was inhibited more than fuzzy brown (fig. 2). The average ZOI against yellow and fuzzy brown was significantly lower than brown across all comparisons (fig. 3a). These inhibition averages correlated with the relative abundances of *Escovopsis* morphotypes in Central America (fig. 3b).

Mean inhibition by Panamanian *Pseudonocardia* was highest against *Escovopsis* from BCI (ZOI=1.50) and GAM (ZOI=1.55), significantly lower against PLR (ZOI=0.980, P-value<0.001) and lower still against LS (0.317696176, p-value<0.001; fig. 4). Within-population versus among-population comparisons showed that the *Pseudonocardia* population on BCI is locally adapted - BCIXBCI ZOI (2.65cm) is greater than cross population comparisons (BCIXPLR ZOI=1.93, p-value<0.001, BCIXGAM ZOI=2.19, p-value-0.007). PLR *Pseudonocardia* inhibition was similar regardless of parasite population (PLRXPLR ZOI= 1.539, PLRXBCI ZOI=1.532, PLRXGAM ZOI=1.435). GAM *Pseudonocardia* was locally adapted only in comparison to PLR (GAMXGAM ZOI=2.750, GAMXBCI ZOI=2.59, GAMXPLR ZOI=2.23, p-value=0.003; fig. 5, table 2).

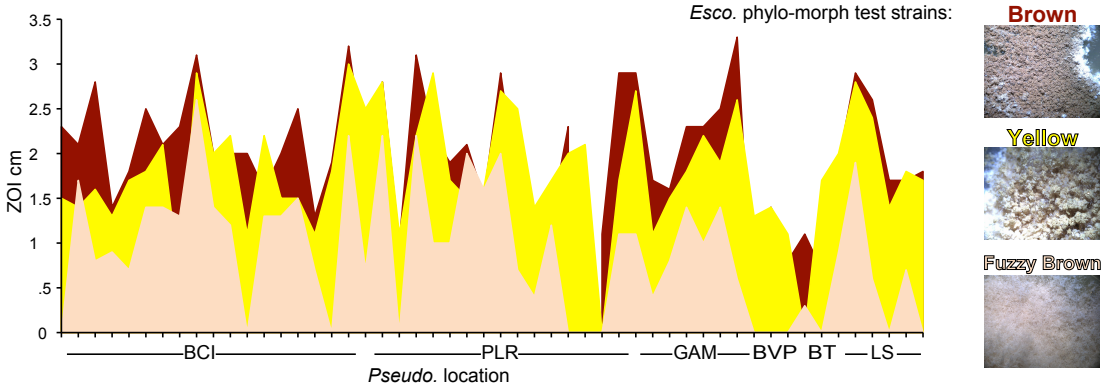


Figure 2. *Pseudonocardia* hierarchical inhibition of three parasite morphotypes Central America. Brown, yellow and fuzzy brown morphotypes represent phylogenetic species. Brown *Escovopsis* inhibition was greater than yellow, which was greater than fuzzy brown in paired t-tests. ZOI corresponds to the length of a zone of inhibition in Petri-plate bioassay experiments.

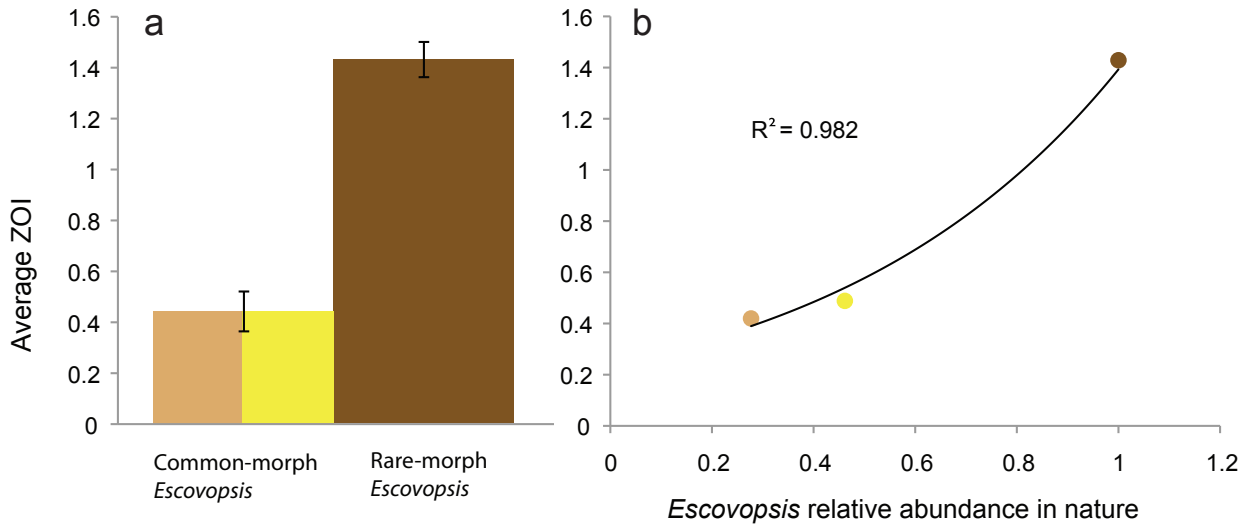


Figure 3. Rare *Escovopsis* pathogen morphotypes experience less inhibition by *Pseudonocardia*. (a) The strength of inhibition corresponds to parasite morphotype abundance in nature, suggesting that parasites experience rare morph advantage (b).

Table 2. Summary of microbial symbiont interactions associated with the fungus-farming ant *Apterostigma dentigerum*

Comparison	F-test p-value	T-test p-value
BCI-BCI X BCI-PLR	0.0001359	8.668e-10
BCI-BCI X BCI-GAM	0.000244	0.0006994
PLR-PLR X PLR-BCI	0.3242	0.9571
PLR-PLR X PLR-GAM	0.6099	0.4747
GAM-GAM X GAM-PLR	0.02777	0.00298
GAM-GAM X GAM-BCI	0.5045	0.3731

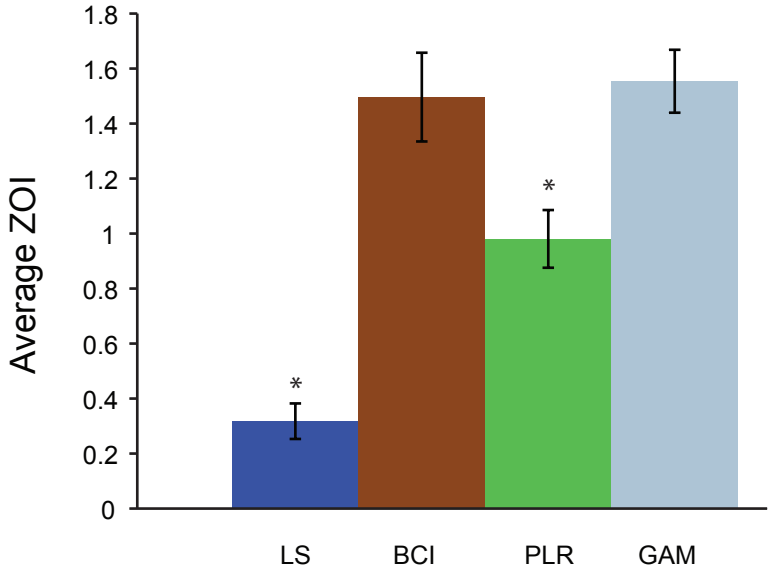


Figure 4. A mosaic of *Escovopsis* inhibition. Panamanian *Pseudonocardia* inhibition of *Escovopsis* from Panama and La Selva biological station in Costa Rica. * indicates mean zone of inhibition was significantly different from all other pairings.

Supplementary figure 1 presents the results of STRUCTURE simulations. K=2 yielded the highest ΔK values (Evanno et al. 2005) for both the linkage (fig. 4a) and no-admixture (not shown) models. Output for both of these models was virtually identical with the exception of admixture in five isolates. These two populations corresponded to the two major clades observed by phylogenetic analysis. As stated in the STRUCTURE documentation, selecting the appropriate K can be difficult when populations conform to isolation by distance (see Caldera & Currie 2012). The highest likelihood scores for the linkage model were observed for K=6 (fig. 4b). For the no-admixture model, Ks larger than six yielded higher likelihood scores; however, the output appeared misleading. For both the linkage and no-admixture models, Ks larger than six did not find additional structure or assign individuals to different populations, but rather only added minimal probability of association to multiple populations for a few isolates. Thus, we also present K=6 for the no-admixture model (sup. fig. 1c). Similar population clusters were identified for both models; however, the linkage model captured a greater distinction between LS, Costa Rica and the Panamanian isolates, and identified a genetic cluster within NPLR that appears admixed with SPLR and GAM. The linkage model identified the sole isolate from GIP as being highly admixed. Finally, for comparison with local adaptation bioassay tests, BCI, PLR and GAM population structure is presented in figure 5e. The subset of fineSTRUCTURE genomic analysis similarly recovered six genetic populations (above), although fineSTRUCTURE was able to separate out a northern region of BCI in contrast to the 6-locus analysis.

Genomic sequencing identified 30 families of secondary metabolite BGCs families. Seven BGCs occurred within a single population; BCI had six unique BGCs and

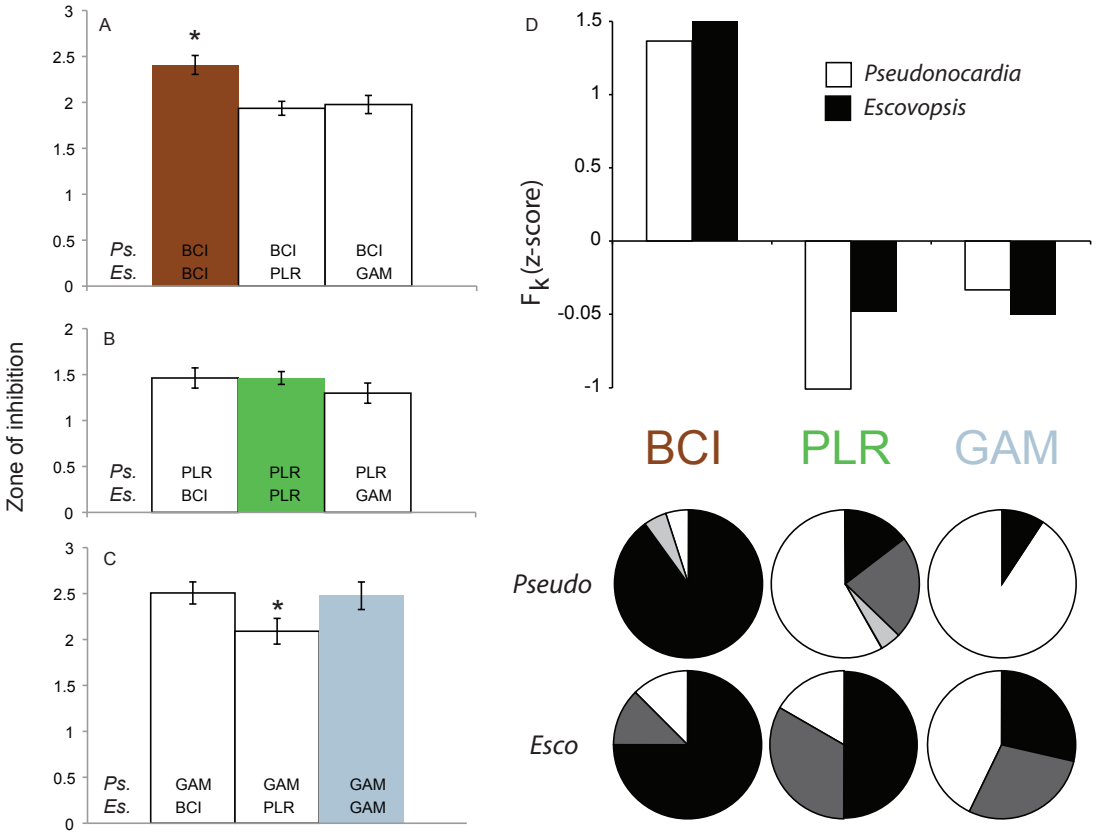


Figure 5. Reciprocal cross tests for local adaptation on (a) BCI, (b) PLR and (c) GAM, with significant local adaptation on BCI. * indicates mean zone of inhibition was significantly different from the other two pairings in the cross. Population STRUCTURE analysis demonstrates that *Escovopsis* and *Pseudonocardia* are genetically distinct populations on BCI, having elevated Fk (d) and greater than 75% or their isolates assigned to one genetic cluster (e).

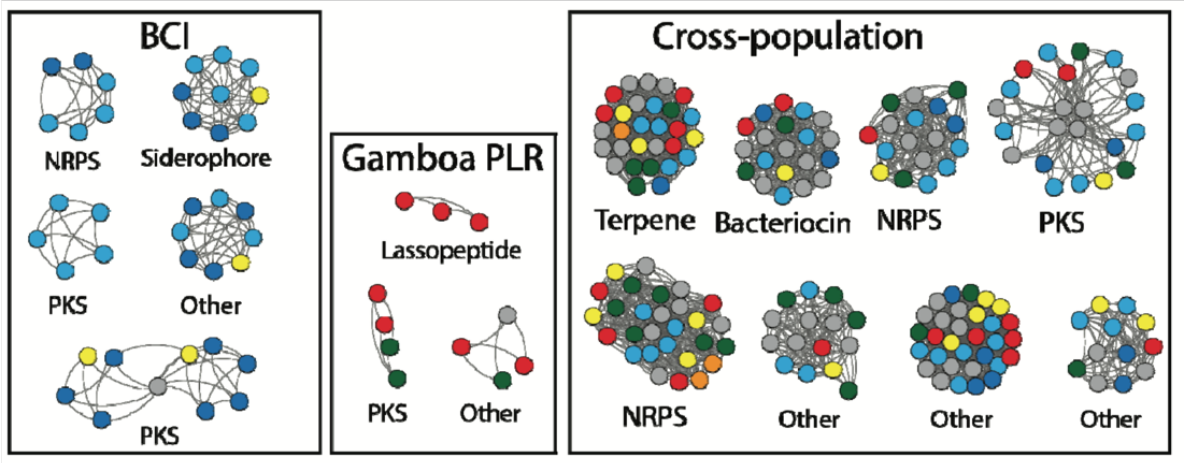


Figure 6. Characterization of biosynthetic gene cluster families in symbiotic *Pseudonocardia* spanning Panama and Costa Rica. Each node resents a contiguous set of genes that are part of a secondary metabolite biosynthesis cluster. Edges represent >80% nucleotide sequence identity and >50% coverage between gene cluster sets. Nodes are colored by their population identification based on fineSTRUCTURE analysis.

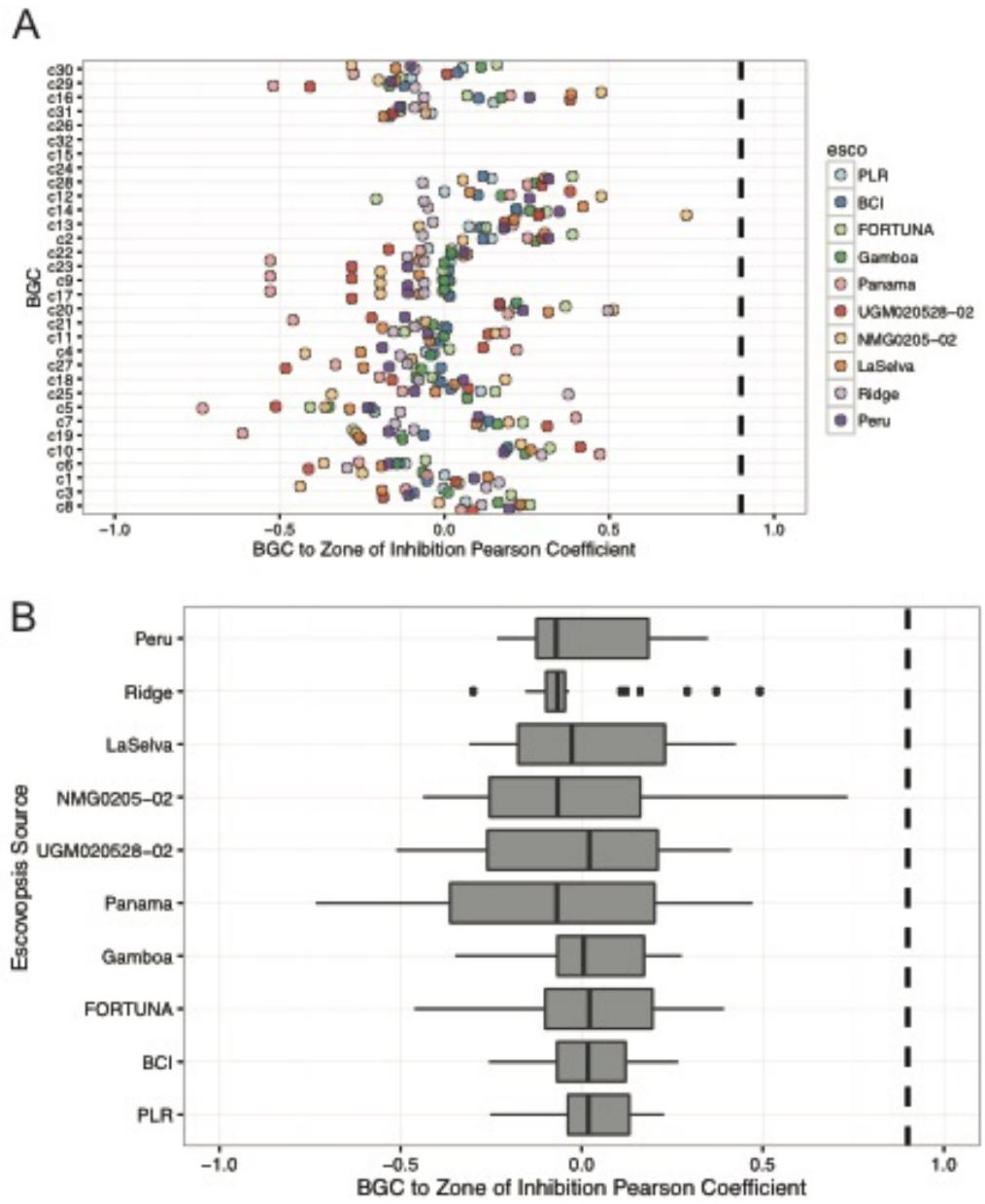


Figure 7. Pearson correlations between the presence/absence of a biosynthetic gene cluster and the observed *Escovopsis* zone of inhibition by *Pseudonocardia*. Groupings based on either BGC (a) or *Escovopsis* source (b) do not reach significance.

GAM had one (fig. 6). A siderophore and a type 2 polyketide were recovered exclusively on BCI. An aminoglycoside was biased towards BCI – seven of the eight occurrences occurred on the island population. GAM and PLR specific BGCs include a lassopeptide found only in isolates from GAM and the southern part of PLR. A nonribosomal peptide was found only in northern PLR. Finally, there was no significant pearson correlation between BGC presence/absence and inhibition by location (fig. 7). Tests for selective sweeps identified at least two BCGs with significant Fst in BCI-mainland comparisons. A bacteriocin appears to have signifiacnt Fst in the following three enzymatic regions (an Acetyl-CoA Carboxylase, a SufE [Cysteine Desufuration] and a Sulfurtransferase; fig. 8a). Figure 8b shows an NRPS BGC had an Fst peak in a cyclization domain.

4.5 Discussion

Pseudonocardia are important mutualist to fungus-farming ants; helping protect their fungal cultivars from the garden pathogen *Escovopsis*. Attine ant use of antibiotic-producing bacteria appears ancient, based on worker ants having glands and exoskeletal structures for maintaining the symbiont and the congruent population structure and co-diversification patterns (Currie et al. 2006; Caldera & Currie 2012; Cafaro et al. 2010). Variation in the antimicrobial inhibitory activity across clades of *Pseudonocardia* suggests possible selection across evolutionary time (Poulson et al. 2010). Here, our population-level study of *Apterostigma dentigerum*–associated symbionts provides evidence that *Pseudonocardia* are under selection to adapt to their community of pathogen species, and are evolving in a manner consistent with the geographic mosaic theory of coevolution at a population scale.

A fundamental concept of the GMC is that coevolved interactions are variable and rarely fix within species. Our initial eight by eight bioassay experiment (fig. 1b) is consistent with this postulation; we observed variation in the interactions across strains of both *Pseudonocardia* and *Escovopsis.* This contrast previous work on the fungal cultivar that identified no variation (fig. 1a), and virtually no indication that the fungi are able to defend themselves from the pathogen *Escovopsis.* These results set up a GMC framework as it helps rule out within-colony recruitment of *Pseudonocardia*. If ants were frequently recruiting free-living *Pseudonocardia* with antibiotics effective against pathogens, one

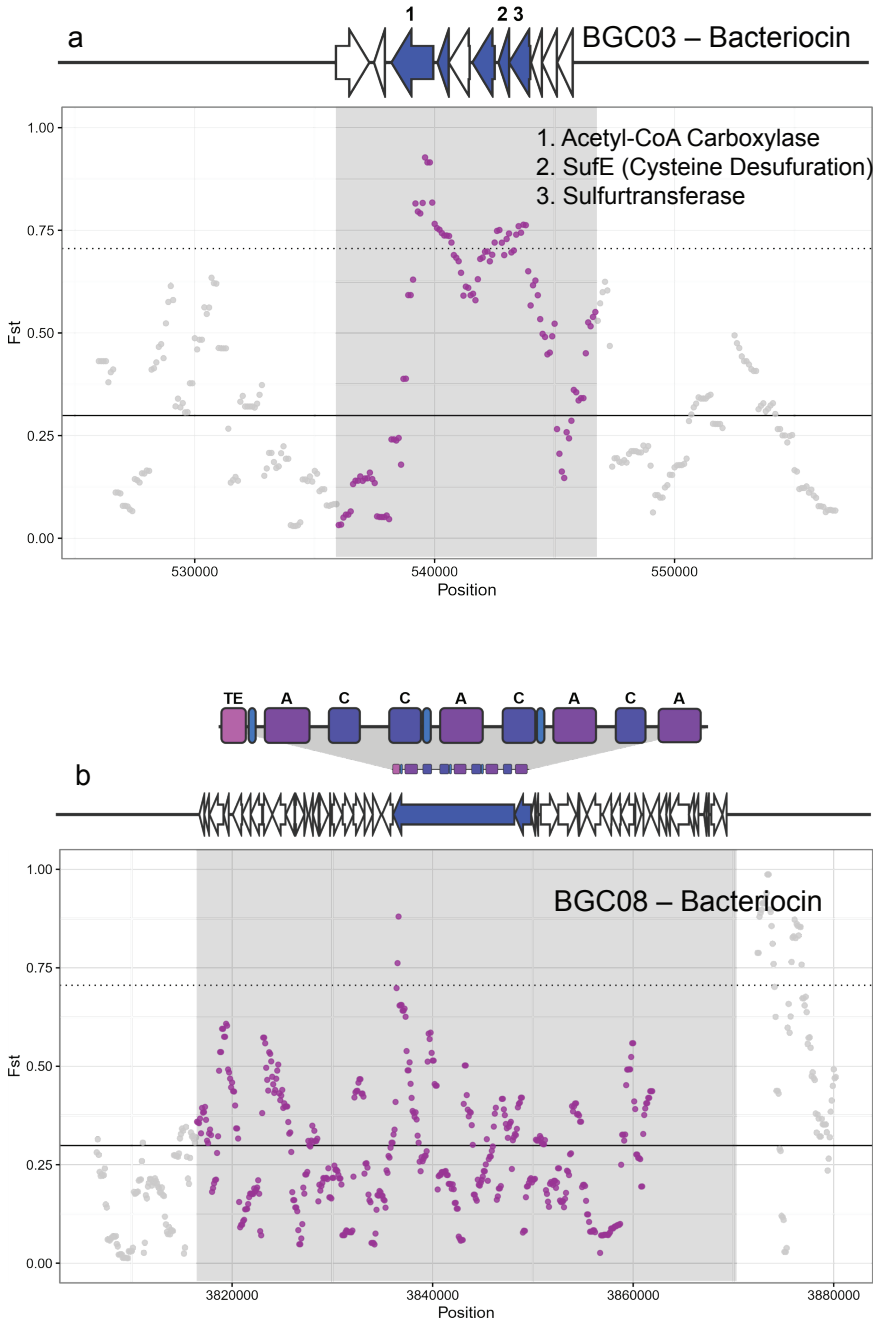


Figure 8. Potential selective sweeps in biosythetic gene clusters. a) Significant Fst within three enzymatic bacteriocin regions in a population of *Psudonocardia* symbionts on Barro Colorado Island, Panama. b) Fst in an NRPS cluster cyclezation region. Comparisons reflects BCI versus all other Central Amerian *Pseudonocardia*.

would expect elevated parasite inhibition within colonies versus between colonies. In fact, within-colony versus between-colony average inhibition and variance were nearly identical. Ruling out colony level symbiont selection suggests that the cross-population level is the appropriate scale for examining *Pseudonocardia-Escovopsis* evolution. Indeed, our larger cross-population assay revealed a geographic mosaic of *Pseudonocardia* antibiotic inhibition across genetically distinct populations in Central America (fig. 4). Moreover, low inhibition by Costa Rican *Pseudonocardia* to Panamanian *Escovopsis* suggests a degree of geographic specialization between the two countries. Taken together, these results provide evidence of predicted empirical patterns of the GMC, including spatial variation in traits mediating interactions among species, trait mismatching among interacting species, and few species-level coevolved traits. These patterns support a GMC between *Pseudonocardia* and *Escovopsis* in *Apterostigma dentigerum*. However, they do not alone provide unequivocal support for the theory, as a thorough demonstration of a GMC requires evidence of selection mosaics and particularly the identification of hot and cold spots of adaptation (Gomulkiewicz et al. 2007). The GMC predicts that populations undergoing reciprocal adaptation (i.e. coevolutionary hot spots) are often nested within cold spots lacking adaptation, in part due to incongruences in population structure (Gibert et al. 2013). Our finding of local adaptation on BCI, but partial adaptation in GAM, and no signature of local adaptation on PLR, thus provides a critical piece of evidence that *Pseudonocardia – Escovopsis* dynamics conform to a GMC (Gomulkiewicz et al. 2007). Local adaptation on BCI is also consistent with predictions that congruent population structure facilitates reciprocal adaptation. *Pseudonocardia* and *Escovopsis* represent distinct genetic populations on

BCI, as they both had an elevated Fk (fig 5d). For comparison, a seminal study of local adaptation across three lake populations of snails and their trematode parasites found local adaptation in all three populations (Lively 1998). Because lakes serve as barriers to gene flow, they helped create the distinct genetic structure that facilitates local adaptation. Anthropogenic disturbances can also erect genetic barriers that facilitate local adaptation (Singer and Thomas 1996). The flooding of the Panama Canal, which created BCI approximately one hundred years ago, may have facilitated local adaptation by creating a barrier to both ant dispersal and *Escovopsis* transmission. Similarly, GAM has an "island nature" that may be facilitating partial local adaptation. GAM forest is nested within the town of Gamboa, and is thus surrounded by paved streets and homes in addition to being surrounded by water on three sides. These subtler anthropogenic barriers may be sufficient to restrict ant dispersal, resulting in the relatively distinct GAM *Pseudonocardia* clade, but not *Escovopsis*, which contains multiple genetic populations within GAM (fig. 5e).

In our attempt to identify signatures of *Pseudonocardia* adaptation to *Escovopsis*, we sought to identify parasite rare morph advantage, a form of negative frequency dependent selection common in host–parasite systems whereby rare parasite have an advantage because hosts have not yet evolved resistance (Takahshi & Kawata 2013). Our test strain bioassay demonstrated that *Pseudonocardia* have different inhibition to various *Escovopsis* morphotypes at this scale, and our large bioassay confirmed that rare parasites experience significantly less inhibition by *Pseudonocardia*. It is worth noting that we previously isolated two *Streptomyces* strains from *A. dentigerum* (Caldera & Currie 2012), and while these isolates had high inhibition to *Escovopsis*, they did not have

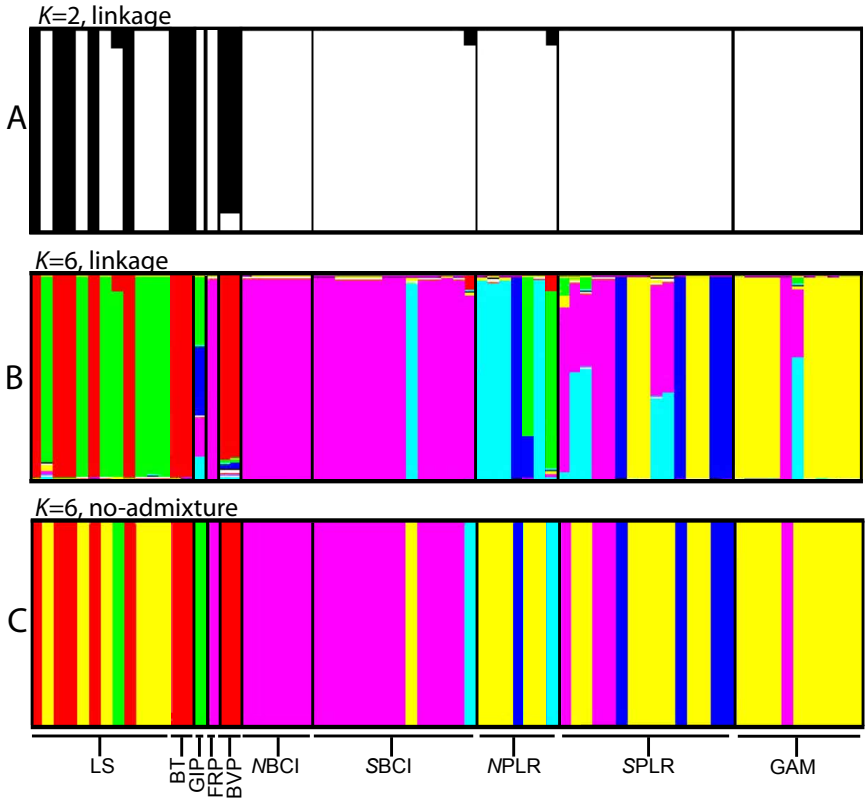
hierarchical inhibition of pathogen morphotypes, suggesting that the occasional observations of attine-associated *Streptomycete* associations may be transient and not coevolved (c.f., Barke et al. 2010).

Genomic analysis revealed extraordinary chemical biosynthetic potential in Central American *Pseudonocardia*. The presence of several unique BGCs on BCI appears to contribute to elevated inhibition on the island population, and a lack of certain BGCs may contribute to lower inhibition in the Costa Rica LS population. Correlations with bioassay inhibition demonstrated that a particular BGC may be associated with either high or low inhibition (fig. 7a); however, given that no pairing reaches significance by location suggests that the presence/absence of BGCs is not driving local adaptation. At least two cross-population BGCs appear to have undergone selective sweeps on BCI. A bactriocin on BCI showed significantly high Fst compared to the mainland populations (fig. 8a). Bactriocins are generally thought to be niche-defending compounds, so while unlikely to be the source of *Escovopsis* inhibition, this could be involved with host-*Pseudonocardia* specificity either directly or indirectly via the killing of potential invader actinobacterial strains. An NRPS BGC may also have undergone a selective sweep on BCI – that Fst peak occurs within a cyclizing domain that could potentially increase secondary metabolite production by freeing up a rate limiting step. These results suggest that adaptation at the local level is occurring within genes and not just through the acquisition of novel BGCs.

4.6 Conclusions

Taken together, these signatures of local adaptation to common parasites and rare pathogen advantage provide evidence of indirect adaptation to a pathogen by its tertiary

target (table 1). The mosaic pattern in symbiont inhibition across geographically structured populations and the nesting of a locally adapted population surrounded by populations lacking adaptation is consistent with a coevolving geographic mosaic. These results also provide evidence of population–level adaptation by *Pseudonocardia* in response to selective pressure imposed as a consequence of its symbiosis with fungus-farming ant hosts.



Supplementary figure 1. Assignment of *Pseudonocardia* isolates to genetic clusters (K) determined using Bayesian simulations in STRUCTURE. Each vertical line corresponds to the proportion of an individual's genome that corresponds to a particular cluster, indicated by color differences. Panel A presents results of the linkage/admixture model (K=2), as determined by calculating ΔK (Evanno et al. 2005). Admixed isolates in A correspond to recombinant sequences identified through tests for recombination and phylogenetic incongruence (Caldera & Currie 2012). B and C show K=6 for the linkage/admixture and no-admixture models, respectively, where the no-admixture model assumes that each isolate derives from a single population and the linkage/admixture model allows individuals to have admixed ancestry.

4.7 References cited

Barke, J., R. F. Seipke, S. Gruschow, D. Heavens, N. Drou, M. J. Bibb, R. J. M. Goss et al. 2010. A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. Bmc Biology 8.

Blin, K., M. H. Medema, D. Kazempour, M. A. Fischbach, R. Breitling, E. Takano, and T. Weber. 2013. antiSMASH 2.0-a versatile platform for genome mining of secondary metabolite producers. Nucleic Acids Research 41:W204-W212.

Brodie, E. D., C. R. Feldman, C. T. Hanifin, J. E. Motychak, D. G. Mulcahy, and B. L. Williams. 2005. Parallel arms races between garter snakes and newts involving tetrodotoxin as the phenotypic interface of coevolution. Journal of Chemical Ecology 31:343-356.

Cafaro, M. J., M. Poulsen, A. E. F. Little, S. L. Price, N. M. Gerardo, B. Wong, A. E. Stuart et al. 2011. Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proceedings of the Royal Society B: Biological Sciences.

Caldera, E. J., and C. R. Currie. 2012. The Population Structure of Antibiotic-Producing Bacterial Symbionts of Apterostigma dentigerum Ants: Impacts of Coevolution and Multipartite Symbiosis. American Naturalist 180:604-617.

Caldera, E. J., M. Poulsen, G. Suen, and C. R. Currie. 2009. Insect Symbioses: A Case Study of Past, Present, and Future Fungus-growing Ant Research. Environmental Entomology 38:78-92.

Carius, H. J., T. J. Little, and D. Ebert. 2001. Genetic variation in a host-parasite association: Potential for coevolution and frequency-dependent selection. Evolution 55:1136-1145.

Chapela, I. H., S. A. Rehner, T. R. Schultz, and U. G. Mueller. 1994. Evolutionary history and the symbiosis between fungus-growing ants and their fungi. Science 266:1691-1694.

Chin, C. S., D. H. Alexander, P. Marks, A. A. Klammer, J. Drake, C. Heiner, A. Clum et al. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nature Methods 10:563-+.

Currie, C.R. 2001. Prevalence and impact of a virulent parasite on a tripartite mutualism. *Oecologia* 128: 99-106

Currie, C. R., U. G. Mueller, and D. Malloch. 1999a. The agricultural pathology of ant fungus gardens. Proceedings of the National Academy of Sciences of the United States of America 96:7998-8002.

Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81-83.

Currie, C. R., J. A. Scott, R. C. Summerbell, and D. Malloch. 1999b. Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701-704.

Currie, C. R., and A. E. Stuart. 2001. Weeding and grooming of pathogens in agriculture by ants. Proceedings of the Royal Society of London Series B-Biological Sciences 268:1033-1039.

Currie, C. R., B. Wong, A. E. Stuart, T. R. Schultz, S. A. Rehner, U. G. Mueller, G. H. Sung et al. 2003b. Ancient tripartite coevolution in the attine ant-microbe symbiosis. Science 299:386-388.

Eddy, S. R. 2011. Accelerated Profile HMM Searches. Plos Computational Biology 7.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14:2611-2620.

Falush, D., T. Wirth, B. Linz, J. K. Pritchard, M. Stephens, M. Kidd, M. J. Blaser et al. 2003. Traces of human migrations in Helicobacter pylori populations. Science 299:1582-1585.

Gardner, P. P., J. Daub, J. G. Tate, E. P. Nawrocki, D. L. Kolbe, S. Lindgreen, A. C. Wilkinson et al. 2009. Rfam: updates to the RNA families database. Nucleic Acids Research 37:D136-D140.

Gerardo, N. M., and E. J. Caldera. 2007. Labile associations between fungus-growing ant cultivars and their garden pathogens. Isme Journal 1:373-384.

Gerardo, N. M., S. R. Jacobs, C. R. Currie, and U. G. Mueller. 2006a. Ancient host-pathogen associations maintained by specificity of chemotaxis and antibiosis. Plos Biology 4:1358-1363.

Gerardo, N. M., U. G. Mueller, and C. R. Currie. 2006b. Complex host-pathogen coevolution in the Apterostigma fungus-growing ant-microbe symbiosis. Bmc Evolutionary Biology 6:88.

Gibert, J. P., M. M. Pires, J. N. Thompson, and P. R. Guimaraes. 2013. The Spatial Structure of Antagonistic Species Affects Coevolution in Predictable Ways. American Naturalist 182:578-591.

Gomulkiewicz, R., D. M. Drown, M. F. Dybdahl, W. Godsoe, S. L. Nuismer, K. M. Pepin, B. J. Ridenhour et al. 2007. Dos and don'ts of testing the geographic mosaic theory of coevolution. Heredity 98:249-258.

Haft, D. H., J. D. Selengut, R. A. Richter, D. Harkins, M. K. Basu, and E. Beck. 2013. TIGRFAMs and Genome Properties in 2013. Nucleic Acids Research 41:D387-D395.

Hyatt, D., G. L. Chen, P. F. LoCascio, M. L. Land, F. W. Larimer, and L. J. Hauser. 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. Bmc Bioinformatics 11.

Kost, C., T. Lakatos, I. Bottcher, W. R. Arendholz, M. Redenbach, and R. Wirth. 2007. Non-specific association between filamentous bacteria and fungus-growing ants. Naturwissenschaften 94:821-828.

Kurtz, S., A. Phillippy, A. L. Delcher, M. Smoot, M. Shumway, C. Antonescu, and S. L. Salzberg. 2004. Versatile and open software for comparing large genomes. Genome Biology 5.

Lawson, Hellenthal, Myers, and Falush (2012), "Inference of population structure using dense haplotype data", PLoS Genetics, 8 (e1002453)

Lively, C. M. 1999. Migration, virulence, and the geographic mosaic of adaptation by parasites. American Naturalist 153:S34-S47.

Lively, C. M., and M. F. Dybdahl. 2000. Parasite adaptation to locally common host genotypes. Nature 405:679-681.

Mikheyev, A. S., U. G. Mueller, and J. J. Boomsma. 2007. Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. Molecular Ecology 16:209-216.

Moran, N., and P. Baumann. 1994. PHYLOGENETICS OF CYTOPLASMICALLY INHERITED MICROORGANISMS OF ARTHROPODS. Trends in Ecology & Evolution 9:15-20.

Mueller, U. G., D. Dash, C. Rabeling, and A. Rodrigues. 2008. Coevolution between attine ants and Actinomycete bacteria: a reevauation. Evolution 62:2894-2912.

Mueller, U. G., H. Ishak, J. C. Lee, R. Sen, and R. R. Gutell. 2010b. Placement of attine ant-associated *Pseudonocardia* in a global *Pseudonocardia* phylogeny (Pseudonocardiaceae, Actinomycetales): a test of two symbiont-association models. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology 98:195-212.

Mueller, U. G., S. A. Rehner, and T. R. Schultz. 1998. The evolution of agriculture in ants. Science 281:2034-2038.

Mueller, U. G., J. J. Scott, H. D. Ishak, M. Cooper, and A. Rodrigues. 2010a. Monoculture of Leafcutter Ant Gardens. Plos One 5:7.

Nawrocki, E. P., and S. R. Eddy. 2013. Infernal 1.1: 100-fold faster RNA homology searches. Bioinformatics 29:2933-2935.

Oh, D. C., M. Poulsen, C. R. Currie, and J. Clardy. 2009. Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nature Chemical Biology 5:391-393.

Poulsen, M., M. J. Cafaro, D. P. Erhardt, A. E. F. Little, N. M. Gerardo, B. Tebbets, B. S. Klein et al. 2010. Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in Acromyrmex leaf-cutting ants. Environmental Microbiology Reports 2:534-540.

Powell, S., D. Szklarczyk, K. Trachana, A. Roth, M. Kuhn, J. Muller, R. Arnold et al. 2012. eggNOG v3.0: orthologous groups covering 1133 organisms at 41 different taxonomic ranges. Nucleic Acids Research 40:D284-D289.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945-959.

Singer, M. C., and C. D. Thomas. 1996. Evolutionary responses of a butterfly metapopulation to human- and climate-caused environmental variation. American Naturalist 148:S9-S39.

Takahashi, Y., and M. Kawata. 2013. A comprehensive test for negative frequency-dependent selection. Population Ecology 55:499-509.

Thompson, J. N. 1994, The coevolutionary process. Chicago, University of Chicago Press.
—. 2005, The geographic mosaic of coevolution. Chicago, University of Chicago Press.

Thompson, J. N., and B. M. Cunningham. 2002. Geographic structure and dynamics of coevolutionary selection. Nature 417:735-738.

Zerbino, D. R., and E. Birney. 2008. Velvet: Algorithms for de novo short read assembly using de Bruijn graphs. Genome Research 18:821-829.

5 Chapter 5: Summary and Future Directions

In this dissertation, I sought to characterize the evolutionary ecology of symbiotic, antibiotic-producing *Pseudonocardia* associated with the fungus-growing ant *Apterostigma dentigerum*. Understanding *Pseudonocardia*'s place in the attine-symbiont system first required a thorough review of symbiont dynamics from a broad phylogenetic scale to a population level (chapter 1) as well as a basic understanding of the population-level cultivar-parasite dynamics that it mediates (chapter 2). Moreover, because coevolutionary dynamics are shaped by population structure, a description of *Pseudonocardia* phylogeography and recombination was also necessary (chapter 3). In chapter 4 I demonstrated that the *Pseudonocardia* – parasite dynamic conforms to a theoretical framework called the geographic mosaic of coevolution, and began building an understanding of the secondary metabolite and molecular genetic mechanisms conferring adaptation. Future work should continue to explore evolutionary genetic change and transmission of secondary metabolites, and the evolutionary dynamics of one particular *Pseudonocardia* strain may provide important insights.

Bioassay inhibition patterns identified a *Pseudonocardia* isolate with phylogeograpic placement that provides an anecdotal example of an "exception that proves the rule." Isolate EC08061908 was collected on BCI but it nests within a PLR clade with high support (fig. 1). Thus, it appears that this strain recently migrated from PLR. Interestingly, this isolate is an outlier within our dataset as having high inhibition, in part because it shows high inhibition to both BCI and PLR (i.e. the strain is not locally adapted). For contrast, EC08061804, which groups robustly within the BCI clade, shows high inhibition to BCI,

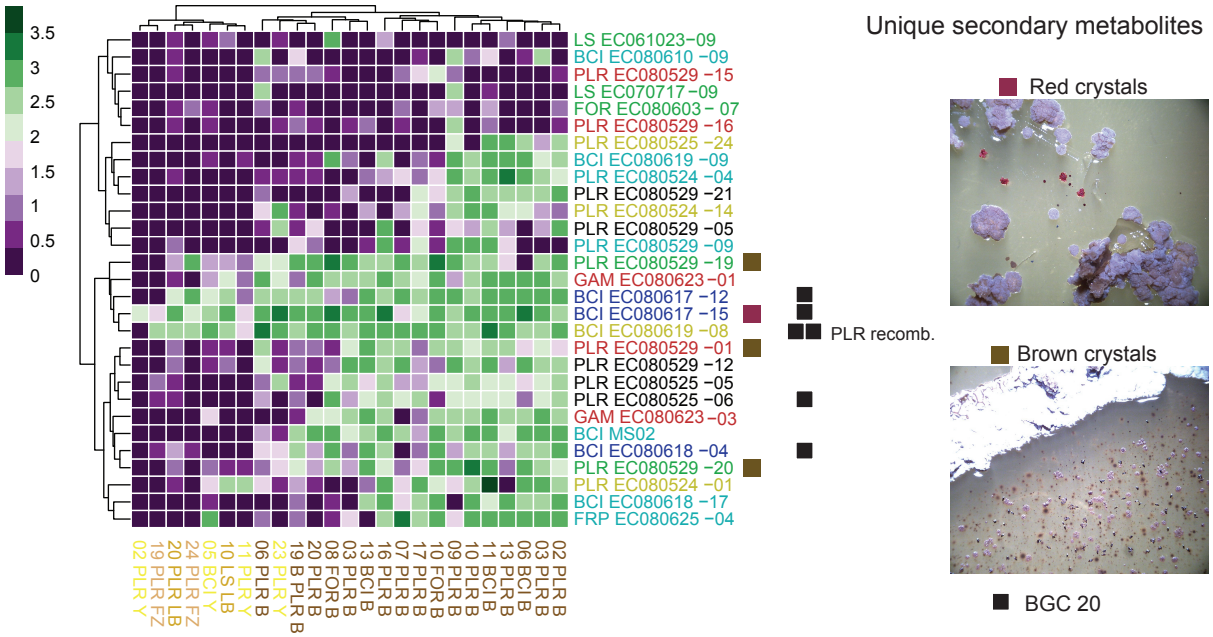


Figure 1. Zones of inhibition in Central American *Escovopsis-Pseudonocardia* challenges. Large zones are shown in dark green and no inhibition shown in dark purple. *Pseudonocardia* (y axis) are color coded by fineSTRUCTURE groupings from whole genome sequencing, and *Escovopsis* (x-axis) are color coded by morphotype.

but lower inhibition to PLR (i.e. the strain is locally adapted). Perhaps during its migration from PLR to BCI, EC08061908 maintained some inhibitory element from PLR, while also acquiring a new element from BCI. The presence of two variants of a particular BCG in also appears to support this (fig 1). Subsequent work should continue to understand examine the differences in BGC variants and seek to link genetic changes to inhibition profiles. EC08061908 also provides an opportunity to explore transmission of secondary metabolites. Because this BCI strain appears to be a recent migrant from PLR, it would be interesting know whether the ant is also a recent migrant. A population genetic study of the ants could utilize exclusion and assignment tests like those used in appendix 6.1 to answer this question.

Further insights into *Pseudonocardia* antibiotic production may also be gained by understanding unique secondary metabolites. While isolating *Pseudonocardia*, I identified two secondary metabolite structures that are easily visible in the media, which I call, “red crystals” and “brown crystals.” The red crystalline structures are associated with high inhibition on BCI, where they are found in only one region, which seems to swamp out local adaptation signatures. Brown crystals are also associated with high inhibition, but they express more variably within strains that are found across populations. A more complete understanding of *Pseudonocardia* secondary metabolite evolution might view the acquisition of unique secondary metabolites as occasional, but valuable inputs of evolutionary utility that are then modified by selection as they spread over geographic space.

This dissertation explored a geographic mosaic dynamic within the canal-zone of Panama, but the potential to detect a geographic mosaic at a larger scale is also possible.

In 2009 I isolated South American *Apterostigma*-associated *Escovopsis* from the Los Amigos station along the Madre De Dios in the Amazon of Peru. I collected multiple G2, G3, and G4 cultivating *Apterostigma* species. Compared to our observations of *Escovopsis* morphotypes in Central America (where brown=common, yellow=less common, and fuzzy brown & light brown=rare) fuzzy brown frequencies were common and we didn't collect any brown or light brown. Unfortunately, I was unable to isolate actinobacteria (in part because I didn't have regular access to electricity or a light powered microscope needed for the appropriate dissection and scraping for isolation. Future work should explore whether these bacteria are genetically distinct from Central American bacterial symbionts and whether Peruvian actinobacteria symbionts are better at inhibiting fuzzy brown *Escovopsis*. Understanding these dynamics might inform a broader geographic mosaic driven by shifts in *Escovopsis* morphotypes from Central America through South America.

6 Appendix

Putative Native Source of the Invasive Fire Ant *Solenopsis invicta* in the USA.

Supplementary Figure 16 *in* The Genome Sequence of the Leaf-Cutter Ant *Atta cephalotes* Reveals Insights into Its Obligate Symbiotic Lifestyle

6.1 Putative Native Source of the Invasive Fire Ant *Solenopsis invicta* in the USA

Previously published in Biological Invasions. 2008, 10(08): 1457-1479.

Eric J. Caldera, Kenneth G. Ross, Christopher J. DeHeer, and D. DeWayne Shoemaker

Author contributions: EJC & DDS performed population genetic analysis and wrote the paper. KGR CJD & DDS conducted the field work and genotyping.

6.1.1 Abstract

The ecological and evolutionary dynamics of newly introduced invasive species can best be understood by identifying the source population(s) from which they originated, as many species vary behaviorally, morphologically, and genetically across their native landscapes. We attempt to identify the source(s) of the red imported fire ant (*Solenopsis invicta*) in the southern USA utilizing data from three classes of genetic markers (allozymes, microsatellites, and mitochondrial DNA sequences) and employing Bayesian clustering simulations, assignment and exclusion tests, and phylogenetic and population genetic analyses. We conclude that the Mesopotamia flood plain near Formosa, Argentina represents the most probable source region for introduced *S. invicta* among the 10 localities sampled across the native South American range. This result confirms previous suspicions that the source population resides in northern Argentina, while adding further doubts to earlier claims that the Pantanal region of Brazil is the source area. Several lines of evidence suggest that *S. invicta* in the southern USA is derived from a single location rather than being the product of multiple invasions from widely separated source localities. Although finer-scale sampling of northern Argentina and Paraguay combined with the use of additional genetic markers will be necessary to provide a highly precise source population assignment, our current results are of immediate use in directing future sampling and focusing ongoing biological control efforts.

6.1.2 Introduction

Invasive species can pose major threats to agricultural and natural environments as well as to the public health (Sax et al. 2005; Wilcove et al. 1998). Considering just insects in agricultural systems, the negative impact of invasives is evident from the fact that a vast number of major pest species in such settings in the USA are of exotic origin (Carruthers 2003). Although the basis for the success of different exotic pests varies, one common contributing element appears to be ecological release from the natural assemblages of competitors, parasites, and predators that occur in their native environments and normally act to suppress their populations (Mitchell and Power 2003; Torchin et al. 2003). Thus, a reasonable approach to managing populations of undesirable exotics is to identify the natural enemies attacking them in their native ranges and to deliberately introduce one or more of these biological control agents into the introduced range.

While biological control has proved successful in suppressing pest populations in a number of cases, one potential difficulty is a failure to recognize biologically important variation among native pest populations that correlates with variation in natural enemy assemblages (Goolsby et al. 2006; Sakai et al. 2001; Waage and Mills 1992). As an example, if strong genetic differentiation exists among native populations of an introduced species, then it is likely either that the species composition of the natural enemy assemblage varies locally over the native range of the exotic or that widespread individual enemy species are locally adapted to it. In such cases, the efficacy and success of natural enemies in controlling their host will likely depend on an interaction between natural enemy and host genotypes (Dybdahl and Storfer 2003; Foitzik et al. 2003;

Goolsby et al. 2006; Kaltz and Shykoff 1998; Kraaijeveld and Godfray 1999; Lively and Dybdahl 2000; Lively et al. 2004; Thrall et al. 2002). If so, efforts to pinpoint the source population of an invasive pest are of paramount importance in identifying appropriate agents of its biological control (Waage and Mills 1992).

In addition to directing efforts toward appropriate locations for identifying potential control agents, determination of the geographical source of an invasive pest may facilitate reconstruction of the invasion history, help reveal important regional genetic variation relevant to its natural history, and shed light on important post-invasion changes in its biology that may affect its pest attributes (Downie 2002; Giraud et al. 2002; Gwiazdowski et al. 2006; Lee 2002; Provan et al. 2005; Ross et al. 1996; Tsutsui et al. 2003). While identification of the source population of an invasive species can be challenging, the development of new molecular tools and statistical methods for analyzing the resulting data have greatly improved the prospects for meaningful results, as illustrated by several recent studies (Gwiazdowski et al. 2006; Harter et al. 2004; Havill et al. 2006; May et al. 2006; Miura et al. 2006).

The red imported fire ant, *Solenopsis invicta*, is an invasive pest of significant economic, agricultural, and medical importance in the USA and elsewhere (Jouvenaz 1990; Lofgren 1986a; Orr 1996; Patterson 1994). This insect was inadvertently introduced into the USA from South America some 75 years ago. Since that time, it has spread throughout the southern part of the country (Callcott and Collins 1996; Lofgren 1986b) and, more recently, to several western states, the Carribean, Australia, and Taiwan (Buckley 1999; Chen et al. 2006; Davis et al. 2001; Huang et al. 2004; MacKay and Fagerlund 1997; McCubbin and Weiner 2002). Solenopsis invicta is of economic

importance in these areas of introduction because: (1) it is an aggressive stinging insect causing mass envenomation incidents and hypersensitivity reactions in humans, (2) it occurs primarily in human- modified habitats, (3) it constructs large mounds that are unsightly and capable of damaging farm machinery, (4) it feeds on several important cultivated plants and tends homopterans that are also plant pests, and (5) it may negatively affect populations of native ants and other ground-dwelling animals (Allen et al. 1998; Carroll and Hoffman 2000; Gotelli and Arnett 2000; Lofgren 1986a; Lofgren et al. 1975; Morrison 2002; Porter and Savignano 1990; Tschinkel 2006; Vinson 1994; Wojcik et al. 2001). These pest traits of *S. invicta* presumably are exacerbated by the relative lack of natural enemies in invasive populations that normally act to suppress fire ant populations, with the effect that population densities in the USA are orders of magnitude greater than in South America (Morrison 2000; Porter et al. 1992, 1997b).

Concerns about the negative economic and ecological impacts of *S. invicta* have led to the development of many different control methods that target individual colonies (e.g., contact insecticides) or are intended to suppress the colonies inhabiting larger areas (e.g., baits containing poisons, growth regulators, or reproductive inhibitors) (Kemp et al. 2000; Williams 1994; Williams and Porter 1994). Because these methods generally have failed to halt the continued spread and enormous population buildups following new introductions, alternative approaches to population management, including those based on biological control by natural enemies from the native range, are being developed (Jouvenaz 1990; Lofgren 1986a; Morrison et al. 2000; Orr 1996; Patterson 1994; Porter 1998, 2000; Porter and Briano 2000; Porter et al. 2004; Williams and deShazo 2004). Clearly, however, sustained success in such novel management approaches requires

detailed knowledge of the biology of this ant in its native habitat, including such crucial ecological information as the identity of key competitors and natural enemies and the nature of their interactions with *S. invicta*. Given that this species occupies a vast native range characterized by profound regional genetic differentiation (Ross et al. 1997, 2007; Ross and Shoemaker 2005), the most scientifically and practically relevant information of this type will come from the native population(s) that served as the source of the USA colonists.

The current study uses genetic data generated from three classes of genetic markers to attempt to identify the native source of *S. invicta* introduced into the USA. Such attempts would have been difficult previously, given the somewhat problematic a-taxonomy of South American fire ants, the lack of a sufficient number of informative molecular markers, and the limited availability of samples from the native range. However, substantial progress in resolving these shortcomings has been made recently (Pitts et al. 2005; Ross et al. 2007; Ross and Shoemaker 2005). An attempt to identify the origin of *S. invicta* is particularly appropriate at this juncture in light of recent studies detailing the nature and extent of geographic population genetic differentiation within the native range (Ross et al. 2007; Ross and Shoemaker 2005). While this recent work provides necessary baseline data for source identification, the observed strong regional differentiation also suggests that natural enemies of *S. invicta* may be locally adapted to their host and that *S. invicta* may differ regionally in some important aspects of its natural history. Thus, identification of the source of the USA colonists is important not only for focusing biological control collections but also to anchor studies of the natural history of *S. invicta* in its native range, an increasingly important endeavor given the emergence of

the species as a prominent model for ecological and evolutionary studies (Gotzek and Ross 2007; Tschinkel 2006).

6.1.3 Materials and Methods

All of the samples used here were subjected to extensive population genetic analyses in several earlier studies (Ross et al. 2007; Ross and Shoemaker 2005; Shoemaker et al. 2006a; Shoemaker et al. 2006b). These previous studies described patterns of genetic variation within native and introduced populations of *S. invicta*, important baseline data for attempting to identify the native source of the USA colonists. Below we summarize briefly the sampling and genetic methods employed in generating these data; additional details can be found in Shoemaker et al. (2006b) and Ross et al. (2007).

Samples

Two social forms of *S. invicta* occur in both the native and introduced ranges. The monogyne (*M*) social form is characterized by the presence of one reproductive queen per colony, while the polygyne (*P*) form is characterized by multiple such queens per colony. The two forms differ not only in colony queen number but in many other important features of their reproductive and dispersal biology that are expected to have important effects on the distribution of genetic variation at various spatial scales (Ross and Keller, 1995, Tschinkel, 2006). Samples of introduced *S. invicta* were collected from 258 colonies at two exemplar localities in the southern USA (Fig.1), with both social forms well sampled at each locality. The Mississippi locality was chosen as an exemplar because of its close proximity to the port of Mobile, Alabama, the suspected initial point of entry of *S. invicta* into the USA (Lofgren 1986a), and because Shoemaker et al.

(2006b) demonstrated that the patterns of diversity within this population appear to closely resemble those of a hypothetical original colonizing population. We included ants from west Louisiana because they appear to be somewhat divergent from other ants sampled throughout the southern USA (Shoemaker et al. 2006b), possibly as the result of a secondary introduction. All sampled colonies at each locality were located within 40 km of one another.

Samples of native *S. invicta* were collected from 567 colonies at ten localities in Brazil and Argentina that span a large portion of the native range (Fig. 2; see also Ahrens et al. 2005; Mescher et al. 2003; Ross et al. 2007). All sampled colonies at a locality were located within 20 km of one another, while distances between pairs of localities ranged from 90 to 1967 km. Samples of each social form were collected in sufficient numbers from the Corrientes and Formosa localities to warrant separate analyses. Following Ross et al. (2007), two distinct populations were distinguished within the Arrorio dos Ratos locality, designated as Arroio X and Y. Three of the Brazilian localities, Pedra Preta, São Gabriel do Oeste, and Campo Grande, lie at the eastern edge of the Pantanal, a large flood plain hypothesized by earlier authors to be the source area for the USA colonists (Allen and Buren 1974; Buren 1972; Buren et al. 1974).

All sampled colonies were identified as *S. invicta* by J. P. Pitts using species-informative morphological characters (Pitts 2002; Trager 1991). The social form of each colony was identified using the methods described in Shoemaker et al. (2006b). Geographic coordinates for the sampling localities and numbers of samples from each are summarized in Appendix I.

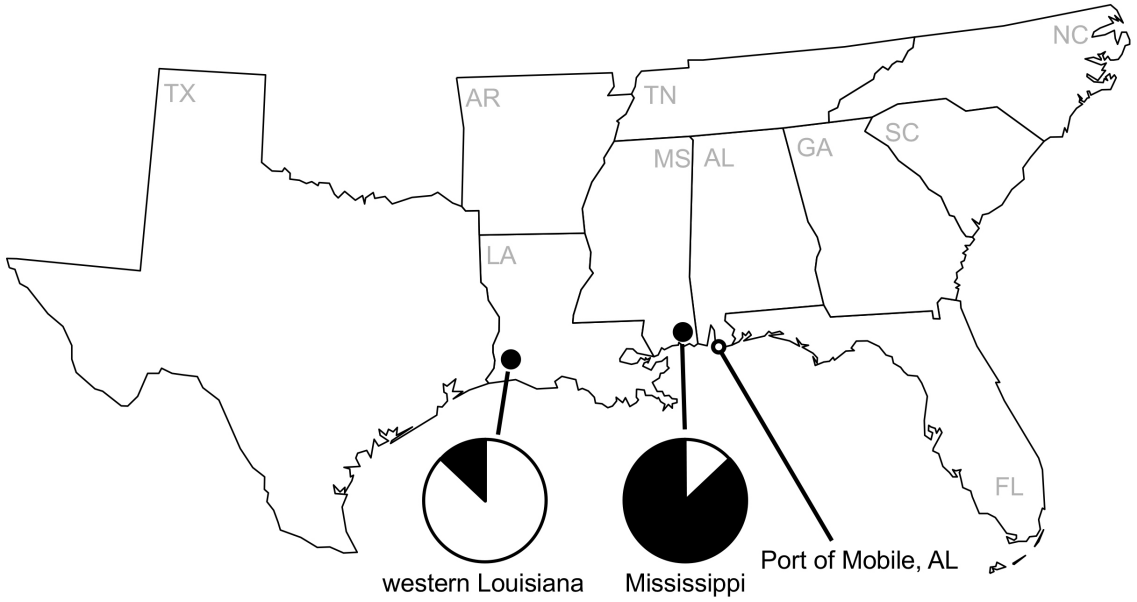


Figure 1. Sampling localities for *S. invicta* within the introduced range in the USA. Pie charts represent the proportions of individuals (colonies) within each geographic locality that were placed in each of two genetic clusters inferred by the program STRUCTURE

Genetic Markers

We genotyped one female alate (winged virgin queen) or dealate (wingless reproductive queen) per colony at 12-14 nuclear loci. Nuclear markers included seven allozyme loci (*Aat-2*, *Acoh-1*, *Acoh-5*, *Est-2*, *G3pdh-1*, *Gpi*, *Pgm-1*) and seven microsatellite loci (*Sol-6*, *Sol-11*, *Sol-18*, *Sol-20*, *Sol-42*, *Sol-49*, *Sol-55*). Methods for allozyme electrophoresis and staining are found in Shoemaker et al. (1992) and Ross et al. (1997). Because the allozyme loci *Est-2* and *Gpi* are monomorphic in *S. invicta* in the USA (Shoemaker et al. 1996), they were not scored in the Mississippi and west Louisiana ants. Primers and procedures for microsatellite amplification and visualization are described in Ross et al. (2007).

We sequenced a 920-bp fragment of the mitochondrial DNA (mtDNA) genome for 644 of the individuals described above using the primers COI-RLR (Simon et al. 1994) and DDS-COII-4 (Ross and Shoemaker 1997; see Appendix II for sample sizes for each population); All sequences for ants from the USA were newly generated for this study (GenBank accession numbers EU352605–EU352610). Primer sequences, PCR reaction conditions, and sequencing methods were identical to those described in Ahrens et al. (2005). All sequences were aligned by eye using sequence data deposited in GenBank (accession number AY2490093), and each was assigned a specific haplotype identification code according to Shoemaker et al. (2006a). For STRUCTURE analyses incorporating mtDNA data from the native range (see below), haplotypes were binned into one of seven well supported clades described in Ross et al. (2007) and Shoemaker et al. (2006a). All unique mtDNA sequences have been deposited in GenBank (see Table 1 of Shoemaker et al. 2006a for GenBank accession numbers).

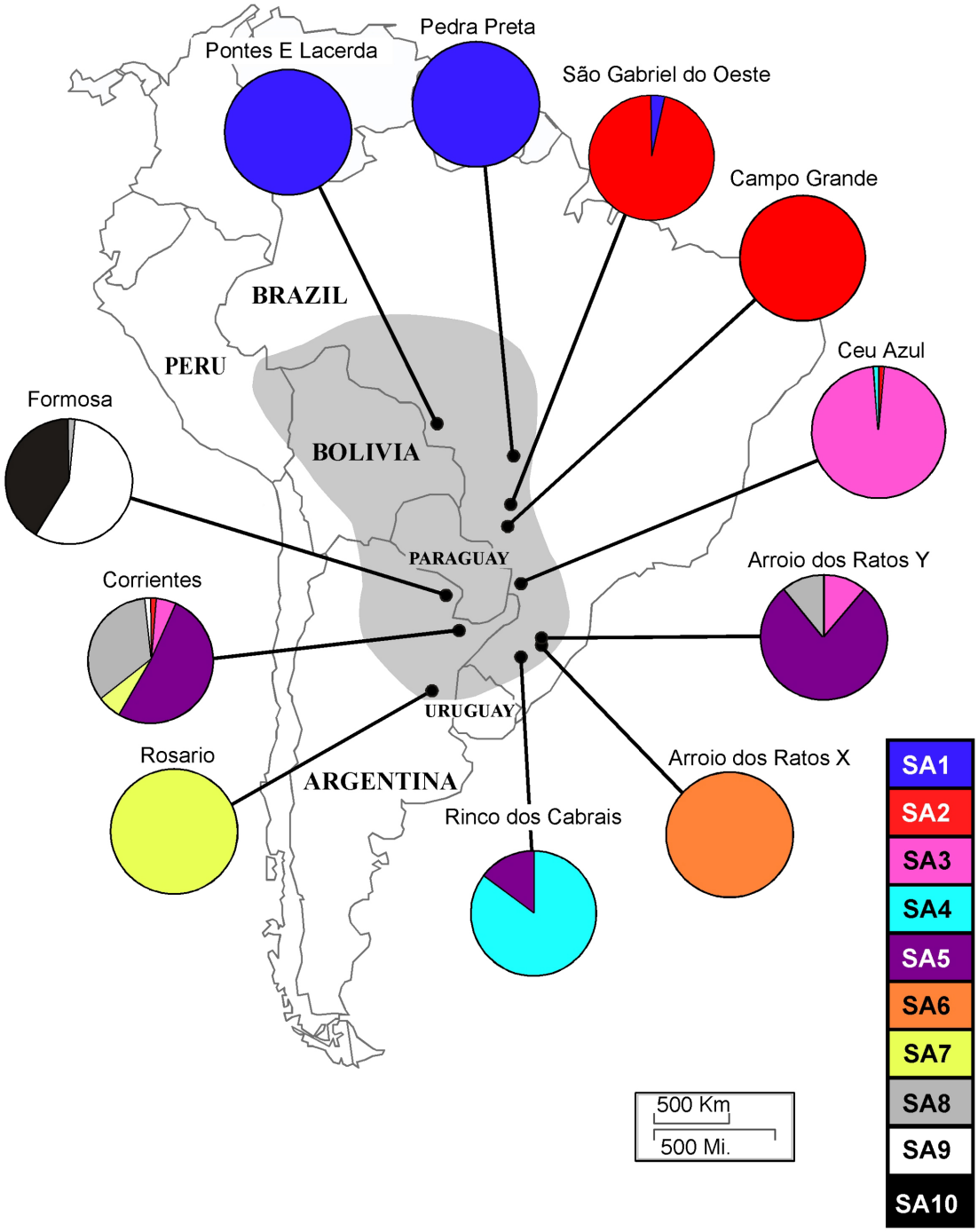


Figure 2. Sampling localities for *S. invicta* within the native range of the species (area shaded gray). Pie charts represent the proportions of individuals (colonies) within each geographic locality that were placed in one of ten genetic clusters inferred by the program STRUCTURE (designated clusters SA1–SA10). The Pantanal and Mesopotamia floodplain regions are depicted with red and green stippling, respectively, while relevant

portions of waterways in the Amazon and La Plata River Basins are depicted with purple and blue lines, respectively. The Parana´ River is indicated by the letter ‘‘P’’

Assignment Tests

We used Bayesian assignment tests implemented in the program GENECLASS2 (Piry et al. 2004) as one approach to determine the likely source(s) of invasive *S. invicta*. Bayesian assignment tests were selected over distance-based tests because the former appear to perform better (Cornuet et al. 1999), and assignment tests utilizing the Rannala and Mountain (1997) methods were selected over approaches employing a uniform prior because the former produce less ambiguous results (Baudouin et al. 2004). Initially, assignment tests for the introduced ants were conducted at both the individual and population levels using the native sampling localities (geographic populations) as reference populations.

Because power in assigning individuals or groups to a reference population can be gained by grouping individuals on the basis of genetic similarity rather than geographic proximity (Baudouin et al. 2004), we also performed the above analyses after grouping ants into distinctive genetic clusters rather than collection localities. To accomplish this, we used the Bayesian program STRUCTURE (Pritchard et al. 2000) to sort individuals from the native or introduced ranges into *K* genetic clusters. We did not include the mtDNA data for these analyses because GENECLASS2 is not equipped to handle data derived from both haploid and diploid markers. All simulations performed in STRUCTURE featured 300,000 runs following a burn-in period consisting of 100,000 runs. We used the admixture model assuming correlated allele frequencies and did not include collection site or social form as priors. Analyses were performed separately for ants from the USA (K=1 to 4) and from South America (K=1 to 19). For both analyses,

ten separate runs were performed for each value of *K*. We ensured accurate estimates of the simulation values by checking that model parameters equilibrated before the end of the burn-in phase and that posterior probabilities were consistent across all ten runs for each data and parameter set. After determining the appropriate value of *K* for each dataset using the Δ*K* method of Evanno et al. (2005), each individual was placed in the cluster in which it had predominant membership (determined from the run with the highest posterior probability for a particular cluster). The resulting new clusters of native ants then were used as reference populations for assignment tests performed on the introduced ants in GENECLASS2. Again, these assignment tests utilizing the STRUCTURE-defined genetic clusters were conducted at both the individual and population levels.

In a somewhat different approach, we used STRUCTURE to determine whether *S. invicta* from Mississippi and west Louisiana grouped predominantly with any distinct genetic cluster comprising ants from the native range. We ran these simulations using the entire set of individuals from both ranges (*K*=1 to 17). Runs were performed with the parameters described above, using only the nuclear DNA data as well as the combined nuclear and mtDNA data.

Once a native source was tentatively identified, we ran STRUCTURE simulations again on a more restricted dataset comprising only this native population and a single geographic population from the USA (Mississippi or west Louisiana). These analyses were repeated with the introduced ants distinguished by membership in the genetic clusters determined from STRUCTURE rather than by locality.

Exclusion Tests

One potential drawback of Bayesian assignment methods is that they assume the actual source population is represented among the reference populations, with the result that individuals of unknown origin will always be assigned with high probability to one or another reference population. In contrast, exclusion tests allow the possibility of excluding all reference populations as potential sources. GENECLASS2 offers three different Monte Carlo sampling algorithms for performing exclusion analyses (Cornuet et al. 1999; Paetkau et al. 2004; Rannala and Mountain 1997). We judged the algorithm of Cornuet et al. (1999) to be the most appropriate for our dataset, given that it assumes that source and introduced populations diverged in the absence of migration. All exclusion analyses were conducted with 100,000 simulated individuals and an alpha level of 0.01.

Phylogenetic Analyses of mtDNA Sequences

To make full use of the information available from the mtDNA, we performed phylogenetic analyses on 92 sequences representing all unique haplotypes from the native and introduced ranges. MtDNA sequences from two other fire ant species, *Solenopsis geminata* and *S. electra* (GenBank accession numbers AY254476 and AY249092, respectively), were used as outgroups for these analyses. We used neighbor-joining (NJ) and Bayesian methods implemented in the programs PAUP 4.08b (Swofford 1999) and MrBayes 3 (Ronquist and Huelsenbeck 2003), respectively, to reconstruct the mtDNA trees. The program MODELTEST (Posada and Crandall 1998) was used to determine the appropriate model of sequence evolution and proportion of invariant sites. The NJ tree was constructed using TrN + I + Γ distances (determined by MODELTEST to have the lowest likelihood ratio; I=0.6970 and Γ=1.8861), with the additional constraint that ties were broken randomly. All branches of zero length were collapsed during searches.

Bootstrap support values for each node within the tree were calculated by performing 10,000 data resamplings. All MCMC searches in the Bayesian analyses employed a uniform prior based on output from MODELTEST, and no priors regarding tree topology were assumed. To ensure that parameter space was thoroughly explored, four separate runs with four heated chains each were performed; each chain featured four million generations (with sampling every 100th generation) following a burn-in period of 100,000 generations. Posterior probabilities were calculated using the trees visited by the Markov chains after burn-in samples were discarded.

Additional Analyses

We constructed a consensus genetic distance tree depicting the relationships of geographic populations at their nuclear genomes. The programs SEQBOOT, GENDIST, and NEIGHBOR within the PHYLIP package (Felsenstein 2004) were used to create replicate data sets, calculate Nei's chord distance (Nei et al. 1983), and construct neighbor-joining (NJ) trees, respectively. Node stability was assessed by performing 1000 bootstrap resamplings. The final consensus tree was produced in the CONSENSE program of PHYLIP by employing the majority rule criterion.

We used the program ARLEQUIN 2.000 (Schneider et al. 2000) to estimate F_{ST} values for all nuclear loci and Φ_{ST} values for the mtDNA between all pairs of native and introduced geographic populations, as well as to estimate allele and haplotype frequencies within geographic populations.

Finally, we employed STRUCTURE to generate F_K values for each geographic population in both the native and introduced ranges based on the nuclear data; this statistic can be interpreted as equivalent to F_{ST} between a sampled population and a

hypothetical population assumed to be ancestral to all the study populations (Falush et al. 2003; Pritchard et al. 2000). We incorporated geographic location and social form as priors for these simulations.

The majority of data format conversions necessary for the different software programs used in this study were conducted with the program CONVERT (Glaubitz 2004). Graphical displays of STRUCTURE output were created using the program DISTRUCT (Rosenberg 2004).

6.1.4 Results

General descriptions of the types and distributions of nuclear genetic and mtDNA variation in native and introduced *S. invicta* populations are provided elsewhere (Ross et al. 2007; Shoemaker et al. 2006a; Shoemaker et al. 2006b). Allele and haplotype frequencies for the populations included in this study are provided in Appendix II (electronic versions available upon request).

Assignment Tests

We first used GENECLASS2 to assign geographic populations from the USA to native geographic populations on the basis of the nuclear marker data. At the population level, both the Mississippi and the west Louisiana populations were assigned to the native Formosa, Argentina population with probabilities greater than 0.999. Population-level assignment probabilities to the remaining native localities were all below 0.0001.

We repeated the above analyses after grouping individuals from the native and introduced ranges into genetic clusters. STRUCTURE simulations revealed that ants from the introduced populations comprise two distinct genetic clusters (K=2) based on calculation of ΔK (Evanno et al. 2005). These clusters correspond roughly to the two

sampling localities (Fig. 1); over 87% of individuals with predominant genetic membership in the first cluster were from west Louisiana, while a similar proportion of individuals with predominant membership in the second cluster were from Mississippi. Thus, we subsequently refer to these two clusters as the "west Louisiana cluster" and the "Mississippi cluster." Very similar results were obtained when the two social forms at each locality were considered separately. For the South American ants, STRUCTURE simulations yielded ΔK values strongly supporting the existence of ten distinct genetic clusters (K=10). We subsequently refer to these as clusters SA1 through SA10 (Fig. 2; see also Ross et al. 2007). As in the introduced range, cluster representation was very similar for the two social forms within Corrientes and Formosa.

After placing each individual in the cluster in which it had predominant membership, population-level assignment tests for the introduced ants were repeated in GENECLASS2. Both the Mississippi and west Louisiana clusters of introduced *S. invicta* were assigned to native cluster SA9 with probabilities greater than 0.999. This cluster is composed almost entirely of individuals from the Formosa population (one individual from the neighboring Corrientes, Argentina population was also placed in this cluster). Thus, GENECLASS2 population analyses of the nuclear data based on both geographic locality and genetic clustering implicate Formosa, Argentina as the source of invasive *S. invicta* in the USA, congruence that is not surprising given that the native genetic clusters are strongly geographically structured (Ross et al. 2007). Notably, while the introduced ants are differentiated into two clusters corresponding roughly to the two sampling localities, their assignment to a single locality and cluster in the native range suggests that all *S. invicta* in the USA may be derived from colonists that originated in the same area.

For the individual-level GENECLASS2 analyses using nuclear genetic clusters as references, 231 (90%) of the individuals from Mississippi and west Louisiana were assigned with high probability (>0.99) to native cluster SA9, the cluster associated almost exclusively with Formosa. The remaining 27 individual assignments were deemed ambiguous because of probabilities <0.99. Nonetheless, the highest assignment probabilities for 21 of these were to cluster SA9. Moreover, among the remaining six ambiguous cases, five assignments were to SA10, a cluster found only in Formosa. The final ambiguous individual was assigned to SA5, a cluster commonly represented in the neighboring Corrientes population as well as the southern Brazil populations of Arroio Y and Rinco dos Cabrais (Fig. 2).

Results of individual-level assignment tests using GENECLASS2 with geographic reference populations were qualitatively similar to the above results using genetic clusters, but a much larger proportion of assignments were deemed ambiguous. Ants from Mississippi and west Louisiana invariably were assigned to the Formosa population, but only 52 (20%) of these assignments were made with probabilities >0.99. This underscores the importance of learning about the boundaries of natural genetic units within the native (and introduced) range in order to extract the most useful information for evaluating potential native sources.

STRUCTURE simulations run using all ants sampled from both ranges yielded evidence for just K=2 clusters, regardless of whether only the nuclear data or both the nuclear and mtDNA data were considered. (This low estimate of K compared to that for just the native ants reflects a bias of the Evanno et al. (2005) method toward detection of the highest level of genetic structure.) Remarkably, native ants from almost all localities

have membership predominantly in just one of the two clusters, while the introduced ants have membership predominantly in the other (Fig. 3). The conspicuous exception to this partitioning of the native and introduced gene pools is the native Formosa population. Ants here appear highly admixed, with average membership coefficients split nearly equally between the two clusters (0.58 and 0.44 with the nuclear markers only; 0.59 and 0.41 with the mtDNA included as well). Thus, this analysis also reveals a stronger genetic link of *S. invicta* in the USA. to the Formosa ants than to any other sampled native population.

Given the multiple lines of evidence of strong genetic affinity between ants from the USA and Formosa, we ran additional STRUCTURE simulations using all 14 nuclear markers for ants from just these areas. Each of the analyses conducted separately on ants from Mississippi and west Louisiana yielded evidence for two clusters (K=2), with the Formosa ants and introduced ants having membership predominantly in separate clusters (Fig. 4). Thus, despite the relative genetic similarity between the Formosa and USA ants when all samples are considered simultaneously, significant genetic differentiation between the putative source and introduced populations is nonetheless detectable. Essentially the same results were obtained when STRUCTURE-defined clusters were substituted for geographically defined populations in the introduced range or when the mtDNA data were incorporated (data not shown).

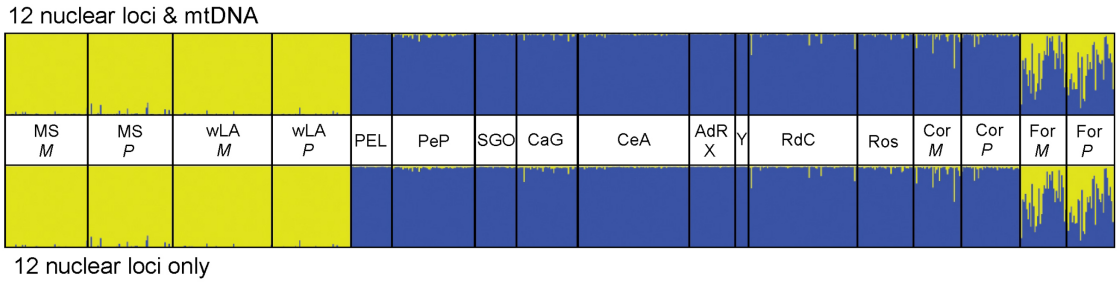


Figure 3. Individual membership coefficients for introduced (USA) and native S. invicta determined from STRUCTURE simulations. The top panel shows results for 14 nuclear markers and the mtDNA combined, whereas the bottom panel shows results for the nuclear markers only. Within each population demarcated by a rectangle, individuals are represented by vertical lines divided into parts proportional to their proposed ancestry in each STRUCTURE-defined genetic cluster. MS, Mississippi; wLA, west Louisiana; PEL, Pontes E Lacerda; PeP, Pedra Preta; SGO, Sa˜o Gabriel do Oeste; CaG, Campo Grande; CeA, Ceu Azul; AdR X, Arroio dos Ratos X; Y, Arroio dos Ratos Y; RdC, Rinco dos Cabrais; Ros, Rosario; Cor, Corrientes; For, Formosa. M, monogyne social form; P, polygyne social form

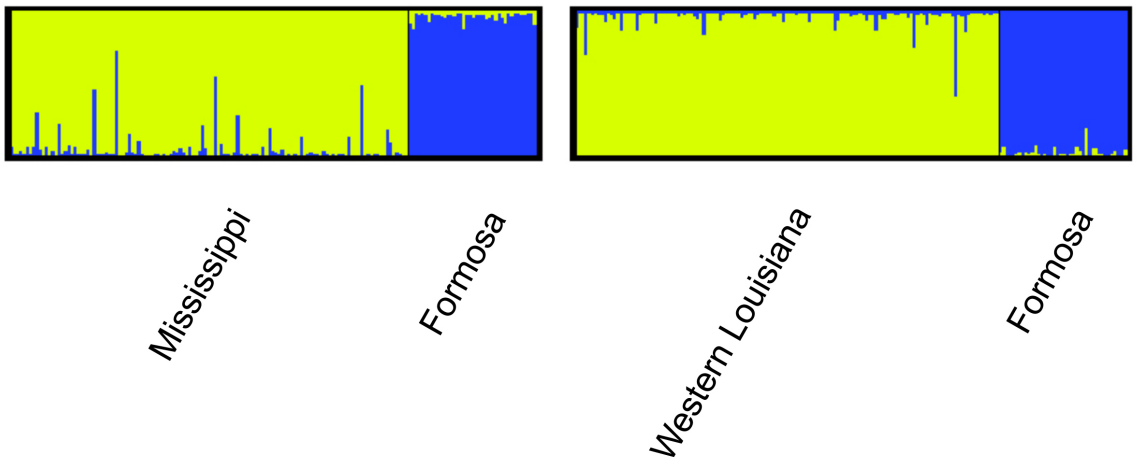


Figure 4. Individual membership coefficients for introduced (Mississippi and west Louisiana, USA) and native (Formosa, Argentina) *S. invicta* determined from STRUCTURE simulations using 14 nuclear markers (analyses conducted separately for each introduced population). Individuals are represented by vertical lines divided into parts proportional to their proposed ancestry in each STRUCTURE-defined genetic cluster

Exclusion Tests

Exclusion tests potentially allow the rejection of all native populations as sources of the USA ants if the actual source was not sampled. Nonetheless, the exclusion tests we performed strongly support our findings from the assignment tests and STRUCTURE simulations that the likely source population for *S. invicta* in the USA occurs near Formosa, Argentina. In the individual-level analyses, only 38 (15%) of the ants from Mississippi and west Louisiana could be excluded as originating from the characteristically Formosan cluster SA9 (at $P<0.01$; Fig. 5). Among these individuals, all but one were excluded from every other potential source cluster as well. Only three other clusters were not excluded entirely as sources for some introduced ants (Fig. 5). These are SA10, a cluster confined to Formosa, SA8, a cluster best represented at the neighboring Corrientes locality, and SA5, a cluster well represented at Corrientes and two southern Brazilian localities (Fig. 2).

It is noteworthy that genetic clusters composed solely or largely of ants from Brazilian localities (SA1, SA2, SA3, SA4, and SA6) were rejected as potential sources for any individual from the USA (Fig. 5). Identical results were obtained when we used geographic populations as the native references; all Brazilian localities invariably were rejected as potential sources.

Phylogenetic Analyses of mtDNA Sequences

The NJ and Bayesian phylogenetic analyses of the mtDNA sequences produced identical trees comprising seven well-supported clades, each of which has a distinctive geographic distribution (Fig. 6; see also Shoemaker et al. 2006a). The six haplotypes found in ants from Mississippi and west Louisiana fall within three clades distributed

chiefly in central and north-central Argentina (designated clades 2, 3, and 4; see Shoemaker et al. 2006a). Four of the six introduced haplotypes are identical to variants from Formosa. A fifth (H5) is identical to a haplotype occurring in Corrientes and a third Argentine locality, Rosario. The sole mtDNA sequence unique to the introduced ants (USA4) differs from a recovered Formosa haplotype by a single point substitution.

Additional Results

The NJ tree depicting the nuclear genetic relationships of the sampled populations is presented in Figure 7, along with estimates of F_K, a measure of the nuclear genetic divergence of each sampled population from a hypothetical population ancestral to all of them. The introduced populations are most closely allied genetically to the Formosa ants among the native populations studied. Values of F_K equal to or exceeding 0.2 are confined to the Brazilian and introduced populations, whereas values less than 0.1 characterize only Corrientes and Formosa. This result reflects the conclusions of Ross et al. (2007) that the more peripheral Brazilian populations are relatively recently derived from ancestral *S. invicta* populations that resided in northern Argentina. More importantly with respect to the present study, it also implies that the introduced populations are among the most divergent relative to this hypothetical ancestral form. Such a pattern is expected given the pronounced changes in the extent and type of genetic variation predicted for bottlenecked populations (Chakraborty and Nei 1977), changes that have been demonstrated in *S. invicta* in the USA for allozyme and sex-determination loci (Ross et al. 1993).

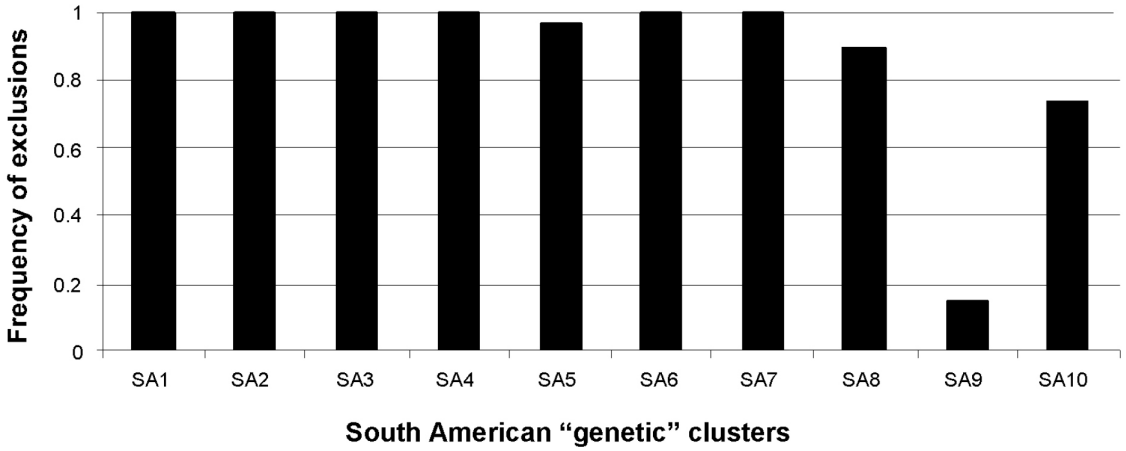


Figure 5. Results of exclusion tests for individual *S. invicta* from the introduced (USA) range based on 14 nuclear markers. The histogram shows the proportions of individuals from both Mississippi and west Louisiana excluded as originating from each STRUCTURE-defined genetic cluster in the native range

Inspection of the nuclear and mtDNA variants in our samples from Formosa and the USA confirms that the variation in the introduced ants generally represents a very restricted subset of the variation found in Formosa, and that most variants in the USA can also be found in Formosa (Fig. 8). Specifically, 43% of the Formosa nuclear alleles and 22% of the Formosa mtDNA haplotypes were detected in the introduced ants, while only four of the 59 total variants from the introduced range (6.8%) were not detected in Formosa (two microsatellite alleles and two mtDNA haplotypes). Importantly, an atypical interrupted-repeat microsatellite allele recovered from both USA localities (*Sol-55*^{152}) was found only in Formosa among the native populations. Because mutations giving rise to such variants occur relatively infrequently (e.g., Jarne and Lagoda 1996), their common occurrence in Formosa and the introduced range is most likely due to a relatively recent genealogical connection between ants in the two areas.

Estimates of F_{ST} (nuclear markers) and Φ_{ST} (mtDNA sequences) between paired native and introduced populations are presented in Figure 9. The lowest F_{ST} values invariably involve the Formosa ants. Estimates of Φ_{ST} yield similar patterns, with one exception; the Mississippi polygyne form is more similar overall in its mtDNA composition to ants from the Argentine populations of Corrientes and Rosario than to ants from Formosa. This result reflects the fact that one common haplotype in the Mississippi polygyne ants occurs only in Corrientes and Rosario in the native range (see Fig. 6).

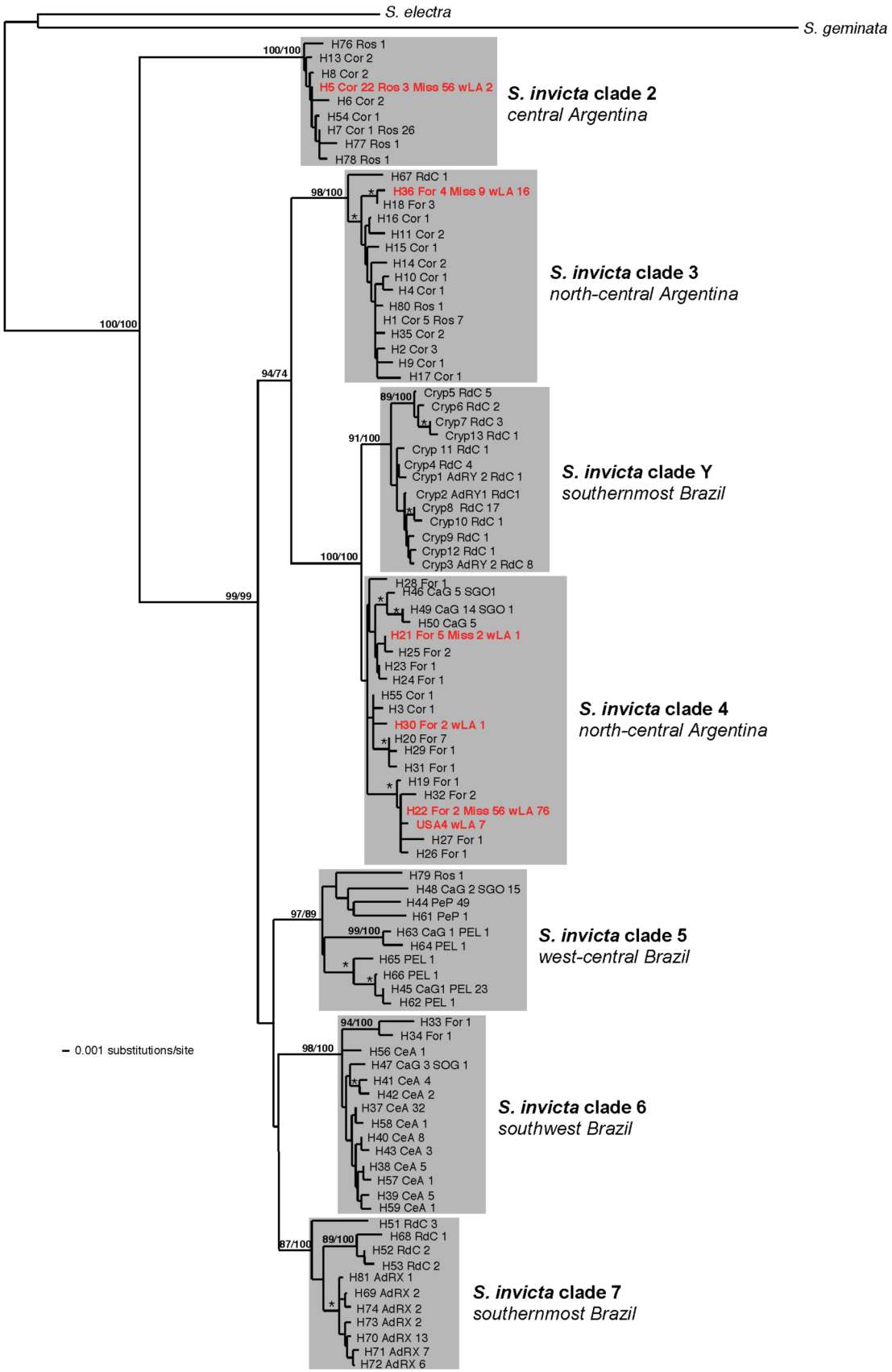

S. electra
S. geminata
100/100
H76 Ros 1
H13 Cor 2
H8 Cor 2
H5 Cor 22 Ros 3 Miss 56 wLA 2
H6 Cor 2
H54 Cor 1
H7 Cor 1 Ros 26
H77 Ros 1
H78 Ros 1
S. invicta clade 2
central Argentina
98/100
H67 RdC 1
H36 For 4 Miss 9 wLA 16
H18 For 3
H16 Cor 1
H11 Cor 2
H15 Cor 1
H14 Cor 2
H10 Cor 1
H4 Cor 1
H80 Ros 1
H1 Cor 5 Ros 7
H35 Cor 2
H2 Cor 3
H9 Cor 1
H17 Cor 1
S. invicta clade 3
north-central Argentina
100/100
94/74
89/100
Cryp5 RdC 5
Cryp6 RdC 2
Cryp7 RdC 3
91/100
Cryp13 RdC 1
Cryp 11 RdC 1
Cryp4 RdC 4
Cryp1 AdRY 2 RdC 1
Cryp2 AdRY1 RdC1
Cryp8 RdC 17
Cryp10 RdC 1
Cryp9 RdC 1
Cryp12 RdC 1
Cryp3 AdRY 2 RdC 8
S. invicta clade Y
southernmost Brazil
100/100
99/99
H28 For 1
H46 CaG 5 SGO1
H49 CaG 14 SGO 1
H50 CaG 5
H21 For 5 Miss 2 wLA 1
H25 For 2
H23 For 1
H24 For 1
H55 Cor 1
H3 Cor 1
H30 For 2 wLA 1
H20 For 7
H29 For 1
H31 For 1
H19 For 1
H32 For 2
H22 For 2 Miss 56 wLA 76
USA4 wLA 7
H27 For 1
H26 For 1
S. invicta clade 4
north-central Argentina
97/89
H79 Ros 1
H48 CaG 2 SGO 15
H44 PeP 49
H61 PeP 1
99/100
H63 CaG 1 PEL 1
H64 PEL 1
H65 PEL 1
H66 PEL 1
H45 CaG1 PEL 23
H62 PEL 1
S. invicta clade 5
west-central Brazil
94/100
H33 For 1
H34 For 1
98/100
H56 CeA 1
H47 CaG 3 SOG 1
H41 CeA 4
H42 CeA 2
H37 CeA 32
H58 CeA 1
H40 CeA 8
H43 CeA 3
H38 CeA 5
H57 CeA 1
H39 CeA 5
H59 CeA 1
S. invicta clade 6
southwest Brazil
0.001 substitutions/site
87/100
89/100
H51 RdC 3
H68 RdC 1
H52 RdC 2
H53 RdC 2
H81 AdRX 1
H69 AdRX 2
H74 AdRX 2
H73 AdRX 2
H70 AdRX 13
H71 AdRX 7
H72 AdRX 6
S. invicta clade 7
southernmost Brazil

Figure 6. Tree depicting phylogenetic relationships of unique *S. invicta* mtDNA sequence haplotypes obtained from both NJ and Bayesian analyses. Codes at terminals indicate geographic localities where each haplotype was found (see Fig. 3 legend). Terminals labeled with white lettering indicate haplotypes found in the USA; haplotype H5 is denoted by a black triangle and haplotype USA4 is denoted by a black circle. Seven major haplotype clades are indicated along with their regional affiliations (see Ross et al. 2007; Shoemaker et al. 2006a). Numbers on branches represent NJ bootstrap support values followed by Bayesian posterior probability values (only values greater than 70% are shown); asterisks indicate additional nodes with bootstrap and posterior probability values greater than 70%

6.1.5 Discussion

The primary objective of this study was to use genetic data generated from three classes of genetic markers (allozymes, microsatellites, and mtDNA sequences) to attempt to identify the native source population(s) from which the fire ant *Solenopsis invicta* in the USA originated. To accomplish this goal, we performed assignment and exclusion tests, as well as population genetic and phylogenetic analyses, utilizing ants collected from diverse native and introduced populations. One result important in paving the way for source identification is that, consistent with earlier genetic studies, significant genetic differentiation was detected among sampled populations within both the native and introduced ranges (Ross et al. 2007; Shoemaker et al. 2006b). The especially marked differentiation among native populations is noteworthy because it constitutes a nearly ideal circumstance for identifying the native source of invasive *S. invicta* by allowing information contained in the unique regional genetic makeups to be exploited. Moreover, such pronounced structure highlights the practical and scientific importance of identifying the source population(s), as it implies that the natural enemies of this pest ant are likely to be locally adapted to their genetically distinct hosts from different areas (see also below).

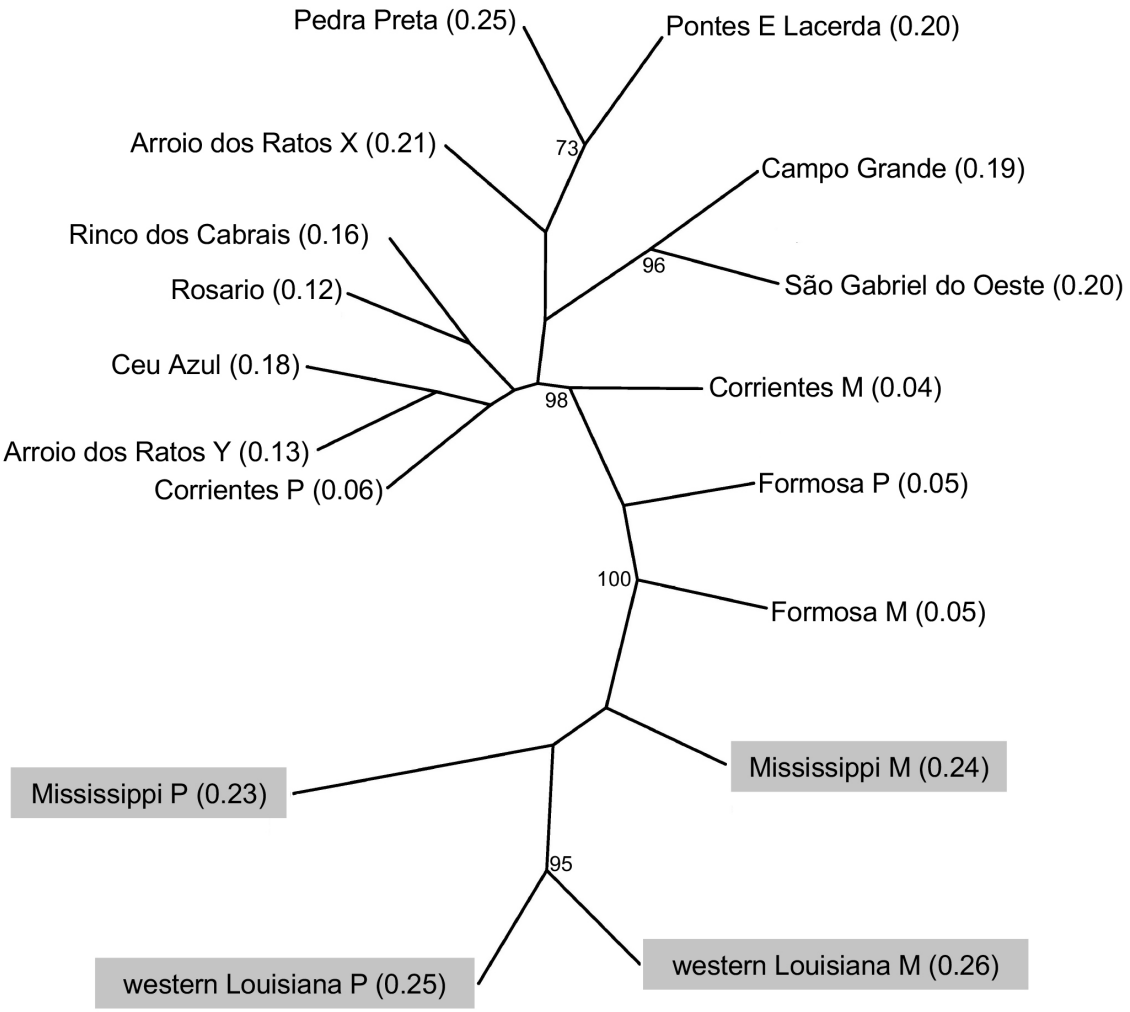


Figure 7. Neighbor-joining (NJ) consensus tree depicting nuclear genetic relationships of native (black lettering) and introduced (white lettering) populations of *S. invicta* based on Nei's chord distance. Numbers at nodes are percentages of bootstrap replicates (out of 1,000) in which population clusters distal to that node were recovered (only values greater than 70% are shown). Numbers in parentheses correspond to population FK values generated by STRUCTURE simulations using the nuclear data. M, monogyne social form; P, polygyne social form

Recognition of the significant differentiation between the two introduced populations, earlier taken to signify possible multiple invasions of *S. invicta* from the native range, is important in that separate analyses involving each population are thus warranted.

A major result consistently obtained by our analyses is that the likely source population for all invasive *S. invicta* in the USA occurs at or near Formosa, Argentina. Furthermore, while some of our analyses did not provide unequivocal evidence implicating this specific source, virtually every analysis ruled out all sampled Brazilian populations as a potential source. These findings are consistent with speculation that northeastern Argentina is more likely to contain the native source population(s) (Mescher et al. 2003; Ross and Trager 1990) than is the earlier suggested Pantanal Region of southwestern Brazil (Allen and Buren 1974; Buren 1972; Buren et al. 1974). While further work will be needed to conclusively pinpoint the source of the USA colonists, our data clearly are of value in geographically focusing such efforts.

Early collections of *S. invicta* in the USA provide a rather detailed picture of the history of the spread of this ant in the USA, but the question of how the original colonists arrived remains unanswered (Tschinkel 2006). One early hypothesis that the ant was introduced from Brazil (Allen and Buren 1974; Buren 1972; Buren et al. 1974) envisioned a scenario in which colonies from the Pantanal either drifted naturally on floodwaters or were carried by commerce to rivers in one of two major drainage systems, the Amazon River Basin to the north or the La Plata River Basin to the south (see Fig. 2). Ultimately, commercial ships would have carried the ants from ports on either of these waterways to the port of Mobile, Alabama, where they first appeared in the USA. Another scenario proposed by Buren (1972) envisioned natural or anthropogenic

movement of colonies southward down the Paraná River (in the La Plata Basin), with eventual transport to the USA from ports near Buenos Aires. Any scenario invoking waterways in the La Plata Basin remains feasible in light of the results of the present study. However, our data further suggest that colonies likely were transported to such a waterway from the flood plains of the Mesopotamia region of northeastern Argentina rather than the flood plains of the Brazilian Pantanal. Mesopotamia is subject to recurrent flooding (as is the Pantanal), an important characteristic posited for the source region by Buren (1972).

Our results are highly relevant to programs intended to control invasive populations of *S. invicta* using natural enemies. Strong genetic differentiation among native populations of *S. invicta* suggests that its various natural enemies, such as parasitic phorid flies, are likely to be locally adapted to their geographically unique ant hosts. The success of such enemies in attacking *S. invicta* thus may depend on genotype matching between the enemies and the ants (Kaltz and Shykoff 1998; Laine 2005; Lively and Dybdahl 2000; Lively et al. 2004; Thrall et al. 2002), and the search for sustainable biological control agents is likely to be more effective if they are collected from the specific local variants of *S. invicta* that were introduced into the USA. Several lines of evidence suggest the possibility of such genotype matching. First, current success in rearing and propagating South American phorid flies on fire ants in the USA is highly variable across trials (Morrison and Porter 2005; Porter and Alonso 1999; Porter et al. 1995; Porter et al. 1997a). Second, two biotypes of the phorid fly *Pseudacteon curvatus* have been shown to differ markedly in their host preferences, with each strongly preferring to attack the fire ant host species on which it was collected (Vazquez et al.

2004). Moreover, the *P. curvatus* biotype most successful in parasitizing *S. invicta* in the USA comes from Formosa, Argentina (Vazquez et al. 2004), the area inferred from our analyses to be the most probable source of these introduced ants. While climate matching between the native and introduced ranges for parasites such as phorids undoubtedly is also an important consideration for choosing potential control agents (Folgarait et al. 2005), this evidence argues that genetic matching of hosts and parasites may be equally or more important. Unfortunately, several studies of the impact of the phorid *P. tricuspis* on introduced *S. invicta* have utilized flies collected from Brazil (e.g., Mehdiabadi and Gilbert 2002; Mehdiabadi et al. 2004; Pereira and Porter 2006), making it more difficult to judge the potential effectiveness of this species in the biological control of fire ants.

Our results are of special significance because *S. invicta* recently has been inadvertently introduced into a number of regions around the world, including the Carribean, Australia, China, and Taiwan (Buckley 1999; Chen et al. 2006; Davis et al. 2001; Huang et al. 2004; MacKay and Fagerlund 1997; McCubbin and Weiner 2002). The extensive dataset we have generated provides necessary baseline information for researchers studying *S. invicta* in any area to readily make initial diagnoses concerning the regional source of the invaders, and then to implement subsequent fine-scale sampling or collection of natural enemies in the implicated region. We expect that such efforts will become increasingly necessary as *S. invicta* continues to be spread globally through commerce.

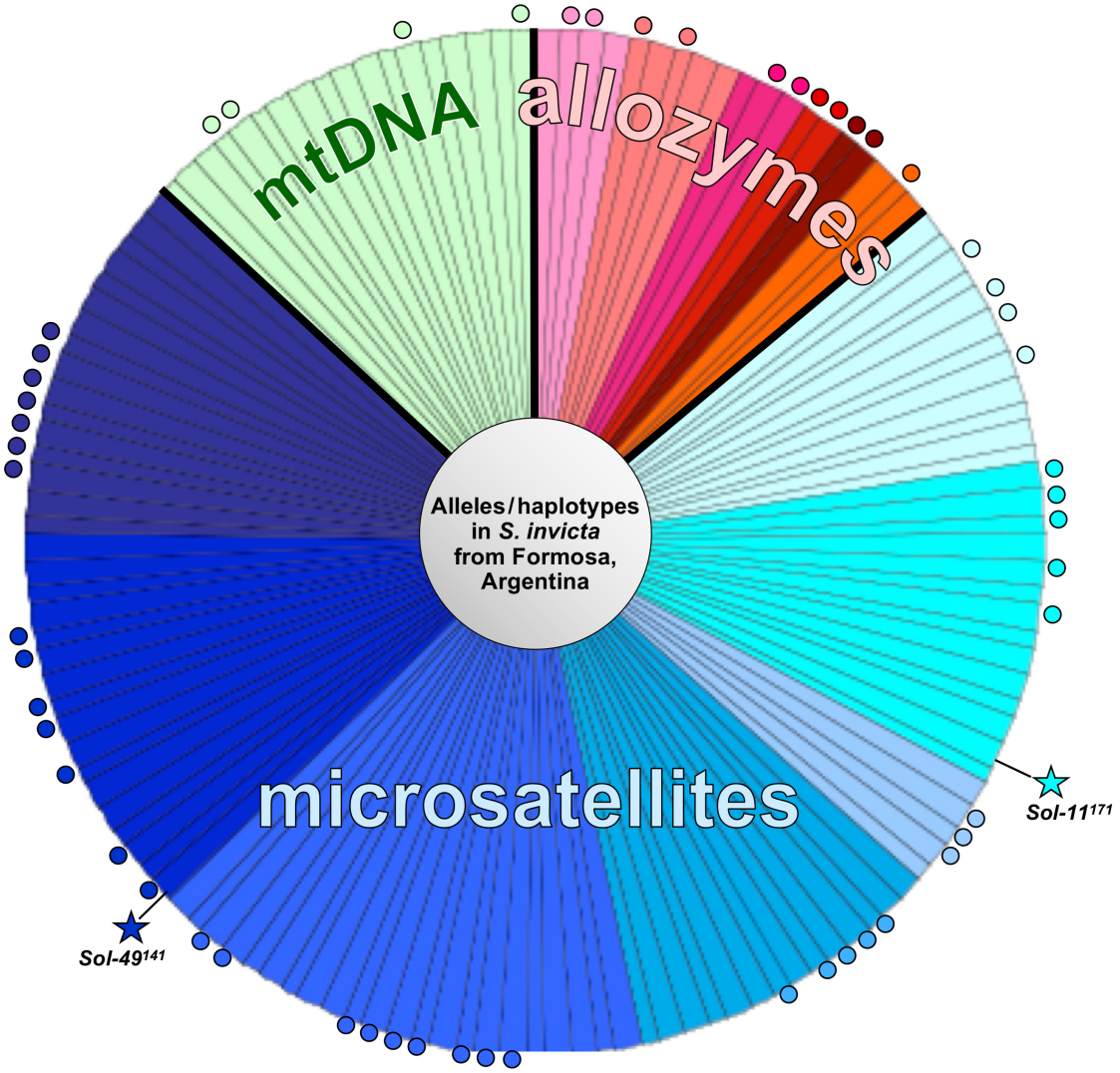


Figure 8. Genetic variation in native Formosa and introduced USA populations of *S. invicta*. Variants detected in Formosa are shown as pie slices, with the nuclear loci and alleles ordered as in Appendix II (the allozyme locus *Gpi* is excluded because it is monomorphic for the same allele in Formosa and the USA). Variants detected in the introduced range are shown outside the pie perimeter, with stars indicating the two microsatellite alleles and two mtDNA haplotypes found in the USA but not Formosa

A recent detailed study of the population genetic structure of introduced *S. invicta* in the USA (Shoemaker et al. 2006b) concluded that at least two introductions may have occurred, one near the presumed original site of entry (Mobile, Alabama) more than 70 years ago, and a second near Port Arthur, Texas at a more recent point. It might be expected that any such secondary introduction involved ants from a different location in the native range than served as the primary source of introduction, yet our data consistently implicate Formosa, Argentina as the native population most closely allied genetically to ants derived from both introductions (as represented in the Mississippi and west Louisiana samples). In addition to the various statistical analyses based on the overall information content of diverse nuclear and mtDNA markers pointing to this conclusion, the unique possession of an atypical interrupted-repeat microsatellite allele in both the Formosa and USA populations constitutes strong corroborating evidence. Furthermore, some added support comes from recent sequence analyses of the candidate social behavior gene *Gp-9* in introduced and native *S. invicta* (Gotzek et al. 2007; Krieger and Ross 2002); only one allele from the USA was found as well in South America, in ants from Formosa and a second Argentine locality between Formosa and Rosario.

Although these various results implicate Formosa as the source region of *S. invicta* in the USA, inspection of our mtDNA haplotype phylogeny suggests the possibility of a more nuanced picture. Most haplotypes from the USA belong to clades 3 and 4, and they either are found exclusively in Formosa or are nearly identical to haplotypes found only there (Fig. 6). However, a single haplotype in clade 2 (H5) found in both introduced populations was not recovered from Formosa ants, but does occur in ants collected from the nearby locality of Corrientes and the more southerly Argentine locality of Rosario.

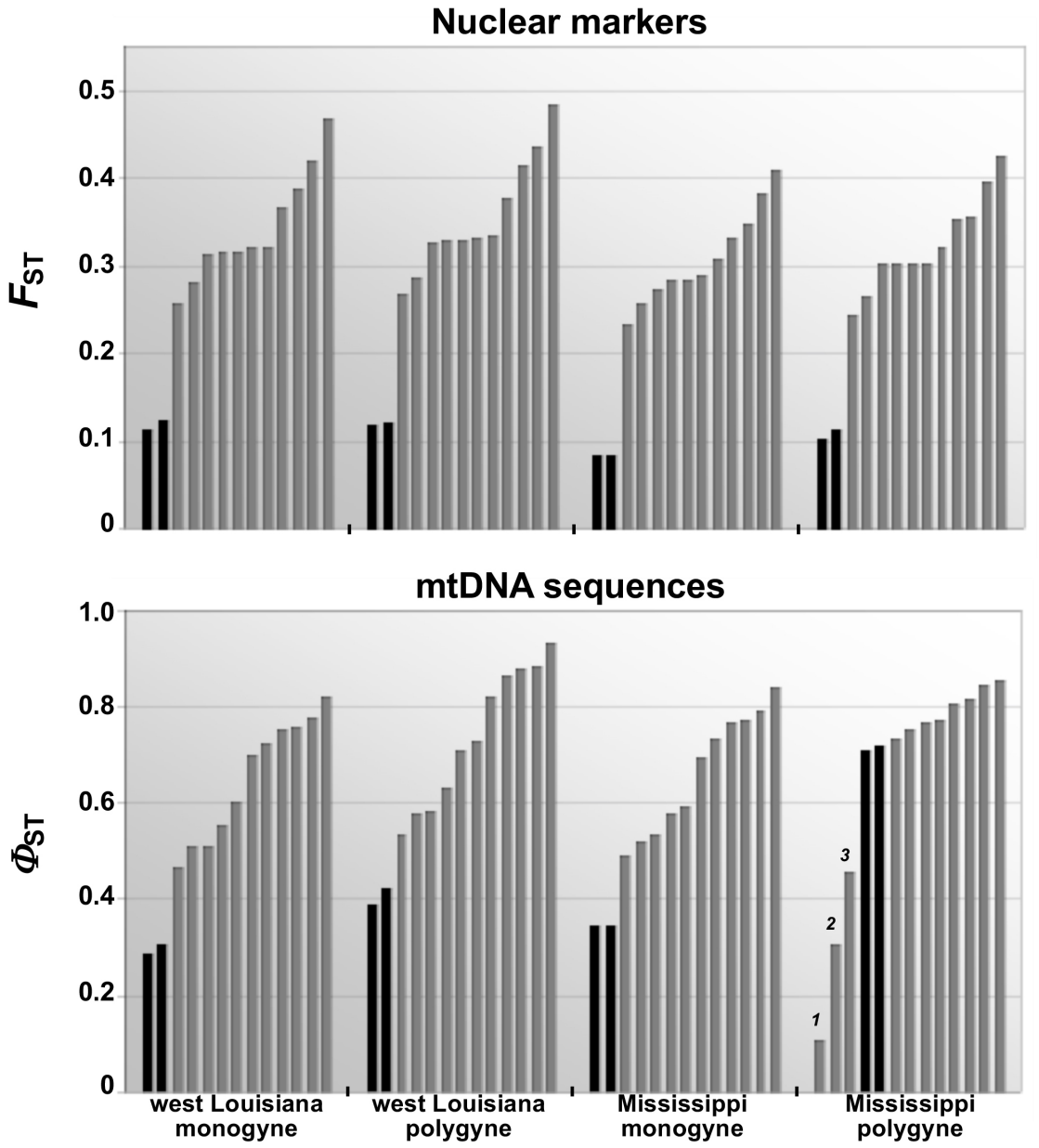


Figure 9. Estimates of FST (nuclear markers) and UST (mtDNA sequences) between paired native and introduced populations of *S. invicta*. Values for comparisons between the introduced ants and the native Formosa, Argentina populations are shown in black. Numbers in the UST graph for the Mississippi P population indicate comparisons to the Corrientes M (1), Rosario (2), and Corrientes P (3) populations. M, monogyne social form; P, polygyne social form

Indeed, all of the clade 2 haplotypes are known only from these latter two localities. While it is possible that clade 2 haplotypes such as H5 occur in Formosa but simply were missed due to sampling error, a perhaps more likely possibility is that the actual source locality lies in an unsampled part of the Mesopotamia region close to both Corrientes and Formosa. The absence of two microsatellite alleles in Formosa that are found in the USA may also support this scenario, although these alleles were not detected anywhere in Argentina (see Appendix II). Despite this uncertainty, one consistent result across our analyses is that all of the sampled Brazilian populations can be excluded as potential source populations. More extensive collections from northeastern Argentina and Paraguay, combined with the use of additional genetic markers currently under development, must now be employed to refine our hypotheses concerning the origin of invasive *S. invicta* in the USA.

6.1.6 Acknowledgements

We thank Laurent Keller and Mark Mescher for help collecting ants and Dietrich Gotzek for comments on an earlier draft of the manuscript. This study was supported by a grant from the United States Department of Agriculture NRICGP (2006-35302-18001; Agreement No. 58-6615-7-137). The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the United States Department of Agriculture or the Agricultural Research Service of any product or service to the exclusion of others that may be suitable. Any opinions, findings, conclusion, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

6.1.7 Appendix Tables

Appendix I. Locations of sampled populations of *S. invicta*. Codes are population abbreviations used in figures and Appendix II. *N* represents the number of individuals (one per nest) sampled from each population.

City	Province or State	Country	Code	N	Latitude	Longitude
Hurley	Mississippi	USA	MS	125	30° 65' 17" N	88° 49' 15" W
De Quincy	Louisiana	USA	wLA	135	37° 06' 25" N	93° 47' 35" W
Corrientes	Corrientes	Argentina	Cor	79	27° 34' 09" S	58° 50' 23" W
Formosa	Formosa	Argentina	For	70	26° 09' 34" S	58° 09' 57" W
Rosario	Santa Fe	Argentina	Ros	42	32° 54' 15" S	60° 47' 13" W
Ceu Azul	Parana	Brazil	CeA	83	25° 08' 30" S	53° 53' 56" W
Pedra Preta	Mato Grosso	Brazil	PeP	62	16° 42' 42" S	54° 34' 22" W
Pontes E Lacerda	Mato Grosso	Brazil	PEL	30	15° 11' 27" S	59° 17' 20" W
Campo Grande	Mato Grosso do Sul	Brazil	CaG	43	20° 21' 10" S	54° 34' 22" W
Rincão dos Cabrais	Rio Grande do Sul	Brazil	RdC	81	29° 43' 60" S	52° 57' 00" W
Arroio dos Ratos	Rio Grande do Sul	Brazil	AdR	44	30° 08' 21" S	51° 30' 11" W
São Gabriel do Oeste	Mato Grosso do Sul	Brazil	SGO	31	19° 17' 24" S	54° 34' 22" W

Appendix II. Nuclear allele and mtDNA haplotype frequencies within 17 sampled sites in the USA and South America

	MS *M*	MS *P*	wLA *M*	wLA *P*	PEL	PeP	SGO	CaG	CeA	AdR X	AdR Y	RdC	Ros	Cor *M*	Cor *P*	For *M*	For *P*
Allozyme loci																	
Pgm-1	**60**	**62**	**73**	**59**	**30**	**62**	**31**	**43**	**83**	**27**	**8**	**80**	**42**	**36**	**43**	**35**	**35**
85	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0.023	0	0.014
90	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0	0	0	0
93	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.012	0	0
96	0.142	0.145	0.034	0.042	0.983	0.621	0.145	0.07	0	0	0.188	0.063	0.119	0.069	0.151	0.171	0.114
98	0	0	0	0	0	0	0	0	0.03	0.37	0	0	0	0	0	0	0
100	0.858	0.855	0.966	0.958	0.017	0.371	0.839	0.93	0.97	0.63	0.813	0.919	0.667	0.903	0.767	0.829	0.857
104	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0	0	0	0.014
107	0	0	0	0	0	0	0	0	0	0	0	0.019	0.214	0.014	0.047	0	0
Acoh-1	**58**	**61**	**70**	**59**	**30**	**62**	**31**	**43**	**82**	**35**	**4**	**81**	**41**	**36**	**43**	**35**	**35**
82	0.086	0.033	0.214	0.093	0	0	0	0	0	0	0	0	0	0	0	0.057	0.071
87	0	0	0	0	0.033	0	0	0	0	0	0	0	0	0	0.012	0	0
92	0	0	0	0	0	0	0	0	0.092	0	0	0	0	0	0	0.029	0
100	0.914	0.967	0.786	0.907	0.967	1	1	1	0.909	0.986	1	1	1	0.986	0.988	0.886	0.929
114	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0.014	0
130	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0
139	0	0	0	0	0	0	0	0	0	0.014	0	0	0	0	0	0	0
Acoh-5	**62**	**60**	**72**	**59**	**30**	**62**	**31**	**42**	**83**	**35**	**4**	**81**	**42**	**36**	**43**	**35**	**35**
84	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0.043
88	0	0	0	0	0	0	0.307	0.202	0.018	0.014	0	0.025	0	0	0	0	0
93	0.234	0.292	0.292	0.195	0.833	0.307	0.355	0.5	0.837	0.986	1	0.877	0.869	0.806	0.861	0.3	0.214
100	0.766	0.708	0.708	0.805	0.167	0.694	0.339	0.298	0.145	0	0	0.099	0.131	0.194	0.14	0.686	0.743
G3pdh-1	**61**	**61**	**73**	**59**	**30**	**60**	**31**	**43**	**81**	**34**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
40	0.41	0.353	0.438	0.424	0	0	0	0	0	0	0	0	0	0	0	0.129	0.143
100	0.59	0.648	0.562	0.576	1	1	1	1	1	1	1	1	1	1	1	0.871	0.857
Aat-2	**62**	**60**	**74**	**59**	**30**	**62**	**31**	**43**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
100	0.927	0.958	0.966	0.949	0	0	0	0	0	0	0	0.068	0.012	0.083	0	0.629	0.443
144	0.073	0.042	0.034	0.051	1	1	1	1	1	0.957	1	0.932	0.988	0.903	1	0.371	0.557
172	0	0	0	0	0	0	0	0	0	0.043	0	0	0	0.014	0	0	0
Gpi	–	–	–	–	**30**	**62**	**31**	**43**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
79	–	–	–	–	0	0	0	0	0	0	0.111	0.025	0	0	0	0	0
88	–	–	–	–	0	0	0	0.023	0	0	0	0	0	0	0	0	0
100	–	–	–	–	1	0.968	1	0.977	1	1	0.889	0.975	1	1	1	1	1
104	–	–	–	–	0	0.032	0	0	0	0	0	0	0	0	0	0	0
Est-2	–	–	–	–	**30**	**62**	**31**	**43**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
58	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0.023	0	0
65	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0.012	0.029	0
68	–	–	–	–	0	0	0	0	0	0	0	0.012	0.012	0	0.012	0	0
73	–	–	–	–	0	0	0	0	0	0	0	0	0	0	0.012	0	0
76	–	–	–	–	0	0	0	0	0	0	0	0	0.048	0	0.012	0	0
81	–	–	–	–	0	0	0	0	0.036	0	0	0.006	0	0	0	0	0
88	–	–	–	–	0	0	0	0	0	0.014	0	0.395	0	0	0	0	0
100	–	–	–	–	1	1	0.903	0.988	0.265	0.014	0	0.303	0.917	0.681	0.326	0.957	1
108	–	–	–	–	0	0	0	0	0	0.971	0	0.185	0.024	0	0	0	0
111	–	–	–	–	0	0	0.097	0.012	0.699	0	1	0.099	0	0.319	0.605	0.014	0

Appendix II continued

	MS *M*	MS *P*	wLA *M*	wLA *P*	PEL	PeP	SGO	CaG	CeA	AdR X	AdR Y	RdC	Ros	Cor *M*	Cor *P*	For *M*	For *P*
Microsatellite loci																	
Sol-6	**62**	**63**	**74**	**58**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
87	0	0	0	0	0	0	0	0	0.265	0	0.056	0.426	0.048	0.139	0.128	0	0
95	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014
97	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0	0
107	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0	0	0.014	0
109	0.129	0.111	0.027	0.017	0	0	0	0	0.06	0	0	0	0	0	0	0.014	0.043
111	0	0	0	0	0.017	0.202	0	0	0	0.3	0.167	0.099	0.262	0.014	0.058	0.014	0.014
113	0.734	0.675	0.628	0.759	0.05	0.339	0.048	0.022	0.199	0.057	0.389	0.253	0.214	0.319	0.267	0.414	0.614
115	0.121	0.183	0.345	0.224	0.017	0.298	0.21	0.344	0.392	0.614	0	0.074	0.012	0.194	0.174	0.229	0.157
117	0	0	0	0	0.067	0.161	0	0.122	0.078	0.029	0.278	0.117	0.083	0.153	0.186	0.129	0.086
119	0.016	0.032	0	0	0.083	0	0	0.167	0	0	0	0	0.31	0.042	0.047	0.071	0.029
121	0	0	0	0	0.55	0	0.694	0.322	0.006	0	0.111	0	0.06	0.083	0.058	0	0
123	0	0	0	0	0.133	0	0	0	0	0	0	0	0	0	0.07	0.071	0.029
125	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0.012	0	0
127	0	0	0	0	0	0	0	0	0	0	0	0.031	0	0.028	0	0.014	0.014
129	0	0	0	0	0	0	0.032	0	0	0	0	0	0	0	0	0.014	0
131	0	0	0	0	0.033	0	0	0	0	0	0	0	0.012	0.014	0	0	0
133	0	0	0	0	0	0	0	0.022	0	0	0	0	0	0	0	0	0
135	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0
Sol-11	**62**	**63**	**73**	**59**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
143	0.21	0.183	0.027	0.068	0	0	0	0	0.006	0.071	0	0	0.191	0.014	0.023	0.1	0.029
145	0.04	0.103	0	0	0	0	0.032	0	0	0	0	0.006	0	0	0	0.071	0.086
147	0.169	0.151	0	0.144	0.533	0	0	0.078	0	0.614	0	0	0.036	0.042	0.035	0.114	0.129
149	0	0	0	0	0	0	0	0	0	0.114	0.111	0.469	0.048	0	0.012	0.114	0.1
151	0.427	0.405	0.623	0.661	0.2	0	0.613	0.767	0.669	0.143	0.444	0.198	0.25	0.486	0.581	0.314	0.457
153	0	0	0	0	0.033	0.016	0.016	0.022	0.121	0.057	0	0.222	0.333	0.208	0.174	0.143	0.014
155	0.153	0.159	0.343	0.127	0.017	0.339	0	0	0	0	0	0.025	0.036	0.056	0	0	0.057
157	0	0	0	0	0	0.073	0	0	0	0	0.056	0	0.06	0.042	0.035	0.071	0.071
159	0	0	0	0	0.067	0.008	0	0	0.048	0	0	0.049	0.024	0.069	0.023	0.014	0
161	0	0	0	0	0.033	0.008	0.032	0.1	0.072	0	0.111	0.019	0.024	0.014	0.023	0	0
163	0	0	0	0	0	0	0	0	0.042	0	0	0	0	0.028	0.012	0	0.014
165	0	0	0	0	0	0	0.032	0.022	0	0	0.111	0	0	0.028	0.035	0.014	0
167	0	0	0	0	0.117	0	0.274	0.011	0.042	0	0.167	0.012	0	0.014	0.023	0	0.029
169	0	0	0	0	0	0.057	0	0	0	0	0	0	0	0	0.023	0.043	0
171	0	0	0.007	0	0	0.186	0	0	0	0	0	0	0	0	0	0	0
173	0	0	0	0	0	0.307	0	0	0	0	0	0	0	0	0	0	0
177	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014
189	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0	0	0	0
Sol-18	**62**	**63**	**74**	**59**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
117	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.012	0	0
121	0	0	0	0	0.05	0.008	0.032	0	0.024	0	0	0	0	0	0	0	0
123	0	0	0	0	0.5	0.774	0.065	0	0	0.714	0.167	0.08	0.107	0.208	0.116	0.043	0.029
125	0.766	0.786	0.919	0.924	0.033	0.218	0.274	0.433	0.843	0.029	0.722	0.883	0.857	0.694	0.744	0.9	0.9
127	0.186	0.151	0.081	0.076	0.15	0	0.629	0.567	0.133	0.257	0.111	0.006	0	0.083	0.093	0.014	0.043
129	0.048	0.064	0	0	0.25	0	0	0	0	0	0	0.031	0	0	0.012	0.043	0
131	0	0	0	0	0.017	0	0	0	0	0	0	0	0.036	0.014	0.012	0	0.029
135	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.012	0	0

Appendix II continued

	MS *M*	MS *P*	wLA *M*	wLA *P*	PEL	PeP	SGO	CaG	CeA	AdR X	AdR Y	RdC	Ros	Cor *M*	Cor *P*	For *M*	For *P*
Sol-20	**62**	**63**	**74**	**59**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
114	0	0	0	0	0	0	0	0	0	0	0	0.012	0	0	0	0	0
116	0	0	0	0	0	0	0	0	0	0	0	0.006	0	0	0	0	0
120	0	0	0	0	0	0.016	0	0	0.036	0	0	0.006	0.071	0.014	0.035	0	0.014
122	0	0	0	0	0	0	0	0	0.06	0	0	0.006	0.06	0.069	0.198	0.043	0.029
124	0.25	0.183	0.115	0.212	0.083	0	0.081	0.167	0.753	0.214	0.722	0.29	0.357	0.347	0.384	0.129	0.157
126	0.589	0.5	0.345	0.322	0.367	0.282	0.258	0.3	0.06	0.086	0.111	0.068	0.191	0.139	0.128	0.357	0.429
128	0.161	0.318	0.264	0.085	0.4	0.694	0.194	0.222	0.078	0.1	0	0.099	0.214	0.111	0	0.214	0.129
129	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0	0
130	0	0	0	0.009	0.033	0.008	0.468	0.311	0	0	0	0.019	0	0.139	0.058	0.1	0.071
131	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0	0	0
132	0	0	0	0	0	0	0	0	0	0	0	0.056	0	0.014	0.035	0.1	0.071
133	0	0	0	0	0	0	0	0	0	0.429	0	0	0	0	0	0	0
134	0	0	0	0	0	0	0	0	0	0	0	0.037	0	0.028	0.012	0	0
135	0	0	0	0	0	0	0	0	0	0.086	0	0	0	0	0	0	0
136	0	0	0.014	0	0	0	0	0	0	0	0.056	0.025	0.06	0	0.047	0.014	0
138	0	0	0	0	0.1	0	0	0	0	0.057	0	0.241	0.048	0.014	0.023	0	0.014
140	0	0	0	0	0.017	0	0	0	0	0.029	0.111	0.124	0	0.028	0.047	0.014	0.014
142	0	0	0	0	0	0	0	0	0.012	0	0	0.012	0	0	0.012	0.014	0.043
144	0	0	0.264	0.348	0	0	0	0	0	0	0	0	0	0.014	0	0	0
146	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.012	0	0.014
148	0	0	0	0	0	0	0	0	0	0	0	0	0	0.056	0	0	0.014
150	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0
152	0	0	0	0.025	0	0	0	0	0	0	0	0	0	0	0.012	0	0
Sol-42	**62**	**63**	**74**	**59**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
91	0	0	0	0	0	0	0	0	0	0	0.111	0	0	0	0	0	0
99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.023	0	0
101	0	0	0	0	0	0	0	0	0	0	0	0	0	0.028	0	0	0
103	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0.035	0	0
105	0	0	0	0	0	0	0	0	0	0	0	0	0	0.014	0.012	0	0
107	0	0	0	0	0.067	0	0.016	0	0	0.071	0	0.025	0.012	0.208	0.244	0.029	0.014
109	0	0	0	0	0	0	0	0	0.006	0	0.056	0.117	0.143	0.139	0.093	0.014	0.014
111	0	0	0	0	0	0	0	0.133	0.115	0.114	0.056	0.025	0.214	0.097	0.035	0	0.029
113	0	0	0	0	0	0	0.032	0.1	0.036	0.029	0	0.124	0	0.042	0.035	0.114	0.029
115	0	0	0	0	0	0	0.274	0.278	0	0.029	0.056	0.006	0.012	0.042	0.116	0.029	0.043
117	0.323	0.294	0.297	0.466	0.133	0	0.113	0.044	0.205	0.143	0	0.019	0	0.083	0.105	0.129	0.114
119	0.113	0.103	0.169	0.22	0	0.016	0.307	0.089	0.06	0.129	0.111	0.099	0.095	0.028	0.058	0.114	0.171
121	0.161	0.079	0.088	0.059	0.017	0.008	0.048	0.044	0.042	0.243	0	0.191	0.095	0.069	0.058	0.086	0.157
123	0	0	0	0	0.033	0.04	0.21	0.089	0.277	0.1	0.056	0.13	0.048	0.014	0.047	0.114	0.1
125	0.016	0.056	0	0.009	0.05	0	0	0.133	0.012	0.029	0.222	0.099	0.036	0.014	0.047	0.043	0.014
127	0	0.008	0	0	0.033	0.04	0	0.067	0.054	0.029	0.111	0.062	0.024	0.056	0.047	0.071	0.057
129	0	0.024	0	0	0	0.161	0	0	0.012	0	0.056	0.093	0.071	0.028	0	0.029	0
131	0.226	0.325	0.284	0.161	0.017	0.516	0	0	0.042	0.043	0	0.006	0.012	0.042	0.012	0.071	0.043
133	0	0	0	0	0.1	0.129	0	0	0.024	0	0	0	0	0.028	0	0.014	0.014
135	0	0	0	0	0.017	0	0	0	0.084	0.043	0	0.006	0.071	0.014	0.012	0	0.014
137	0	0	0	0	0	0.089	0	0	0	0	0.167	0	0.06	0.014	0.012	0.014	0.029
139	0	0	0	0	0.183	0	0	0	0	0	0	0	0.083	0	0	0.029	0.014

Appendix II continued

	MS *M*	MS *P*	wLA *M*	wLA *P*	PEL	PeP	SGO	CaG	CeA	AdR X	AdR Y	RdC	Ros	Cor *M*	Cor *P*	For *M*	For *P*
141	0	0	0	0	0.2	0	0	0.022	0	0	0	0	0	0.028	0	0.014	0.057
143	0.153	0.095	0.155	0.085	0	0	0	0	0	0	0	0	0.024	0	0	0.014	0.014
145	0.008	0.016	0.007	0	0	0	0	0	0	0	0	0	0	0.014	0.012	0.029	0.043
147	0	0	0	0	0.033	0	0	0	0	0	0	0	0	0	0	0	0
149	0	0	0	0	0.05	0	0	0	0	0	0	0	0	0	0	0.014	0.014
151	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.029	0.014
159	0	0	0	0	0.017	0	0	0	0.03	0	0	0	0	0	0	0	0
Sol-49	**62**	**63**	**73**	**59**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
141	0.129	0.127	0	0	0.883	0.532	0.048	0	0	0	0	0	0	0	0	0	0
142	0	0	0.096	0.085	0	0	0	0	0	0	0	0	0	0.028	0.023	0.086	0.057
146	0	0	0	0	0	0	0	0	0	0.1	0.111	0	0	0	0.012	0	0.014
148	0.073	0.064	0.151	0.22	0	0	0	0	0	0	0	0	0	0	0	0.029	0.043
150	0	0	0	0	0	0	0.113	0.111	0	0	0.111	0.049	0.238	0.069	0.047	0.029	0.029
152	0	0	0	0	0	0	0.032	0	0	0.057	0	0.043	0.012	0	0.047	0.114	0.043
154	0	0	0	0	0	0	0.452	0.289	0.169	0	0	0.006	0.012	0.097	0.047	0.071	0.057
156	0	0.008	0	0	0	0.073	0.032	0	0.133	0.3	0	0.049	0	0.042	0.081	0.043	0.057
158	0	0	0	0	0	0.008	0	0	0.06	0.229	0.111	0.148	0.024	0.111	0.105	0.029	0.071
160	0.444	0.516	0.404	0.373	0	0.024	0.016	0.222	0.241	0.014	0	0.142	0.036	0.167	0.093	0.071	0.214
162	0.145	0.151	0.021	0.042	0.033	0.024	0.048	0.078	0.024	0.086	0.167	0.049	0.191	0.125	0.186	0.057	0.129
164	0	0	0	0	0.05	0.323	0.048	0.189	0.018	0.029	0.222	0.117	0.06	0.139	0.105	0.114	0.086
166	0.21	0.135	0.322	0.28	0.017	0.016	0	0.022	0.072	0.029	0	0.08	0.131	0.042	0.128	0.143	0.086
168	0	0	0.007	0	0.017	0	0	0	0.09	0.014	0.278	0.031	0.024	0.069	0.07	0.057	0.029
170	0	0	0	0	0	0	0	0.011	0.084	0.1	0	0.167	0.262	0.056	0.023	0.086	0.029
172	0	0	0	0	0	0	0.016	0	0.102	0	0	0.062	0.012	0.028	0.023	0.014	0
174	0	0	0	0	0	0	0.145	0.056	0.006	0.014	0	0.031	0	0.014	0	0.043	0.029
176	0	0	0	0	0	0	0	0.022	0	0	0	0.025	0	0	0.012	0.014	0.029
178	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0.014	0	0	0
180	0	0	0	0	0	0	0	0	0	0.029	0	0	0	0	0	0	0
182	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0	0	0	0
184	0	0	0	0	0	0	0.016	0	0	0	0	0	0	0	0	0	0
Sol-55	**62**	**63**	**72**	**59**	**30**	**62**	**31**	**45**	**83**	**35**	**9**	**81**	**42**	**36**	**43**	**35**	**35**
145	0	0	0	0	0	0	0	0	0.09	0	0	0	0	0	0.058	0	0.014
147	0	0	0	0	0	0	0	0.056	0.03	0	0.167	0.006	0.214	0.069	0.093	0.014	0.257
149	0.419	0.77	0.597	0.636	0	0.008	0	0.044	0	0.086	0	0.006	0	0.097	0.267	0.114	0.1
151	0.04	0.008	0.063	0.042	0.067	0	0.113	0.011	0.127	0	0	0.068	0.048	0.222	0.058	0.1	0.143
152	0.105	0.024	0.097	0.076	0	0	0	0	0	0	0	0	0	0	0	0.029	0
153	0.04	0.056	0.007	0	0.05	0.557	0.145	0.178	0.398	0.557	0.611	0.21	0.155	0.222	0.174	0.257	0.171
155	0.137	0.04	0.049	0.085	0.233	0.218	0.645	0.511	0.115	0.114	0.111	0.154	0.345	0.069	0.105	0.071	0.071
157	0.032	0.024	0.007	0.025	0.017	0	0	0.178	0.163	0	0	0	0.083	0.056	0.012	0.071	0.014
159	0.226	0.079	0.181	0.136	0.017	0	0	0.011	0.03	0.014	0	0.161	0.012	0.056	0.081	0.114	0.129
161	0	0	0	0	0.033	0.073	0	0	0.006	0.1	0	0.235	0.107	0.111	0.128	0.071	0.043
163	0	0	0	0	0.433	0.024	0.097	0.011	0.042	0.086	0	0.006	0.012	0.056	0.012	0.043	0.029
165	0	0	0	0	0.133	0.024	0	0	0	0	0.056	0.117	0.012	0	0.012	0.043	0.014
167	0	0	0	0	0	0	0	0	0	0.014	0	0.031	0	0	0	0.029	0.014
169	0	0	0	0	0.017	0	0	0	0	0	0	0	0	0.028	0	0.014	0
171	0	0	0	0	0	0	0	0	0	0	0	0.006	0	0	0	0	0
173	0	0	0	0	0	0	0	0	0	0.014	0	0	0	0.014	0	0.014	0

Appendix II continued

	MS *M*	MS *P*	wLA *M*	wLA *P*	PEL	PeP	SGO	CaG	CeA	AdR X	AdR Y	RdC	Ros	Cor *M*	Cor *P*	For *M*	For *P*
175	0	0	0	0	0	0	0	0	0	0.014	0	0	0	0	0	0.014	0
177	0	0	0	0	0	0	0	0	0	0	0	0	0.012	0	0	0	0
181	0	0	0	0	0	0	0	0	0	0	0.056	0	0	0	0	0	0
183	0	0	0	0	0	0.048	0	0	0	0	0	0	0	0	0	0	0
187	0	0	0	0	0	0.008	0	0	0	0	0	0	0	0	0	0	0
189	0	0	0	0	0	0.04	0	0	0	0	0	0	0	0	0	0	0
mtDNA clade	**61**	**63**	**72**	**31**	**28**	**50**	**18**	**31**	**64**	**33**	**9**	**55**	**41**	**31**	**21**	**20**	**16**
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0.05	0.841	0.014	0.032	0	0	0	0.032	0	0	0	0	0.78	0.71	0.381	0	0
3	0.148	0	0.222	0	0	0	0	0	0	0	0	0.018	0.195	0.226	0.619	0.3	0.063
4	0.803	0.159	0.764	0.968	0	0	0.125	0.742	0	0	0	0	0	0.065	0	0.7	0.813
5	0	0	0	0	0.179	0.02	0.833	0.097	0	0	0	0	0.024	0	0	0	0
6	0	0	0	0	0.821	0.98	0.056	0.129	1	0	0	0	0	0	0	0	0.125
7	0	0	0	0	0	0	0	0	0	1	0	0.145	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	1	0.836	0	0	0	0	0

Sample sizes [number of individuals (=nests)] are indicated separately for the nuclear loci and mtDNA in bold

6.1.8 References

Ahrens ME, Ross KG and Shoemaker DD (2005) Phylogeographic structure of the fire ant *Solenopsis invicta* in its native South American range: Roles of natural barriers and habitat connectivity. Evolution 59: 1733-1743

Allen CR, Lutz RS and Demarais S (1998) Ecological effects of the invasive nonindigenous ant, *Solenopsis invicta,* on native vertebrates: The wheels on the bus. Transactions of the North American Wildlife and Natural Resources Conference 63: 56-65

Allen GE and Buren WF (1974) Microsporidan and fungal diseases of *Solenopsis invicta* Buren in Brazil. Journal of the New York Entomological Society 82: 125-130

Baudouin L, Piry S and Cornuet JM (2004) Analytical Bayesian approach for assigning individuals to populations. Heredity 95: 217-224

Buckley A (1999) Fire ants in California. American Bee Journal 139: 88

Buren WF (1972) Revisionary studies on the taxonomy of the imported fire ants. Georgia Entomological Society 7: 1-26

Buren WF, Allen GE, Whitcomb WH, Lennartz FE and Williams RN (1974) Zoogeography of the imported fire ants. Journal of the New York Entomological Society 82: 113-124

Callcott AMA and Collins HL (1996) Invasion and range expansion of imported fire ants (Hymenoptera: Formicidae) in North America from 1918-1995. Florida Entomologist 79: 240-251

Carroll CR and Hoffman CA (2000) The pervasive ecological effects of invasive species: Exotic and native fire ants. In: Coleman DC and Hendrix PF (eds) Invertebrates as Web-Masters in Ecosystems, pp 221-232, Wallingford, Oxon, UK

Carruthers RI (2003) Invasive species research in the United States Department of Agriculture - Agricultural Research Service. Pest Management Science 59: 827-834

Chakraborty R and Nei M (1977) Bottleneck effects on average heterozygosity and genetic distance with the stepwise mutation model. Evolution 31: 347-356

Chen JSC, Shen CH and Lee HJ (2006) Monogynous and polygynous red imported fire ants, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), in Taiwan. Environmental Entomology 35: 167-172

Cornuet JM, Piry S, Luikart G, Estoup A and Solignac M (1999) New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153: 1989-2000

Davis LR, Vander Meer RK and Porter SD (2001) Red imported fire ants expand their range across the West Indies. Florida Entomologist 84: 735-736

Downie DA (2002) Locating the sources of an invasive pest, grape phylloxera, using a mitochondrial DNA gene genealogy. Molecular Ecology 11: 2013-2026

Dybdahl MF and Storfer A (2003) Parasite local adaptation: red queen versus suicide king. Trends in Ecology and Evolution 18: 523-530

Evanno G, Regnaut S and Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14: 2611-2620

Falush D, Stephens M and Pritchard JK (2003) Inference of population structure using multilocus genotype data: Linked loci and correlated allele frequencies. Genetics 164: 1567-1587

Felsenstein J (2004) 2004. PHYLIP (Phylogeny Inference Package) version 3.6. Department of Genome Sciences, University of Washington, Seattle.

Foitzik S, Fischer B and Heinze J (2003) Arms races between social parasites and their hosts: geographic patterns of manipulation and resistance. Behavioral Ecology 14: 80-88

Folgarait PJ, Bruzzone O, Porter SD, Pesquero MA and Gilbert LE (2005) Biogeography and macroecology of phorid flies that attack fire ants in south-eastern Brazil and Argentina. Journal Of Biogeography 32: 353-367

Giraud T, Pedersen JS and Keller L (2002) Evolution of supercolonies: the Argentine ants of southern Europe. Proceedings of the National Academy of Sciences, USA

Glaubitz JC (2004) CONVERT: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. Molecular Ecology Notes 4: 309-310

Goolsby JA, DeBarro PJ, Makinson JR, Pemberton RW, Hartley DM and Frohlich DR (2006) Matching the origin of an invasive weed for selection of a herbivore haplotype for a biological control programme. Molecular Ecology 15: 287-297

Gotelli NJ and Arnett AE (2000) Biogeographic effects of red fire ant invasion. Ecology Letters 3: 257-261

Gotzek D and Ross KG (2007) Genetic regulation of colony social organization in fire ants: An integrative overview. Quarterly Review of Biology 82: 201-226

Gotzek D, Shoemaker D and Ross KG (2007) Molecular variation at a candidate gene implicated in the regulation of fire ant social behavior. PLOS One 2: e1088. doi:10.1371/journal.pone.0001088

Gwiazdowski RA, Van Driesche RG, Desnoyers A, Lyon S, Wu Sa, Kamata N and Normark BB (2006) Possible geographic origin of beech scale, Cryptococcus fagisuga (Hemiptera: Eriococcidae), an invasive pest in North America. Biological Control 39: 9-18

Harter AV, Gardner KA, Falush D, Lentz DL, Bye RA and Rieseberg LH (2004) Origin of extant domesticated sunflowers in eastern North America. Nature 430: 201-205

Havill NP, Montgomery ME, Yu G, Shiyake S and Caccone A (2006) Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introduction to eastern North America. Annals of the Entomological Society of America 99: 195-203

Huang TC, Chou YC and Chou HC (2004) The infestation and control of the red imported fire ant in Taiwan. Proceedings of the Symposium on the Control of the Red Imported Fire Ant, Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan, Taipei, Taiwan

.

Jarne P and Lagoda PJL (1996) Microsatellites: From molecules to populations and back. Trends in Ecology and Evolution 11: 424-429

Jouvenaz DP (1990) Approaches to biological control of fire ants in the United States. In: Vander Meer RK, Jaffe K and Cedeno A (eds) Applied Myrmecology: A World Perspective, pp 620-627, Westview Press, Boulder, Colorado

Kaltz O and Shykoff JA (1998) Local adaptation in host-parasite systems. Heredity 81: 361-370

Kemp SF, deShazo RD, Moffitt JE, Williams DF and Buhner WA (2000) Expanding habitat of the imported fire ant (*Solenopsis invicta*): A public health concern. Journal of Allergy and Clinical Immunology 105: 683-691

Kraaijeveld AR and Godfray HCJ (1999) Geographic patterns in the evolution of resistance and virulence in *Drosophila* and its parasitoids. The American Naturalist 153: S61-S74

Krieger MJB and Ross KG (2002) Identification of a major gene regulating complex social behavior. Science 295: 328-332

Laine AL (2005) Spatial scale of local adaptation in a plant-pathogen metapopulation. Journal of Evolutionary Biology 18: 930-938

Lee CE (2002) Evolutionary genetics of invasive species. Trends in Ecology and Evolution 17: 386-391

Lively CM and Dybdahl MF (2000) Parasite adaptation to locally common host genotypes. Nature 405: 679-681

Lively CM, Dybdahl MF, Jokela J, Osnas EE and Delph LF (2004) Host sex and local adaptation by parasites in a snail-trematode interaction. American Naturalist 164

Lofgren CS (1986a) History of imported fire ants in the United States. In: Lofgren CS and Vander Meer RK (eds) Fire Ants and Leaf Cutting Ants: Biology and Management, pp 36-49, Westview Press, Boulder, Colorado

Lofgren CS (1986b) The economic importance and control of imported fire ants in the United States. Economic Impact and Control of Social Insects, pp 227-256, Praeger, New York

Lofgren CS, Banks WA and Glancey BM (1975) Biology and control of imported fire ants. Annual Review of Entomology 20: 1-30

MacKay WP and Fagerlund R (1997) Range expansion of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), into New Mexico and extreme western Texas. Proceedings of the Entomological Society of Washington 99: 757-758

May GE, Gelembiuk GW, Panov VE, Orlova MI and Lee CE (2006) Molecular ecology of zebra mussel invasions. Molecular Ecology 15: 1021-1031

McCubbin KI and Weiner JM (2002) Fire ants in Australia: A new medical and ecological hazard. Medical Journal of Australia 176: 518-519

Mehdiabadi NJ and Gilbert LE (2002) Colony-level impacts of parasitoid flies on fire ants. Proceedings of the Royal Society - Biological Sciences (Series B) 269: 1695-1699

Mehdiabadi NJ, Kawazoe EA and Gilbert LE (2004) Phorid fly parasitoids of invasive fire ants indirectly improve the competitive ability of a native ant. Ecological Entomology 29: 621-627

Mescher MC, Ross KG, Shoemaker DD, Keller L and Krieger MJB (2003) Distribution of the two social forms of the fire ant *Solenopsis invicta* (Hymenoptera: Formicidae) in the native South American range. Annals of the Entomological Society of America 96: 810-817

Mitchell CE and Power AG (2003) Release of invasive plants from fungal and viral pathogens. Nature 421: 625-627

Miura O, Torchin ME, Kuris AM, Hechinger RF and Chiba S (2006) Introduced cryptic species of parasites exhibit different invasion pathways. Proceedings of the American Academy of Arts and Sciences, USA 103: 19818-19823

Morrison LW (2000) Mechanisms of interspecific competition among an invasive and two native fire ants. Oikos 90: 238-252

Morrison LW (2002) Long-term impacts of an arthropod-community invasion by the imported fire ant, *Solenopsis invicta*. Ecology 83: 2337-2345

Morrison LW, Kawazoe EA, Guerra R and Gilbert LE (2000) Ecological interactions of *Pseudacteon* parasitoids and *Solenopsis* ant hosts: environmental correlates of activity and effects on competitive hierarchies. Ecological Entomology 25: 433-444

Morrison LW and Porter SD (2005) Phenology and parasitism rates in introduced populations of *Pseudacteon tricuspis*, a parasitoid of *Solenopsis invicta*. Biocontrol 50: 127-141

Nei M, Tajima F and Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. II. Gene frequency data. Journal of Molecular Evolution 19: 153-170

Orr MR (1996) Host manipulation by *Wolbachia* is a neutral trait within single populations. Animal Behaviour 51: 1183-1185

Paetkau D, Slade R, Burdents M and Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Molecular Ecology 13: 55-65

Patterson RS (1994) Biological control of introduced ant species. In: Williams DF (ed) Exotic Ants: Biology, Impact, and Control of Introduced Species, pp 293-308, Westview Press, Boulder, Colorado

Pereira RM and Porter SD (2006) Range expansion of the fire ant decapitating fly, *Pseudacteon tricuspis*, eight to nine years after releases in North Florida. Florida Entomologist 89: 536-538

Piry S, Alapetite A, Cornuet J-M, Paetkau D, Budouin L and Estoup A (2004) GENECLASS2: A software for genetic assignment and first-generation migrant detection. Journal of Heredity 95: 536-539

Pitts JP (2002) A cladistic analysis of the *Solenopsis saevissima* species-group (Hymenoptera: Formicidae). Ph.D., Entomology, University of Georgia, Athens, GA

Pitts JP, McHugh JV and Ross KG (2005) Cladistic analysis of the fire ants of the *Solenopsis saevissima* species-group (Hymenoptera: Formicidae). Zoologica Scripta 34: 493-505

Pitts JP, McHugh JV and Ross KG (2007) Revision of the fire ants of the *Solenopsis saevissima* species-group (Hymenoptera: Formicidae). Zootaxa in press

Porter SD (1998) Host-specific attraction of *Pseudacteon* flies (Diptera : Phoridae) to fire ant colonies in Brazil. Fla. Entomol. 81: 423-429

Porter SD (2000) Host specificity and risk assessment of releasing the decapitating fly *Pseudacteon curvatus* as a classical biocontrol agent for imported fire ants. Biological Control 19: 35-47

Porter SD and Alonso LE (1999) Host specificity of fire ant decapitating flies (Diptera : Phoridae) in laboratory oviposition tests. Journal of Economic Entomology 92: 110-114

Porter SD and Briano JA (2000) Parasitoid-host matching between the little decapitating fly *Pseudacteon curvatus* from Las Flores, Argentina and the black fire ant *Solenopsis richteri*. Florida Entomologist 83: 422-427

Porter SD, de Sa LAN and Morrison LW (2004) Establishment and dispersal of the fire ant decapitating fly *Pseudacteon tricuspis* in North Florida. Biological Control 29: 179-188

Porter SD, Fowler HG and MacKay WP (1992) Fire ant mound densities in the United States and Brazil (Hymenoptera: Formicidae). Journal of Economic Entomology 85: 1154-1161

Porter SD, Pesquero MA, Campiolo S and Fowler HG (1995) Growth and development of *Pseudacteon* phorid fly maggots (Diptera: Phoridae) in the heads of *Solenopsis* fire ant workers (Hymenoptera: Formicidae). Environmental Entomologist 24: 475-479

Porter SD and Savignano DA (1990) Invasion of polygyne fire ants decimates native ants and disrupts arthropod community. Ecology 7: 2095-2106

Porter SD, Williams DF and Patterson RS (1997a) Rearing the decapitating fly *Pseudacteon tricuspis* (Diptera: Phoridae) in imported fire ants (Hymenoptera: Formicidae) from the United States. Journal of Economic Entomology 90: 135-138

Porter SD, Williams DF, Patterson RS and Fowler HG (1997b) Intercontinental differences in the abundance of *Solenopsis* fire ants (Hymenoptera: Formicidae): Escape from natural enemies? Environmental Entomology 26: 373-384

Posada D and Crandall KA (1998) MODELTEST: Testing the model of DNA substitution. Bioinformatics 19: 817-818

Pritchard JK, Stephens M and Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945-959

Provan J, Murphy S and Maggs CA (2005) Tracking the invasive history of the green alga *Codium fragile* ssp. *tomentosoides*. Molecular Ecology 14: 189-194

Rannala B and Mountain JL (1997) Detecting immigration by using multilocus genotypes. Proceedings of the National Academy of Science USA 94: 9197-9201

Ronquist F and Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572-1574

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes 4: 137-138

Ross KG, Krieger MJB, Keller L and Shoemaker DD (2007) Genetic variation and structure in native populations of the fire ant *Solenopsis invicta*: Evolutionary and demographic implications. Biological Journal of the Linnaean Society 92: 541-560

Ross KG, Krieger MJB, Shoemaker DD, Vargo EL and Keller L (1997) Hierarchical analysis of genetic structure in native fire ant populations: Results from three classes of molecular markers. Genetics 147: 643-655

Ross KG and Shoemaker DD (1997) Nuclear and mitochondrial genetic structure in two social forms of the fire ant *Solenopsis invicta*: Insights into transitions to an alternate social organization. Heredity 78: 590-602

Ross KG and Shoemaker DD (2005) Species delimitation in native South American fire ants. Molecular Ecology 14: 3419-3438

Ross KG and Trager JC (1990) Systematics and population genetics of fire ants (*Solenopsis saevissima* complex) from Argentina. Evolution 44: 2113-2134

Ross KG, Vargo EL and Keller L (1996) Social evolution in a new environment: The case of introduced fire ants. Proceedings of the National Academy of Sciences, USA 93: 3021-3025

Ross KG, Vargo EL, Keller L and Trager JC (1993) Effect of a founder event on variation in the genetic sex-determining system of the fire ant *Solenopsis invicta*. Genetics 135: 843-854

Sakai AK, Allendorf FW, Holt JS, Lodge DM, Molofsky J, With KA, Baughman S, Cabin RJ, Cohen JE, Ellstrand NC, McCauley DE, O'Neil P, Parker IM, Thompson JN and Weller SG (2001) The population biology of invasive species. Annual Review of Ecology and Systematics 32: 305-332

Sax DF, Stachowicz JJ and Gaines SD (2005) Species invasions: Insights into ecology, evolution, and biogeography. Sinauer Associates, Sunderland, Massachussetts, 495 pp
Schneider S, Roessli D and Excoffier L (2000) Arlequin ver. 2.000: A software for population genetic data analysis. Genetics Biometry Laboratory, University of Geneva, Switzerland

Shoemaker DD, Ahrens M and Ross KG (2006a) Molecular phylogeny of fire ants of the *Solenopsis saevissima* species-group based on mitochondrial DNA sequences. Molecular Phylogenetics and Evolution 38: 200-215

Shoemaker DD, Costa JT and Ross KG (1992) Estimates of heterozygosity in two social insects using a large number of electrophoretic markers. Heredity 69: 573-582

Shoemaker DD, DeHeer CJ, Krieger MJB and Ross KG (2006b) Population genetics of the invasive fire ant *Solenopsis invicta* (Hymenoptera: Formicidae) in the USA. Annals of the Entomological Society of America 99: 1213-1233

Shoemaker DD, Ross KG and Arnold ML (1996) Genetic structure and evolution of a fire ant hybrid zone. Evolution 50: 1958-1976

Simon C, Frati F, Beckenbach A, Crespi B, Liu H and Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of

conserved polymerase chain reaction primers. Annals of Entomological Society of America 81: 651-701

Swofford DL (1999) PAUP*. Phylogenetic Analysis Using Parsimony (*and other methods), version 4.0. Sinauer Associates, Sunderland, MA

Thrall PH, Burdon JJ and Bever JD (2002) Local adaptation in the *Linum marginale-Melampsora lini* host-pathogen interaction. Evolution 56: 1340-1351

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ and Kuris AM (2003) Introduced species and their missing parasites. Nature 421: 628-630

Trager JC (1991) A revision of the fire ants, *Solenopsis geminata* group (Hymenoptera: Formicidae: Myrmicinae). Journal of the New York Entomological Society 99: 141-198
Tschinkel WR (2006) The Fire Ants. Harvard University Press, Cambridge, Massachusetts, 752 pp

Tsutsui ND, Suarez AV and Grosberg RK (2003) Genetic diversity, asymmetrical aggression, and recognition in a widespread invasive species. Proceedings of the National Academy of Science, USA 100: 1078-1083

Vazquez RJ, Porter SD and Briano JA (2004) Host specificity of a biotype of the fire ant decapitating fly *Pseudacteon curvatus* (Diptera : Phoridae) from northern Argentina. Environmental Entomology 33: 1436-1441

Vinson SB (1994) Impact and invasion of *Solenopsis invicta* (Buren) on native food webs. In: Williams DF (ed) Exotic Ants: Biology, Impact, and Control of Introduced Species, pp 293-308, Westview Press, Boulder, Colorado

Waage JK and Mills NJ (1992) Biological control. In: Crawley MJ (ed) Natural Enemies: the Population Biology of Predators, Parasites, and Diseases, pp 412-430, Blackwell Scientific Publications, Cambridge, MA

Wilcove DS, Rothstein D, Dubow J, Phillips A and Losos E (1998) Quantifying threats to imperiled species in the United States. Bioscience 48: 607-615

Williams DF (1994) Control of the introduced pest *Solenopsis invicta* in the United States. In: Williams DF (ed) Exotic ants. Biology, impact, and control of introduced species, pp 282-292, Westview Press, Boulder, Colorado

Williams DF and deShazo RD (2004) Biological control of fire ants: an update on new techniques. Annals of Allergy Asthma and Immunology 93: 15-22

Williams DF and Porter SD (1994) Fire Ant Control. Science 264: 1653-1653

Wojcik D, Allen CR, Brenner R, . J., Forys EA, Jouvenaz D and Lutz RS (2001) Red imported fire ants: Impact on biodiversity. American Entomologist 47: 16-23

6.2 Supplementary (16) *in* The Genome Sequence of the Leaf-Cutter Ant *Atta cephalotes* Reveals Insights Into Its Obligate Symbiotic Lifestyle

Previously published as a supplement to Suen et al. 2011. PLoS Genetics. 7(2): 1-11

Eric Caldera, Meredith C. Naughton, Chris R. Smith, and Garret Suen

Author contributions. EJC facilitated *Atta cephalotes* ant collection and dna extraction, built the hexamerin phylogeny and helped write the paper. GS, MCN & CRS performed all other experiments and wrote the paper.

6.2.1 Hexamerins

In insects like *Apis mellifera*, *Nasonia vitripennis*, and *Drosophila melanogaster*, hexamerins are synthesized during the larval stage and serve as amino acid sources during development into adults [1]. A total of 4 hexamerins are found across insects, including *hex 70a*, *hex 70b*, *hex 70c,* and *hex 110*. Using the *Apis mellifera* gene models for these 4 genes, we searched for their presence in *Atta cephalotes*. We found that most are present, with the exception of *hex 70c*. We then investigated the presence/absence of these 4 genes across sequenced hymenopteran genomes, including the two ants *Camponotus floridanus* and *Harpegnathos saltator* [2]. All 4 hexamerins are found in each of these genomes with varying copy and gene synteny (Fig. 3 from the main text). In *Apis mellifera*, these hexamerins appear to be expressed at different times, as *hex 70a* and *hex 110* are expressed highly during the larval, pupal and adult stage of workers, while

hex 70b and *hex 70c* are expressed only during the larval stage [1]. To accurately place the *hex* genes in *Atta*, we conducted a phylogenetic analysis as shown in Fig.1. We obtained the set of *hex* genes from 4 other hymenopteran genes and created a phylogenetic tree that includes other known *hex* genes from across insect lineages, as previously reported by Martins *et al*. [1]. Protein sequences were aligned using default parameters in MUSCLE [3], and Baysean phylogenetic construction utilized MrBayes v3.1.2 [4]. The Blosum model with a gamma distribution of substitution rates was used. Simulations featured 4 Metropolis-coupled MCMC chains with 300,000 generations, and a burin of 100,000. Analysis of this tree indicates that *hex* genes of the same class cluster together according to taxonomic order. Specifically, all of the *hex 70a*, *hex 70b*, *hex 70c,* and *hex 110* in hymenoptera group together with the majority of the ants *hex* genes clustering together.

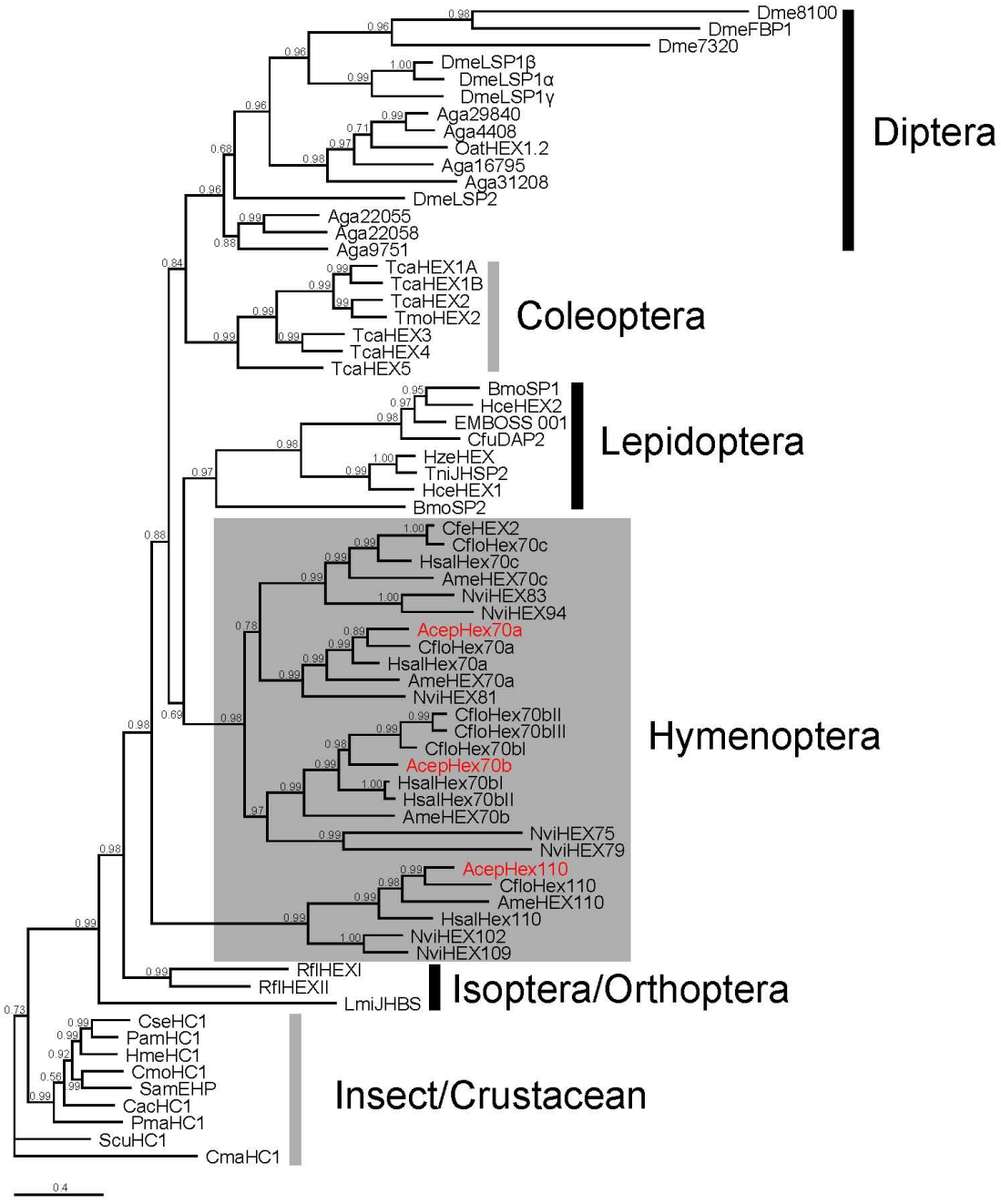


Figure 1. Phylogenetic analysis of the hexamerin genes across different insect orders. Values at nodes represent Bayesian posterior probabilities.

6.2.2 References

Bonasio R, Zhang G, Ye C, Mutti NS, Fang X, et al. (2010) Genomic Comparison of the Ants Camponotus floridanus and Harpegnathos saltator. Science 329: 1068.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Research 32: 1792.

Martins J, Nunes F, Cristino A, Simoes Z, Bitondi M (2010) The four hexamerin genes in the honey bee: structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. BMC Molecular Biology 11: 23.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572.