The Dissertation Committee for Alexander Sergeyevich Mikheyev Certifies that this is the approved version of the following dissertation:

The role of host switching in the evolution of the fungus-gardening ant symbiosis

Committee:

Ulrich Mueller, Supervisor

Tom Juenger

Larry Gilbert

Rodrigo Sierra

Mike Singer

The role of host switching in the evolution of the fungus-gardening ant symbiosis

by

Alexander Sergeyevich Mikheyev, B.A., M.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2009

Acknowledgements

This work would not have been possible without the help of my mentors, collaborators and friends, particularly Patrick Abbot and Ulrich Mueller. I am deeply grateful to both of them. I would also like to thank members of the Mueller lab and my friends in EEB who have been a great help, as well as a source of inspiration throughout my dissertation. I am grateful to Rachelle Adams, Deepa Agashe, Natalia Biani, Stephan Bresson, Henrik De Fine Licht, Evan Economo, Nicole Gerardo, Abbie Green, Anna Himler, Barrett Klein, Krushnamegh Kunte, Matthew Kweskin, Christian Rabeling, Jenny Rocca, Sergio Sanchez-Pena, Ruchira Sen, Jarrod Scott, Anna Schmidt, Scott Solomon, Meredith Swett, Brian Wee, and Zoe Yek. I would also like to thank Duur Aanen, Maurício Bacci, Koos Boomsma, Sean Brady, Sofia Bruschi, Cameron Currie, Michael Paulsen, Steve Rehner, Ted Schultz, Aline Silva and Jim Wetterer for ideas, specimens, field support, and other generous aid. Finally, I would like to thank members of my committee, my adviser Ulrich Mueller, as well as, Larry Gilbert, Tom Juenger, Rodrigo Sierra and Mike Singer, for their useful advice during the dissertation process and for trusting my intuition and allowing me much-needed freedom choose my own path as I navigated the dissertation process. Last but not least, I would like to thank Sandy Monahan, who has done an excellent job facilitating all of the paperwork, in spite of my various fellowship obligations, missed deadlines and last-minute requests.

Much of Chapter 1 is based on a manuscript currently being written by me in collaboration with Ulrich Mueller and Patrick Abbot.

Chapter 2 has previously been published in *Biological Invasions* (2008, **10:**467-473)**.** I thank Nicole Gerardo for help advice on how to collect *Escovopsis* and, along with Stephen Taerum, for help in identifying *Escovopsis*. Jarrod Scott and Patrick Abbott provided valuable advice for the molecular analyses. I would like to thank Scott Solomon, Jarrod Scott, Evan Economo and Christian Rabeling for their companionship and help during our travels through Mexico and the Caribbean. I am also grateful to Scott Solomon and Anna Schmidt for reading and substantially improving the manuscript through their comments. The manuscript has benefited from the excellent comments of two anonymous reviewers. I would also once again like to thank my Ph.D. advisor Ulrich Mueller for generously allowing the use of funds from his NSF CAREER grant (DEB-9983879) for this project. Additional funding for this research has been funded by a fellowship from the US EPA and research grants from the University of Texas section of Ecology, Evolution and Behavior, as well as from a NSF Dissertation Improvement Grant (DEB-0508613).

Chapter 3 has previously been published in *Proceedings of the National Academy of Sciences* (2006, **103**:10702-6) with Ulrich Mueller and Patrick Abbot as co-authors. We thank J. Scott for developing the microsatellite library and sharing his expertise, Vikram Chhatre for assisting with molecular bench work, and Nancy Moran for helpful suggestions on previous drafts. This work was supported by National Science Foundation Grant DBI-0102094 and funds from Vanderbilt University (to P.A.), National Science Foundation Integrated Research Challenges in Environmental Biology Grant

DEB-0110073 (to U.G.M.), and National Science Foundation Dissertation Improvement Grant DEB-0508613 (to A.S.M. and U.G.M.). Additional funds were provided by fellowships from the U.S. Environmental Protection Agency, the Dolores Zohrab Liebmann Fund, and research grants from the University of Texas Section of Integrative Biology (to A.S.M.).

Chapter 4 has previously been published in *Molecular Ecology* (2007, **16:**209–216) with Ulrich Mueller and and Koos Boomsma as co-authors. Funding for this research has been provided by NSF (IRCEB DEB-0110073) to UGM and by a NSF Dissertation Improvement Grant (DEB-0508613)to ASM. JJB was supported by the Danish Natural Science Research Council. Additional funding for this research was funded by fellowships from the US EPA, the Dolores Zohrab Liebmann Fund, as well as research grants from the University of Texas section of Ecology, Evolution and Behavior to ASM. A two-month stay of ASM in Copenhagen was funded by the Danish Ph.D. School ISOBIS. We would like to thank Sylvia Mathiasen and Jarrod Scott for advice on microsatellite techniques and general laboratory help and Boris Baer, Scott Solomon and Michael Poulsen, as well as other researchers at the University of Copenhagen for helping to collect the material used in this study and generously sharing it. We are deeply greatful to Steve Rehner for sharing his ideas and unpublished findings, which seeded the current work. This paper has been considerably improved by discussion with Mark Kirkpatrick and Patrick Abbot, as well as by comments on the manuscript made by Natalia Biani, Barrett Klein, Alan Herre, Anna Schmidt and an anonymous reviewer.

Chapter 5 has previously been published in *Molecular Ecology* (2008, **17:**4480-4488) with Tanya Vo and Ulrich Mueller as co-authors. We would like to thank A. Yang, S. Beshers and J. Grehen for providing information on collecting sites and A. M. Green, A. Himler, N.J.R. Plowes, J. Seal, A.M. Schmidt, J.J. Schubart, T.R. Schultz for donating specimens or helping with collection in the field. We are grateful to R.M.M. Adams, H. Ishak, N. Biani, B. Klein, C. Rabeling, J. Rocca, R. Sen, S.E. Solomon, S.H. Yek and three anonymous reviewers for their comments on the manuscript. Funding for this research has been provided by NSF (IRCEB DEB-0110073) to UGM and by a NSF Dissertation Improvement Grant (DEB-0508613) to ASM. Additional funding for this research was funded by fellowships from the US Environmental Protection Agency, the Dolores Zohrab Liebmann Fund, as well as research grants from the University of Texas, Section of Ecology, Evolution and Behavior to ASM.

The role of host switching in the evolution of the fungus-gardening ant symbiosis

Publication No._____

Alexander Sergeyevich Mikheyev, Ph.D.

The University of Texas at Austin, 2009

Supervisor: Ulrich G. Mueller

The fungus-growing ants have long provided a spectacular example of co-evolutionary integration between distantly related taxa. Their ecological success has been thought to depend largely on the evolutionary alignment of reproductive interests between ants and fungi, following vertical transmission and the ancient suppression of fungal sexuality. In my dissertation I explored the role of lateral cultivar switching on the evolution of the fungus-gardening ant mutualism. First, I provided the first evidence for sexual reproduction in the attine cultivars, together with evidence of extensive independent long-distance horizontal transmission of fungal genes. In fact, fungi have greater gene flow relative to their host ants, crossing the Gulf of Mexico between Latin America and Cuba, over which the ants cannot readily disperse. Second, for the special case of leaf-cutting ants, I show that the cultivar population was largely unstructured with respect to host ant species, and leaf-cutting ants interact largely with a single species of fungus. Finally, I examined the effect of post-glacial expansion on the population structure of the northern fungus-gardening ant *Trachymyrmex septentrionalis* and compared it with

that of its two microbial mutualists: a community of lepiotaceous fungal cultivars and associated antibiotic-producing *Pseudonocardia* bacteria. This comparison allowed me to examine the effect of historical biogeographic forces, such as climate-driven range shifts, on the population structure of the ants and their microbial symbionts. While neither the cultivar nor the *Pseudonocardia* genetic structure was correlated with that of the ants, they were significantly, though weakly, correlated with each other. These results suggest that biogeographic forces may act differently on macro- and microscopic organisms, even in the extreme case where some microbial mutualists may be vertically transmitted from generation to generation and share the same joint ecological niche. Thus, binding forces that appear to enforce host fidelity are relatively weak and pairwise associations between cultivar lineages and ant species have little opportunity for evolutionary persistence. Taken together, my studies suggest that mechanisms other than long-term pairwise interactions between ants and fungi (so-called partner fidelity feedback) govern the evolution of the mutualism over evolutionary time.

Table of Contents

CHAPTER 1

The fungus-gardening ant symbiosis as a model system for the study of coevolution

1.1 MODELS FOR COEVOLUTION OF MUTUALISTS

Coevolutionary analysis has traditionally focused on intimate long-term association between interacting taxa as the key mechanism dictating evolutionary change, predicting patterns of synchronously codiversified associations between interacting clades (Becerra 2003; Brooks & McLennan 1991; Feinsinger *et al.* 1983). Specifically, correspondences between phylogenies of interacting taxa have been widely interpreted as evidence for ecological processes such as specialization, reciprocal adaptation and co-evolution (De Vienne *et al.* 2007). By contrast, ecological observations of existing communities reveal that most organisms are involved in a web of interactions with other organisms. Direct interactions, such as predation or mutualism, can combine to produce complex interaction networks, such that seemingly isolated events can have far-reaching consequences across entire communities (Polis & Winemiller 1996; Wootton (2002). The topology of these interaction networks determines both their resilience to ecological change and should affect the evolutionary potential of constituent species (Bascompte & Jordano 2007; Bascompte *et al.* 2006; Guimaraes *et al.* 2007; Rezende *et al.* 2007). However, how community interaction networks actually change across evolutionary timescales remains largely unknown, thus limiting our understanding of how stable

communities are assembled and, ultimately, how they can be conserved (Montoya *et al.* 2006).

One of the best studied mutualistic association, the nutritional mutualism between ants and fungi have been resolved in the past decade through a series of phylogenetic and population-genetic analyses (Chapela *et al.* 1994; Hinkle *et al.* 1994; Mikheyev *et al.* 2006; Mikheyev *et al.* 2007; Mikheyev *et al.* 2008; Mueller *et al.* 1998; Munkacsi *et al.* 2004; Schultz & Brady 2008; Schultz & Meier 1995; Silva-Pinhati *et al.* 2004; Wetterer *et al.* 1998b). Ant-fungus interactions are compartmentalized into subwebs (Figure 1.1), characterized by ant-specific cultivar types and correspondingly unique fungal morphologies. Attine ants have a range of behaviors and morphological adaptations that are finely tuned for the maintenance of their fungus gardens (Currie *et al.* 2006; Currie & Stuart 2001). As subwebs tend to involve distinct fungal types, fungal morphology-specific ant adaptations, such as 'weeding' of contaminates, likely serve as barriers to cultivar exchange between subwebs. There exist few ant-cultivar associations that bridge subwebs. By contrast, ant species readily share cultivars within each subweb; the most dramatic example of this phenomenon occurring in the leaf-cutting ants, where two genera of ants interact with a single fungal species (Mikheyev *et al.* 2006; Mikheyev *et al.* 2007). Lower-attine ants sample from two large pools of already 'domesticated' cultivars, but also novel fungi from their environment (Mueller *et al.* 1998; Vo *et al.* in press), whereas higher-attine fungi appear to exist obligately within the symbiosis. Thus, a range a specificities exists within the symbiosis, from the loosely interacting subwebs of the lower attines that are connected to free-living fungal populations, to the more specialized obligate subwebs of higher attine ants (Figure 1.1).

Traditionally, coevolution within the symbiosis was believed to be driven by long-term associations between ant and fungal lineages (Chapela *et al.* 1994; Mueller 2002). In particular, ants were considered masters of their gardens, propagating anciently asexual fungal cultures. However, the extent to which these pairwise interactions persist over evolutionary time has not been tested. Also, although fungal fruiting was indeed rarely observed, its actual contribution to fungal population structure remained unknown. The goal of this dissertation was to understand the population biology of the cultivar fungi and to examine whether coevolution between ants and fungi indeed happens in a one-to-one fashion, or whether widespread sharing gives rise to diffuse network-like dynamics between the symbionts.

1.3 FIGURE

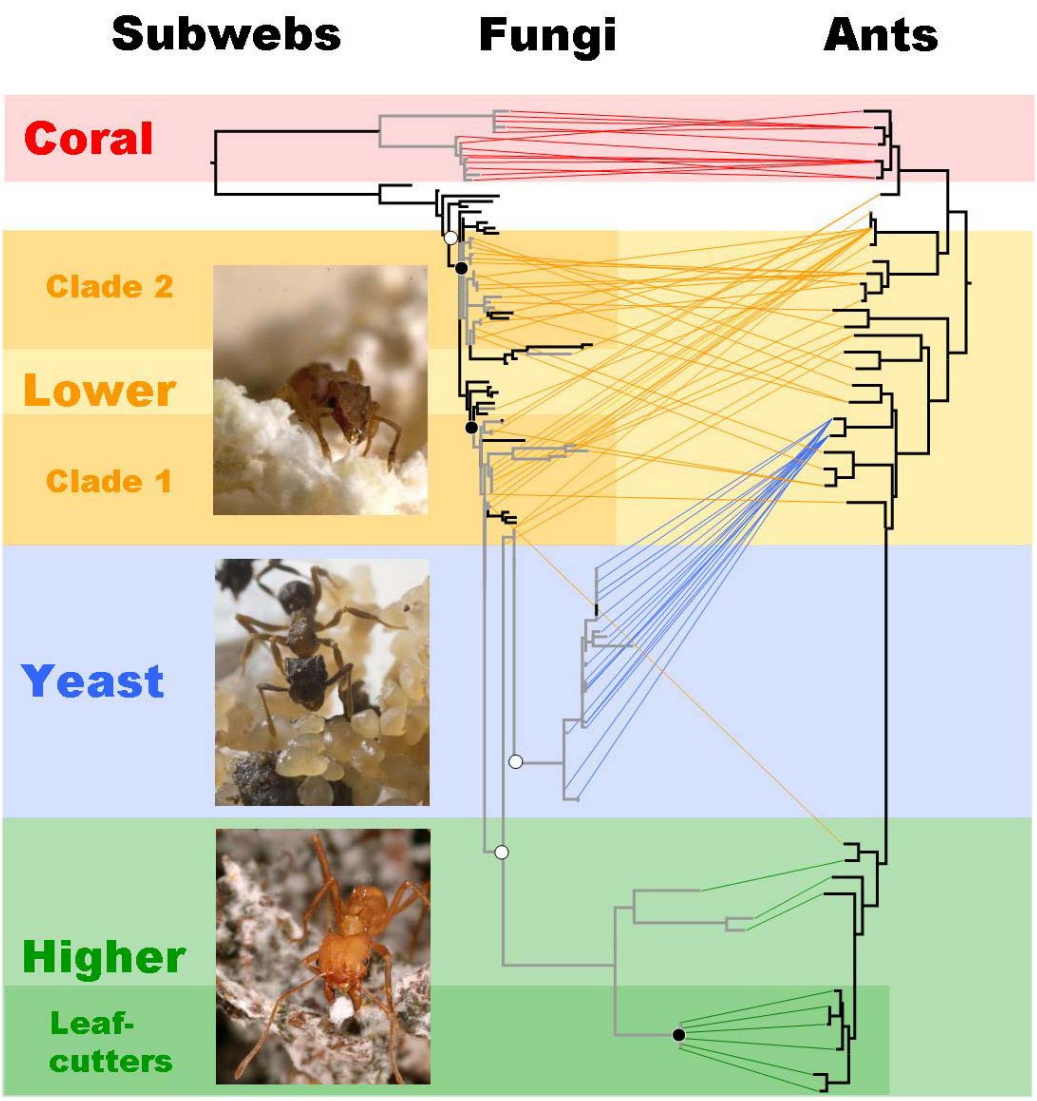

Figure 1.1. Mutualist phylogenies and network structure in the attine ant-cultivar symbiosis. Over the 50-million-year history of the symbiosis, there have been repeated domestications of cultivars (grey) from a pool of free-living fungi (black), followed by lateral spread within defined ant clades. The network of interactions is compartmentalized into several largely independent subwebs. The **lower attine** subweb

consists of at least two independently acquired cultivar clades ("Clade 1" and "Clade 2") (Mueller *et al.* 1998). Although some ants appear to specialize on one or the other of the two lower attine clades, others ants use fungi across both of them (Mehdiabadi *et al.* 2006; Mueller *et al.* 1998). The lower-attine cultivars maintain a connection to free-living relatives and have no known modifications adapting them to ant cultivation (Mueller *et al.* 1998; Vo *et al.* in press). (photo: *Cyphomyrmex wheeleri* in its garden). Cultivars in the **yeast** subweb are grown in single-celled (yeast) form by the ants, unlike the rest of the cultivars, which grow as filamentous fungi by the ants. Despite this derived single-celled growth form, these yeast-cultivars are nonetheless capable of a free-living filamentous existence (Mueller *et al.* 1998) (photo: *Cyphomyrmex rimosus* attending balls of yeast garden). Cultivars in the **higher attine** subweb appear to lack a free-living existence. Grape-like clusters ("gongylidia") produced by higher attine fungi act as the primary food source of the ant larvae (Quinlan & Cherrett 1979). The leaf-cutting ants form a monophyletic group in the higher attines and primarily associate with a single species of cultivar fungus (Mikheyev *et al.* 2006). The leaf-cutting ants and their cultivar form a subweb within the larger higher attine subweb. (photo: a minor worker of *Atta cephalotes* harvesting gongylidia). In addition to the major lepiotaceous fungi associated with the fungus-gardening ants, distantly related **coral** fungi have also been domesticated by a lineage of phylogenetically primitive fungus-gardening ants in the genus *Apterostigma* (Munkacsi *et al.* 2004). Although rare, connection between subwebs do exist and more will certainly be uncovered with increased sampling. The stem nodes of subwebs dated in this study are indicated by white circles. Nodes of interest within the subwebs, such as the clades of lower attine fungi and origin of the leaf-cutter fungus, are shown by black circles.

CHAPTER 2

History, genetics and pathology of a leaf-cutting ant introduction: a case study of the Guadeloupe invasion.

Abstract: As dominant herbivores and notorious pests in their native Neotropics, introduced leaf-cutting ants have the potential for ecological and economic harm. Although a large-scale invasion of leaf-cutting ants has not occurred, an isolated introduction in the Caribbean islands of Guadeloupe provides useful insight into the progress of such an invasion. Since being first detected in 1954, *Acromyrmex octospinosus* has colonized virtually all available land area, defying an aggressive control campaign and damaging agriculture. I attempted to reconstruct the origins and spread of the invasion, as well as screen for the presence of garden pathogens, which could be used for biological control. Mitochondrial sequencing of the *A. octospinosus* complex throughout the Caribbean showed that the probable source of the invasion lies on Trinidad and Tobago or northeast South America. Using historical records and field surveys, the invasion's rate of spread was estimated at 0.51 km/year. Microsatellite genotyping further confirmed the limited dispersal abilities of *A. octospinosus*, showing the presence of isolation by distance (even in a relatively small geographic area) and suggested ubiquitous local inbreeding. Although the invasion likely resulted from the introduction of a single colony, microsatellites showed a high level of genetic variation in the introduced population, likely as a consequence of multiple mating by the queen. A survey showed that the specialized fungus garden pathogen *Escovopsis* exists on the

islands, suggesting that the successful spread of the ants was not due to escape from this parasite. Given that chemical control has failed in the past and that biological control using specialized garden pathogens seems improbable, only vigorous quarantine and inspection programs may prevent wide-scale leaf-cutting ant invasions in the future.

2.1 INTRODUCTION

Ants are present in large numbers in many ecosystems and drive numerous ecological processes (Hölldobler & Wilson 1990). Consequently, introduced ant species, especially a few high-impact ant invaders, may cause fundamental changes in their new habitat to the detriment of native organisms. For example, by competitively excluding native seed-dispersing ants, invasive Argentine ants have caused major shifts in plant compositions in the South African shurblands (Christian 2001). Likewise, by eliminating the native ants, which serve as food for horned lizards, Argentine ants have likely led to declines in lizard populations in California (Suarez & Case 2002). Unfortunately, little is known about these high-impact invaders before they become established and initiate major ecological changes. Although efforts to elucidate criteria by which potentially invasive ants could be determined are underway (*e.g.*, McGlynn (1999b) and Suarez *et al.* (2005)), no high risk taxa have yet been identified prior to major invasions.

Leaf-cutting ant genera *Acromyrmex* and *Atta* are dominant Neotropical herbivores. *Atta* nests may contain millions of workers and consume hundreds of kilograms of leaves per year (see a review by Wirth et al (2003)). Their ability to consume a diverse array plants results from an ancient obligate mutualistic interaction with a fungus cultivar

(Weber 1972; Wheeler 1907). Because of their voraciousness and impressive dietary breadth, the leaf-cutters are major agricultural pests of the New World (Cherrett 1968). Hölldobler and Wilson (1990) wrote: "If any leafcutter ants, especially *Atta*, were to be established in sub-Saharan Africa or some other part of the Old World tropics, the result might be an ecological catastrophe. The terrestrial ecosystems of these continents are unprepared for these highly organized insects." Although not commonly considered invasive, leaf-cutters can travel with commercial and military cargo and at least two *Acromyrmex* species have been intercepted by US Customs (Suarez *et al.* 2005). Although the Old World was spared a leaf-cutter invasion, a population of the leaf-cutter ant *Acromyrmex octospinosus* became established in the Caribbean, on Guadeloupe. These islands, previously free of native leaf-cutter ants (Cherrett 1968), provide a unique opportunity to study the dynamics of a leaf-cutter invasion in advance of a possible ecological catastrophe. This invasion may also provide useful insights into basic leaf-cutting ant biology.

A. octospinosus (locally known as *la fourmi-manioc*, or the manioc ant) occupied a 600-hectare area in the vicinity of the Morne-à-l'eau community on the island of Grande Terre at the time of its discovery in 1954 (Blanche 1954). Two years later the local government began an eradication campaign, which focused largely on control through a variety of pesticides, although the use of psychotropic drugs to "impair the vigilance" of *Acromyrmex* was also considered (Kermarrec & Mauleon 1990). Attempts to prevent the westward spread of the ants to the rest of Guadeloupe failed and they also colonized the nearby island of Basse Terre. Subsequently, large-scale efforts to combat the spread of

Acromyrmex were abandoned, leaving the ants to freely spread throughout the rest of the islands (Figure 2.1).

I attempted to answer a number of general questions aimed at understanding (a) how a leaf-cutter ant invasion originates, (b) how it progresses, and (c) whether biological control may be feasible using the specialized fungus garden pathogen *Escovopsis* (Currie & Stuart 2001; Currie *et al.* 2003b). To determine the origin the introduced ants, I conducted a biogeographic study of the *A. octospinosus* complex throughout the Caribbean. The rate of spread of *Acromyrmex* in a novel habitat was estimated from field surveys and historical accounts of the invasion's extent at various points in time. Dispersal patterns were further investigated using a microsatellite analysis of population genetic structure and diversity. Finally, in order to ascertain whether the success of the *Acromyrmex* invasion may have been aided by escape from virulent fungal pathogens, which may eventually be used for biological control, I surveyed the leaf-cutter colonies for *Escovopsis*, as well as for *Pseudonocardia* actinomycete bacteria used to combat the parasitic fungi (Currie *et al.* 2003b; Currie *et al.* 1999b).

2.2 MATERIALS AND METHODS

Collection. Several dozen nest workers of all sizes were sampled from 60 colonies throughout the Guadeloupe islands of Basse Terre and Grande Terre in December of 2003 (Figure 2.1B). Ants and fungi were preserved in 95% ethanol and stored at -80 ˚C when brought to the lab several weeks later.

Origin of the invasion. I used the primers COI and COII, anchored in cytochrome oxidase sub-units I and II, respectively (see Sumner 2004b, for primer sequences and PCR conditions) for reconstructing ant phylogenetic relationships. The amplified region also included an intergenic spacer, which was too variable for unambiguous alignment across the species complex and was excluded from the analysis. A neighbor-joining tree was calculated by CLUSTAL with support provided by 1000 bootstrap replicates. The sequences have been deposited in GenBank (accession numbers EF485021-EF485028).

Rate of spread. From the point of initial detection, the invasion expanded relatively unhindered from central Grande Terre to the north, west, and east (Figure 2.1). Using maps of the *A. octospinosus* distribution from Malato *et al.* (1977) and Therrien (1986), I conducted a regression analysis of the ants' spread in each of these three directions as a function of time. A lack-of-fit F-test was confirmed that expansion of the invasion fronts was linear with respect to time. Minitab (v. 11) was used for regression analyses. All regression estimates are presented ± 95% confidence interval.

Gene flow. One individual from each colony was selected haphazardly for DNA extraction, which was accomplished using a guanidinium isothiocyanate-based extraction protocol (Mikheyev *et al.* 2006). DNA was amplified using three pairs of primers re-designed from loci originally isolated by Ortius-Lechner *et al.* (2002) from a Panamanian population of *A. octospinosus* (the original primers did not amplify DNA from the Guadeloupe population). 10 µl PCR reactions contained 1 µl of genomic DNA, 1 x reaction buffer, 1 mM dNTPs, 0.01 µM M13-tailed primer, 0.15 µM other primer (Table 2.1), 0.18 µM of fluorescently labeled M13 tail, 5 mM $MgCl_2$ and 0.1 U Taq polymerase.

PCR reaction conditions involved an initial denaturing step of 94 ˚C for 2 min, followed by 30 cycles of 94 ˚C for 10 s, 60 ˚C for 20 s, and 72 ˚C for 10 s. The reaction was incubated at 72 ˚C for a 1 h final extension step. The length of the amplified microsatellite fragments was read on an ABI PRISM® 3100 genetic analyzer. Four samples did not amplify at one or more loci and were excluded from the analysis. Allelic diversity, Hardi-Weinberg equilibrium and isolation by distance computations were carried out in GenAlEx (v. 6) (Peakall & Smouse 2006). Arlequin (v. 3) was used for the F_{st} calculations (Excoffier *et al.* 2005).

Fungus garden pathology. *Escovopsis* screening was conducted by placing 1-2 cm^2 ant-free samples of the fungus garden into a sterile 1.5 ml tubes. Garden contaminants were allowed to colonize the fungus over the course of two weeks at room temperature. I searched for ants covered with a characteristic white bloom during colony excavation in order to screen for the presence of mutualistic *Pseudonocardia* bacteria (Currie *et al.* 2003a; Currie *et al.* 2006).

2.3 RESULTS

Origins of the invasion. Phylogenetic analyses demonstrated that *A. octospinosus* from Guadeloupe belong to a clade distinct from most Central American and Cuban *A. octospinosus*, while being most closely related to ants from Trinidad and Tobago (Figure 2.3). The affinity of Guadeloupe ants with Trinidad and Tobago is further supported by near-identity of the highly variable mitochondrial spacer region, which was excluded from phylogenetic analysis.

Rate of spread. A linear regression model provided a significant fit to the spread estimates, explaining 82% of the variance (regression $F_{1,10}$=46.6, P=4.6×10^{-5}; Lack-of-fit $F_{2,8}$=2.7, P=0.13). The average rate of spread for *A. octospinosus* was 0.51±0.20 km/year and an initial introduction was extrapolated to 1950±1.8. According to the linear model, given this rate of spread, the ants would not have colonized the extreme south of Basse Terre, which lay some 30 km south of the invasion front in the mid-1980s, at the time of my surveys. Indeed, I was unable to find ants south of Capesterre-Belle-Eau during my survey. Additional evidence came from interviews with local residents who claimed that no *fourmi-manioc* were present in the extreme south of Basse Terre. According to temperature and precipitation data published by Therrien (1986), these habitats should be well within the range of environments tolerated by *A. octospinosus* on Guadeloupe, making it likely that the ants still have not reached the extreme south of Guadeloupe.

According to the microsatellite analysis, although there was no significant population structure between the two islands (F_{st} = 0.022, P=0.10), there was evidence of evidence of weak isolation by distance (R_{xy} = 0.06, P = 0.029), probably as a result of the ants' poor dispersal abilities. In addition, populations on both islands exhibited substantial heterozygote deficiency at all microsatellite loci, suggesting common local inbreeding (Table 2.2).

Fungus garden pathology. A variety of fungi colonized the cultivars when the ants were removed, including the specialized garden pathogen *Escovopsis*, which was observed in 7 out of the 60 garden samples (~12%). The identity of *Escovopsis* was further confirmed using spore morphology and through sequencing a section of the *EF1-α* gene (N. Gerardo

and S. Taerum, unpublished data). Although no forager bore visible amounts of *Pseudonocardia* symbionts, workers inside the garden were often covered with an easily visible film of these bacteria (Figure 2.2) (Currie *et al.* 2006).

2.4 DISCUSSION

Origins of the invasion. Without exhaustively sampling the Caribbean using microsatellite markers, the exact origin of *A. octospinosus* will remain unknown. However, an introduction from Central America or Cuba can be ruled out. Panamanian *A. octospinosus* may even be a different species (Sumner *et al.* 2004). Without providing any further justification for their claim, Malato *et al.* (1977) suggested that the ants were introduced via unregulated commerce from an unspecified English-speaking country in the Caribbean. Phylogenetic data support this hypothesis, since the ants are closely related to those on Trinidad and Tobago, although northeastern South America is also a possibility (Figure 2.3).

It is most likely that the ants were introduced either as newly-mated queen(s) or an incipient colony associated with potted vegetation, since mature nests are too large and too aggressive to be transported inadvertently. Recent work has shown that probably only one cultivar genotype was introduced on Guadeloupe (Mikheyev *et al.* 2006). As genetically identical cultivars genotypes are rare on the mainland (Mikheyev *et al.* 2007), a single-queen introduction seems most likely. Often invaders suffer considerable loss of genetic diversity, relative to source populations, which, paradoxically, may provide a competitive edge to some ants, such as the Argentine ants, by allowing the formation of

wide-ranging super-colonies (Holway *et al.* 1998; Tsutsui *et al.* 2000). However, for other ants, like the imported red fire ant, secondary introductions may have been important for successful establishment (Ross & Fletcher 1985; Shoemaker *et al.* 2006). Ant queens acquire all the sperm they will use during a single mating flight and the rates of polyandry vary between species (Hölldobler & Wilson 1990). In contrast to the singly-mating Argentine and fire ants (Krieger & Keller 2000; Ross *et al.* 1988), every *Acromyrmex* queen has the potential to bring greater genetic diversity through mating with numerous males (Murakami *et al.* 2000). Consequently, the levels of diversity on Guadeloupe, are comparable to those seen on the mainland (Ortius-Lechner *et al.* 2002).

Rate of spread. Historical accounts of *Acromyrmex* spread on Guadeloupe provided a unique insight into the dispersal rate of these ants, which spread at the surprisingly low rate of 0.51±0.20 km/year. The slow and even expansion of the invasion suggests mating flights as a dominant dispersal mechanism, unlike the usual jump dispersal and colony fission associated with other invasive ants (Suarez *et al.* 2001). The factors that limited within-Guadeloupe spread of *A. octospinosus* by humans are likely the same that limit international transport – that the ants cannot nest opportunistically, requiring large excavated cavities for their fungus garden (Wetterer *et al.* 1998a), and are substantially larger and more visible than most invasive ants (McGlynn 1999a). Low dispersal ability can also be inferred from molecular data, which show isolation by distance (even over a few tens of kilometers on Guadeloupe) and reduced heterozygosity, which suggests inbreeding (Table 2.2).

Fungus garden pathology. Considerable evidence exists indicating that invading species gain an ecological advantage by escaping their natural enemies (Keane & Crawley 2002, Mitchell & Power 2003). *A. octospinosus* provides a test of this hypothesis, since garden pathogens of attine cultivars are relatively well-known (Currie *et al.* 1999a). However, the success of *A. octospinosus* on Guadeloupe is unlikely due to escape from garden pathogens, as evidenced by both presence of *Escovopsis* and abundance of metabolically costly actinomycetes on the bodies of garden workers, suggesting an ongoing need to protect the garden from pathogens (Poulsen *et al.* 2003) (Figure 2.2). The presence of *Escovopsis* in the attine gardens is notable, since dispersing virgin queens appear not to carry the pathogen along with the parental cultivar they take on their mating flight, but instead acquire it only after the onset of foraging (Currie *et al.* 1999a). Thus, *Escovopsis* must either be sufficiently common in the environment to have been introduced independently, or, less likely, A. *octospinosus* was introduced as a foraging colony, rather than a newly-mated queen.

Implications for further invasions. Although *A. octospinosus* damaged agriculture in Guadeloupe (Blanche 1954), whether they affect natural ecosystems is unknown. The South of Basse Terre, where the ants still appear to be absent, may be a location where the progressive ecological effects of a leaf cutting ant invasion can be still observed. While most modern-day invasive ants are cryptic and easily transported hidden within goods, *A. octospinosus* appears transport-limited, making introductions a rare event (McGlynn 1999b). On the other hand, even a single queen may carry sufficient genetic diversity to initiate a successful invasion. On Guadeloupe, history has shown that chemical control against such an invasion would be ineffective once it has become fully

established (Febvay *et al.* 1988; Gomel *et al.* 1989). Given the presence of *Escovopsis* on Guadeloupe, biological control using specialized garden pathogens does not appear promising. However, the limited dispersal ability of *A. octospinosus* may allow the elimination of an incipient population. Such an effort should be rapid and thorough, possibly involving the destruction of all available plant food sources in the vicinity of the introduced *A. octospinosus* population. However, undoubtedly the best defense involves an awareness of the invasive potential of leaf-cutting ants and in addition to an investment into thorough agricultural inspection and quarantine services (Suarez *et al.* 2005). Although a large number of countries already have such services, their track record is not perfect (*e.g.*, major invasives, such as the red imported fire ant *Solenopsis invicta*, established despite the activity of the US Customs service). The situation can no doubt be improved by comprehensive studies of the relationship between different inspection and quarantine practices and their relative success at keeping out invaders, and, if necessary, changes in quarantine law.

2.5 TABLES AND FIGURES

Locus	Primer sequence (5' → 3')
	TCTGTTAGTCATAAGTATGTCACTGTCG
Ech4225	AACCGATATGTGTCGATATGTATTTGTTAG
	GAATGAATTAAATAACGCTAGTGAAAGTAG
Ech3385	GTGATTATCCACTGATTCAGATTTAAAGAG
	GATCATTTATCCTCAATTCAATAGTTTG
Ech3197	GTTTATGGAACCAGTAGACCCTAATGAAG

Table 2.1. Primers used in the microsatellite survey, based on loci originally developed by Ortius-Lechner *et al.* (2002). Note that the first primer in each pair was synthesized with a fluorescently labeled M13 tail for microsatellite analyses.

Locus	Alleles	H_o-H_e	P
Ech4225	6	-0.38	4×10^{-13}
Ech3385	3	-0.036	0.10
Ech3197	7	-0.31	4×10^{-20}

Table 2.2. Alleleic diversity on Guadeloupe. The table shows the name of the microsatellite locus according to Ortius-Lechner *et al.* (2002), the number of alleles, the difference between expected and observed heterozygocity values and their χ^2 probability.

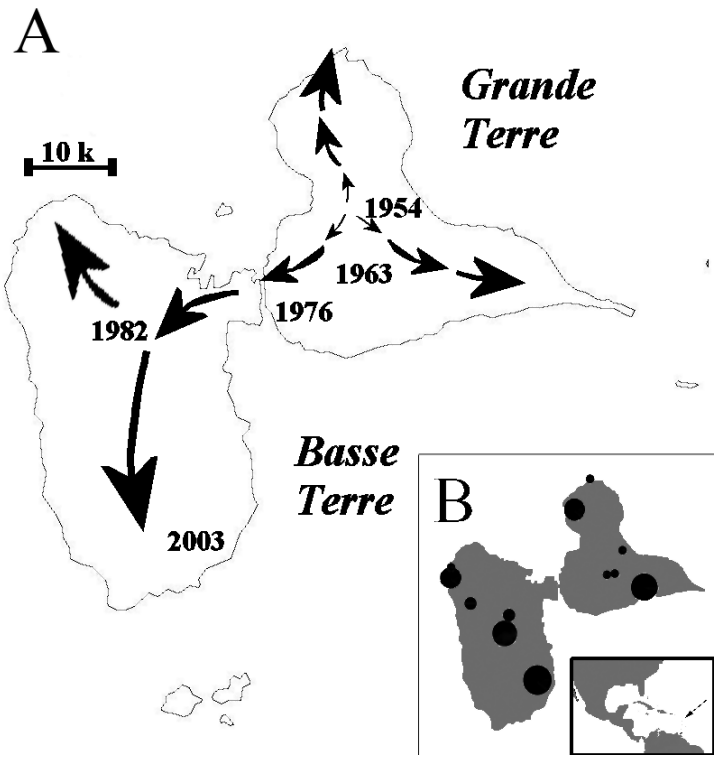

Figure 2.1. History of the invasion of Guadeloupe by *Acromyrmex*. After their initial discovery at the community of Morne-à-l'eau on Grande Terre, the ants slowly colonized the both major islands.

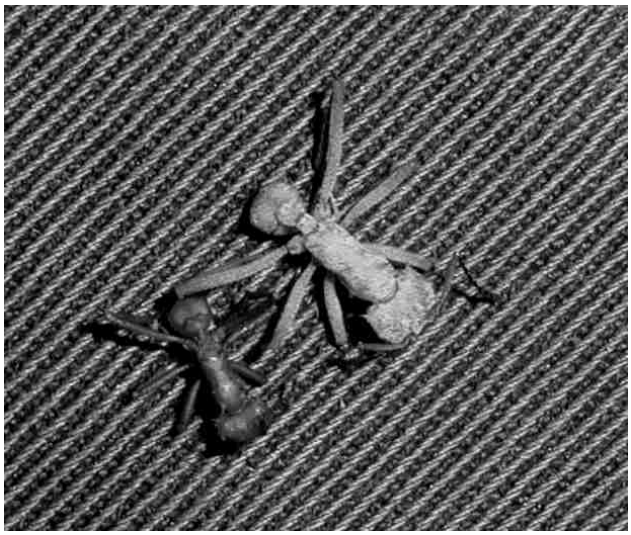

Figure 2.2. *Acromyrmex* workers with and without actinomycete symbionts. Both workers came from the same colony; the one the left is a forager and has no visible actinomycetes, while the one on the right is a garden worker, and has a white actinomycete bloom.

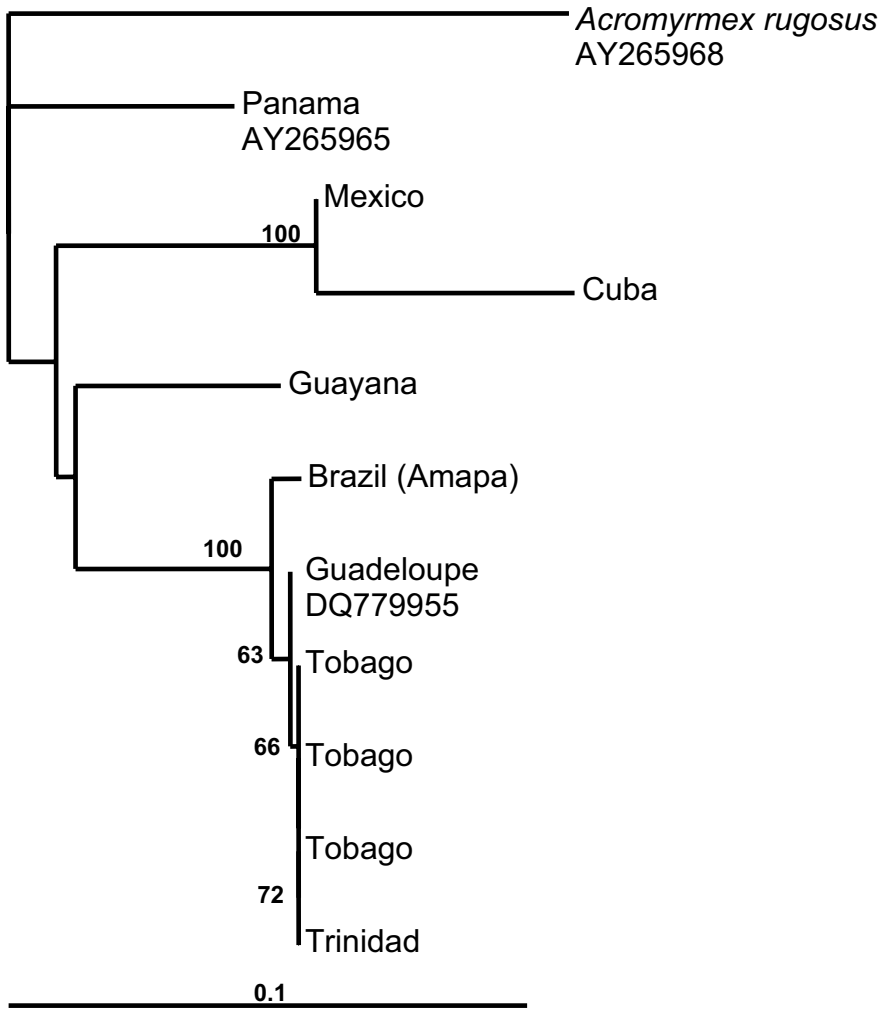

Figure 2.3. Phylogeny of the *Acromyrmex octospinosus* species complex based on cytochrome oxidase subunits I and II. While distinct from populations inhabiting Central America, the Guadeloupe population shows a marked affinity with populations from Trinidad and Tobago and eastern South America. These genetic affinities were further supported by nearly identical intergenic spacer sequences. Genbank accession numbers accompany reference sequences.

CHAPTER 3

The discovery of cryptic sex and many-to-one co-evolution in the cultivars of leaf-cutter ants

Abstract: The fungus-growing ants have long provided a spectacular example of co-evolutionary integration between distantly related taxa. Their ecological success is thought to depend largely on the evolutionary alignment of reproductive interests between ants and fungi following vertical transmission and the ancient suppression of fungal sexuality. Here we provide the first evidence for recombination in attine cultivars, based on conservation of meiosis-specific genes, the existence of recombination between nuclear loci and direct observations of recombination in an isolated invasive population of the leaf-cutting ant *Acromyrmex octospinosus*. In addition, we document extensive long-distance horizontal transmission of fungal genes between leaf-cutter taxa resident on mainland Central and South America and those endemic to Cuba, suggesting both lack of specificity in ant/cultivar co-evolutionary interactions and independent dispersal by the cultivar. The leaf-cutters and their fungal symbionts exhibit unique co-evolutionary dynamics, in which ant species that diverged millions of years ago cultivate a single sexual and geographically-widespread symbiont species. Moreover, strict fungal clonality and vertical transmission evidently are not critical components of leaf-cutter success, despite widely-held perceptions of obligate clonality and pair-wise co-evolutionary specificity.

3.1 INTRODUCTION

Attine ants evolved agriculture based on the cultivation of fungi some 50-60 million years ago. By allowing the exploitation of food sources unavailable to most other ants, agriculture allowed the fungus-gardeners to become a taxonomically diverse group, with various sub-groups differing widely in the extent of specialization and nutritional dependency (Mueller *et al.* 2001; Wheeler 1907). The most derived group, the "higher attines", includes the leaf cutters, nearly fifty species in the genera *Atta* and *Acromyrmex* (Schultz & Meier 1995), and are perhaps the most ecologically dominant organisms in the New World tropics outside of humans (Hölldobler & Wilson 1990; Wheeler 1907). Typically, following mating and dispersal from their natal nest, young queens cultivate mycelia brought from parental gardens, a behavior believed to stabilize the mutualism by restricting horizontal cultivar exchange (Bot *et al.* 2001). However, horizontal host switches are known to occur in some congeneric taxa (Green *et al.* 2002; Mueller *et al.* 1998). For example, in the primitive fungus-gardening ants (the "lower attines"), horizontal transfers occur directly between the cultivar lineages, or via free-living fungal populations acting as bridges (Green *et al.* 2002). The higher attines, by contrast, possess no known free-living relatives, and the presumption has long been that these cultivars are propagated by strictly clonal inheritance (Chapela *et al.* 1994; Mueller *et al.* 1998). Although infrequent observations of fruiting body formation in gardens of higher attines (Mueller (2002) suggest either the possibility of a cryptic sexual stage, or else its vestigial expression, there has been no direct test of the long-standing asexuality hypothesis. Consequently, nearly all of the growing body of work on this complex mutualism has been predicated on the assumption of clonality.

Assuming clonal reproduction as our null hypothesis, we tested for asexuality and admixture in the leaf cutter cultivars by seeking genetic signatures of clonality. First, we characterized protein evolution along fragments of the eukaryote *recA* homologs *DMC1* and *RAD51*. Both mediate homologous DNA pairing and strand exchange, and are necessary for completion of the meiotic cell cycle (Bishop *et al.* 1992; Shinohara *et al.* 1992; West-Eberhard 2003). *RAD51*, the fundamental eukaryotic recombinase, is required for both mitotic and meiotic DNA metabolism, whereas *DMC1* is solely expressed during meiosis. (Mikosch *et al.* 2001; Nara *et al.* 1999). One prediction of long-term asexuality is that disuse of the meiotic machinery, typically under strong purifying selection in eukaryotes, would be associated with relaxed functional constraints on coding nucleotides that alter amino acid sequences (Normark *et al.* 2003). Long-term loss of sex would thus result in either elevated rates of protein evolution in genes underlying meiosis, or perhaps degradation and elimination from genomes not requiring meiotic cell function (Paoletti *et al.* 2005; Ramesh *et al.* 2005). Next, we tested for evidence of interlocus recombination between *RAD51*, *DMC1*, and elongation factor 1-alpha (*EF1-α)*. Clonality predicts negligible levels of phylogenetic incongruence across genomes, because fully linked genetic markers used to infer taxonomic relationships share an identical evolutionary history (Smith & Smith 1998). Recombination acts to break down linkage and can cause conflicting genealogical relationships between loci.

In order to establish whether long-term congruence exists between ant and cultivar genealogies, we examined the population genetics of cultivars on two island populations,

in which the biogeographic histories of the ant hosts are established. First, we used an invasive population of the leaf-cutter *Acromyrmex octospinosus*, which colonized the French Caribbean islands of Guadeloupe approximately 50 years ago (Malato *et al.* 1977), to detect ancestral cultivar genotype(s) and follow their subsequent evolution. Next, we derived the phylogenetic relationship for cultivars from leaf-cutter taxa on Cuba and on the mainland, from Central and South America (Panama and Brazil). Cuban leaf-cutter ant fauna consists of two endemic species of *Atta* (Fontenla Rizo 1993) and an endemic (Mikheyev, pers. observations) *Acromyrmex* species, which is unusual for Caribbean islands, where they are largely absent (Cherrett 1968). Thus, differences in rates of endemism and genetic affinity with mainland populations between the ants and cultivars provide an insight into differential dispersal abilities and long-term genealogical incongruence between the two symbiont taxa.

3.2 MATERIALS AND METHODS

Amplification of ITS, *DMC1*, *RAD51*, *EF1-α* and *BIP*. Except for ITS4 and ITS5 primers (White *et al.* (1990)), commonly used for fungal ribosomal ITS amplification, primer sequences were developed from conserved regions of gene homologs in GenBank (Table 3.1). DNA was extracted according to Kweskin (2003) or by incubating a single cluster of fungal gongylydia (structures used to feed ant larvae) in 100 µl 5% aqueous Chelex resin for 1.5h at 60 ˚C and then at 99 ˚C for 10 minutes.

One µl of the extract was used as template in 10 µl reaction volumes. The PCR contained 1 x reaction buffer, 1 mM dNTPs, 0.5 µM primers, 5 mM $MgCl_2$ with 0.1 U of

Bioline Taq polymerase. Average reaction conditions involved an initial denaturing step of 94 ˚C for 2 min, followed by 30 cycles of 94 ˚C for 10 s, 60 ˚C for 20 s, and 72 ˚C for 30 s. PCR products were approximately 500 base pairs long, except for the DMC1-FB/RF primer combination, which produced fragments slightly over one kb in size. The annealing temperature was varied slightly to correspond with primer Tm. Two µl of the products were run on 1.5% agarose gels and visualized by staining with ethidium bromide. Reactions that yielded strong bands were cleaned by polyethylene glycol precipitation (a 1:1 PCR product/20% PEG mixture was incubated for 15 min at 37 ˚C for, followed by a 10 min centrifugation at 4,000 rpm and two washes with 80% ethanol). Purified products were cycle-sequenced using the ABI BigDye Terminator Kit (v. 3.1) and sequenced on an ABI PRISM® 3100 genetic analyzer according to the manufacturer's instructions. To insure the accuracy of sequence information, both the forward and reverse sequences were generated.

Population genetics of the Guadeloupe invasion. As above, fungal DNA was extrected by boiling gongylydia in Chelex resin. And DNA was extracted by crushing legs of workers in 50 µl of extraction buffer (50g guanidinium isothiocyanate, 50 ml PCR-grade water, 5.3 ml 1 M Tris-HCl (pH 7.6), 5.3 ml 0.2 M EDTA, 10.6 ml of 20% sodium lauryl sarcosinate and 1 ml 2-mercaptoethanol) and incubating for 1h at 70 ˚C with occasional vortexing. The extractions were briefly centrifuged and the supernatant was collected. The extraction was chilled for 2 or more hours at -20 ˚C, following addition of 50 µl isopropanol. Afterwards the extraction was centrifuged at 4,000 rpm for 20 min and the supernatant was discarded. The pellet was washed with 150 µl cold ethanol, dried and re-dissolved in 50 µl PCR-grade water.

The intergenic spacer between cytochrome oxidase subunits I and II was amplified using primers (COI and COII) and conditions previously published by Sumner *et al.* (Sumner *et al.* 2004). PCR products were purified and sequenced as above.

Cultivars were genotyped using M13-tailed primers B0150B, A0460, C606 and B0358 (Scott et al, in prep). PCR reagent concentrations were as above, except that forward reverse primer were added up to 0.01 and 0.15 μM, respectively. Additionally, 0.18 μM of fluorescently labeled M13 tail was included in the reaction. PCR reaction conditions involved an initial denaturing step of 94 ˚C for 2 min, followed by 20 cycles of 94 ˚C for 10 s, 60 ˚C for 20 s, and 72 ˚C for 10 s. Subsequently, 15 more cycles were performed with the annealing temperature at 53 ˚C. The length of the amplified microsatellite loci was read using an ABI PRISM® 3100 genetic analyzer.

Statistical analyses. *Positive selection analysis.* HyPhy (Kosakovsky Pond *et al.* 2005) was used to test for relaxed selection on *DMC1* and *RAD51* and to calculate dN/dS values and 95% confidence intervals. First, an appropriate codon model was selected independently for both genes (HKY85 was the best model for both). Subsequently, we tested whether *DMC1* and *RAD51* evolved under a different under a different selection regime in the putatively asexual clade of higher attine cultivars. The analysis was carried out using a complete model of site-to-site rate variation with four rate classes, a polarity/charge/hydrophobicity-based amino acid model.

Recombination between genes. Recombination analyses were carried out on a concatenated alignment of DMC1, RAD51 and *EF1-α* sequences (1504 bp) for eight *Atta* and *Acromyrmex* cultivars. The PAUP's *homopart* test (Swofford 1993) used 18 parsimony-informative characters out of 37 total variable characters. The presence of recombination was tested independently using TOPALi (Milne & Abbott 2004), with defailt settings and a 500 bp window sliding at 10 bp per step. The significance threshold was calculated using 100 bootstrap replicates.

3.3 RESULTS

Conservation of meiosis-specific genes. We found that both *DMC1* and *RAD51* were strongly conserved at the amino acid level, and essentially identical to free-living lepiotaceous fungi. Ratios of nonsynonymous to synonymous nucleotide changes indicated no evidence of relaxed functional constraints (dN/dS = 0.033±0.012 for *DMC1*; dN/dS = 0.015±0.006 for *RAD51*) and no evidence for accelerated rate of evolution in the higher attine clade (P = 0.87 for *DMC1*; P = 0.75 for RAD51). In particular, no changes localized to functional regions unambiguously crucial to activity (*e.g.*, Walker motifs; Figure 3.1). In contrast to the conserved coding gene regions, introns in the sequenced fragments of *DMC1* (6 introns) and *RAD51* (2 introns) diverged to the point of unalignability, indicating that strong purifying selection must be acting on homologous exons in the various taxa we surveyed.

Interlocus recombination between nuclear genes. The phylogenetic signals in the *DMC1*, *Rad51* and *EF1-α* partitions showed significant lack of homogeneity (P = 0.026), according to a parsimony-based analysis (Swofford 1993). An independent sliding-window analysis (Milne & Abbott 2004) of a three-gene alignment also showed the presence of recombination, and automatically partitioned the data into incongruent sections corresponding to boundaries in the three genes (Figure 3.2). While we cannot exclude the possibility of other processes generating conflicting gene-tree topologies (incomplete lineage-sorting or hybridization, for example (Maddison 1997)), these alternatives to recombination are equally at odds with the hypothesis of long-term vertical transmission and clonal reproduction.

Genealogical congruence between ants and their cultivars on island populations. Assuming clonality and co-evolutionary matching between ants and fungi, the *A. octospinosus* cultivars on Guadalupe should have experienced demographic sorting of post-invasion genetic diversity roughly similar to its ant host, with the ancestral cultivar propagule modified solely by mutation. For the ants, a highly variable 223-bp fragment of non-coding mtDNA (Sumner *et al.* 2004) revealed just one *A. octospinosus* haplotype from 59 colonies. Consistent with the invasion history (Malato *et al.* 1977), the population was founded by a single introduction of either a lone female or perhaps a group sisters. However, for the cultivar, a quarter of the colonies on Guadeloupe appeared to have acquired recombinant strains. In accord with a small founding population, 77% (33/43 gardens) of the nests harbored an identical genotype heterozygous at four microsatellite loci. However, we detected seven other low frequency genotypes homozygous at one or more loci, arranged into a loop-like haplotype network

(Figure 3.3). Simple mutational mechanisms and genotyping errors, such as the failure to amplify one allele at a heterozygous locus, can alter genotype distributions or their estimation, but such errors are not expected simultaneously across multiple, independently-assayed loci, and neither are likely to produce loop-like haplotype networks. Moreover, no new alleles were observed in the low-frequency cultivars, indicating mutation has not played significant role in determining genotype frequencies. Rather, in the course of 50 years since the introduction of *A. octospinosus* to Guadeloupe, the cultivar appears to have undergone recombination.

Whereas the ants have experienced an extensive period of evolutionary isolation on Cuba, the cultivars exhibit no evidence of endemism. In fact, they are nearly identical to their mainland counterparts in all four intron-rich nuclear genes (*DMC1*, *Rad51*, *EF1-*α and *BIP*) and in the rDNA internal transcribed spacer (ITS; Figure 3.2). Although it is conceivable that, like Guadeloupe, Cuba recently received one of the mainland fungal strains that replaced (rather than recombined with) the indigenous cultivar, the complete obliteration of the multi-locus signature of an indigenous strain seems unlikely. The pattern suggests a panmictic symbiont population structure, involving recurrent long-range dispersal and gene flow between Cuban and mainland populations of leaf-cutter cultivars. Panmixia between island and mainland populations is not predicted by clonal and strictly vertical propagation, and contradicts the assumption of long-term co-evolutionary fidelity between different leaf-cutter ant and fungal lineages.

3.4 DISCUSSION

Although the attine cultivars are clearly not clonal, these data do not directly reveal the mechanisms of dispersal and recombination in the cultivars. In addition to sexual fruiting and meiosis, most fungal groups express a variety of irregular reproductive modes. Various "parasexual" processes involving anastomosis, heterokaryosis, mitotic recombination and sporolation are common in filamentous fungi (Pagnocca *et al.* 2001; Pontecorvo 1956). To date, outside of laboratory settings, parasexuality has not been described from natural populations of basidiomycete fungi (Abomo-Ndongo *et al.* 2002; Anderson & Kohn 1998; Carvalho *et al.* 1995; Johannesson & Stenlid 2004; Polak *et al.* 1997; Selosse 2001; Taylor *et al.* 1999; Xu *et al.* 1996). Vegetative incompatibility probably limits opportunities for hyphal fusion, and thus reduces the natural frequency of parasexual mechanisms that depend upon anastomosis (Anderson & Kohn 1998). While the rareness of parasexuality in basidiomycetes, the conservation of the meiotically-expressed *DMC1*, and the occasional observations of sexual fruiting bodies (basidiocarps) in the fungus gardens (Mueller (2002) implies that meiotic sporolation is a principle cause of recombinant cultivars, it may well be that both meiotic and mitotic processes contribute to admixture in attine cultivars. We envision a scenario similar to the following: (a) meiosis occurs during spore production in the basidiocarp in attine cultivars, (b) the spores then travel to ant-tended gardens and undergo heterokaryosis via mitotic recombination. Consistent with the existence of nuclear admixture in the attine symbiosis, like other mitotic recombinants leaf-cutter cultivars occasionally exhibit polyploidy (Kweskin 2003 ; Mikheyev *et al.* 2007). Alternatively, recombination may conceivably occur via a free-living population of higher attine cultivars, as with the

lower attines, but such a population has not yet been detected, despite intensive surveys (Mueller *et al.* 1998). Whether sexual or parasexual mechanisms are at work, the population genetic and evolutionary consequences are indistinguishable: recombination shuffles genetic variation in attine cultivars and results in non-clonal, reticulate propagation.

Collectively, these data reveal that a single biological species of fungus, previously named *Leucoagaricus gongylophorus* (Möller 1893; Pagnocca *et al.* 2001), co-evolves with two different genera of leaf-cutting ants. In addition to crossing generic boundaries in host ant species, recombination within *L. gongylophorus* homogenizes genetic variation over great geographic distances, acting over relatively short periods of time. This conclusion is consistent with previous findings that Brazilian *Acromyrmex* and *Atta* cultivars show little or no differentiation in ribosomal ITS regions over thousands of kilometers (Silva-Pinhati *et al.* 2004). Thus, the evolutionary dynamics between ants and the cultivar fungus may be qualitatively different than currently believed. Rather than a one-to-one correspondence between ant and cultivar lineages, a many-to-one relationship exists, in which a single general-purpose fungal mutualist acts as a nexus for indirect interactions between divergent ant lineages.

Initially, long-term clonal propagation was proposed as a central mechanism promoting ant-fungus coevolution (Chapela *et al.* 1994). *Atta* and *Acromyrmex*, for example, exhibit behaviors such as vertical transmission and garden monocultures thought to function in the alignment of reproductive interests (Mueller (2002). This interpretation was revised, at least in the lower fungus-gardening ants, whose cultivars were discovered

to exist as free-living forms (Mueller *et al.* 1998). Subsequently, the presumed long-term asexuality of the higher attine cultivars, predicted to reduce fungal fitness by accumulation of deleterious mutations, has been invoked to explain a number of phenomena occurring at the transition between lower and higher attines, such as greater virulence of the specialized garden parasite *Escovopsis* (Currie *et al.* 1999a; Currie & Stuart 2001) and the evolution of multiple mating by queens of the leaf-cutting ants (Villesen *et al.* 1999; Villesen *et al.* 2002). Our data, which strongly support the existence of recombination in the cultivars, require a re-evaluation of these hypotheses, and implicit support of their alternatives. As well, recombinant attine cultivars require reassessment of the contrasts typically drawn between attines and other insect farmers (Aanen *et al.* 2002; Mueller *et al.* 2005). The termites and beetles farm sexual fungal crops, which presumably express pathogen resistance. The ants rely on antibiotics from mutualistic actinomycete bacteria that rapidly co-evolve with garden pathogens, a mechanism hypothesized to compensate for fungal asexuality in the evolutionary arms race with parasites (Mueller (2002). The discovery of on-going sexual reproduction suggests that ant cultivars require no novel explanations for the maintenance of immune function (although such explanations are not prohibited). However, because clonal propagation was a major point of difference between attines and other insect farmers (Mueller 2002; Mueller *et al.* 2005), the bridging of this gap suggests an opportunity for the development of a more unitary understanding of insect agriculture.

More importantly, the discovery of horizontal exchange and sexual reproduction in attine cultivars mirrors an on-going and fundamental change in our understanding of co-evolution. Unlike parasitic interactions, one-to-one specificity in mutualistic

interactions is evidently not common, as once thought, even in exemplary systems where specificity was the once rule (Machado *et al.* 2005). Such specificity rarely occurs in any but the most integrated interactions, such as those between aphids and their primary endosymbionts or various mycorrhizal associations (Clark *et al.* 2000). Rather, molecular studies of virtually all other well-characterized mutualisms, from lichens (DePriest 2004) to plant-pollinator mutualisms (Stanton 2003; Strauss & Irwin 2004), reveal evolutionary diffuseness. Why co-evolution either requires or promotes a certain evolutionary promiscuity in biological interactions remains poorly understood (Bell *et al.* 2005), but it seems possible that insect agriculture, as well as other multi-guild mutualistic interactions, could operate under an emerging principle of diffuse co-evolution between participants.

3.5 TABLE AND FIGURES

Primer name	Sequence (5' 3')
BIP3-F	GAT GTY AGC AAG AAC CTY CG
BIP3-R	TTG TCC TTG GTG AGS GRA CG
EF1*α* -F	GTT GCT GTC AAC AAG ATG GAC ACT AC
EF1*α*-R	GCC TTG ATG ATA CCA GTC TCG ACA CG
RAD51-F	GGC AAA TGT TTG TAT ATA GAT ACT G
RAD51-R	CAC CGA TAG GTT TCT TCT CAT TAC C
DMC1-FB	GGT ATG TCG GAA GCC AAA G
DMC1-RF	TCG GCC CAA CCT CCT TCG TC
DMC1-F	AAG CTG CAC ACA AAA TCT TGG TTA G
DMC1-R	GTC AAT GTC AAG AGA TCG GAT ACA C

Table 3.1. Primer sequences used for amplifying fungus-gardener cultivar genes.

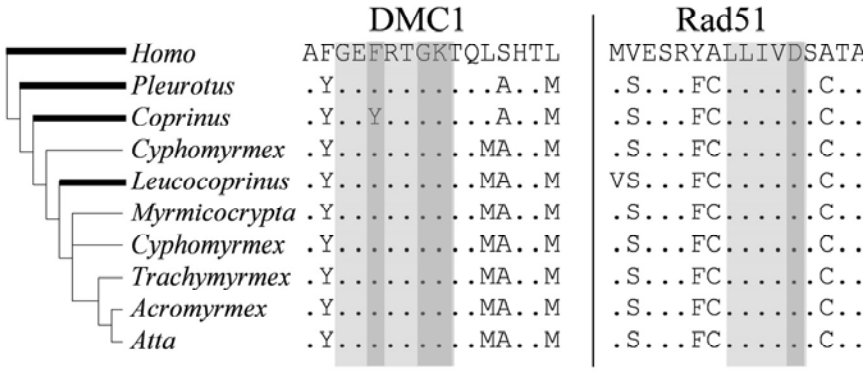

Figure 3.1. Conservation of ATP-binding Walker motifs in DMC1 and RAD51 at the amino acid level. Synonymous substitutions are indicated by plus signs below the alignment. Free-living taxa are indicated by bold highlighting of branches on the phylogeny.

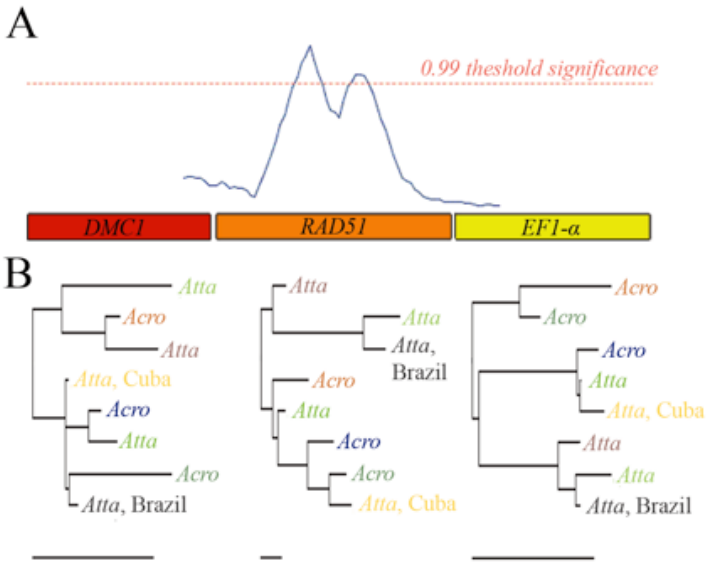

Figure 3.2. Evidence for recombination in the alignment of DMC1, RAD51 and EF1-α. (A) PDM local divergence plot using a 500 base pair sliding window. (B) Bayesian trees for partitions automatically generated from local divergence scores. Unless otherwise specified, all samples were collected in Panama. Bars below below each tree are scaled to 0.01 substitutions/site.

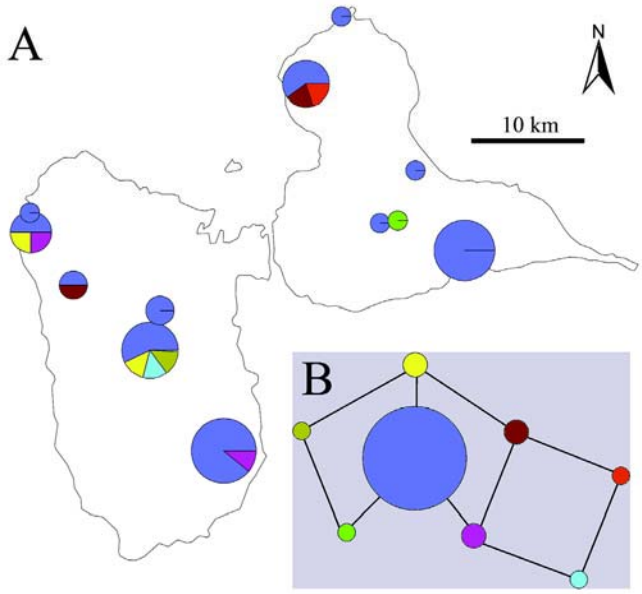

Figure 3.3. Distribution of collections and relationships among cultivar genotypes on the islands of Guadeloupe. The grey inset contains a parsimony network connecting the observed genotypes. Pie charts and network vertices are scaled according to relative cultivar frequencies.

CHAPTER 4

Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi.

Abstract: Switching of symbiotic partners pervades most mutualisms, despite mechanisms that appear to enforce partner fidelity. To investigate the interplay of forces binding and dissolving mutualistic pairings, we investigated partner fidelity at the population level in the attine ant-fungal cultivar mutualism. The ants and their cultivars exhibit both broad-scale co-evolution, as well as cultivar switching, with short-term symbiont fidelity maintained by vertical transmission of maternal garden inoculates via dispersing queens and by the elimination of alien cultivar strains. Using microsatellite markers, we genotyped cultivar fungi associated with five co-occuring Panamanian attine ant species, representing the two most derived genera, leaf-cutters *Atta* and *Acromyrmex*. Despite the presence of mechanisms apparently ensuring the co-transmission of symbiont genotypes, different species and genera of ants sometimes shared identical fungus garden genotypes, indicating widespread cultivar exchange. The cultivar population was largely unstructured with respect to host ant species, with only ten percent of the structure in genetic variance being attributable to partitioning among ant species and genera. Furthermore, despite significant genetic and ecological dissimilarity between *Atta* and *Acromyrmex*, generic difference accounted for little, if any, variance in cultivar population structure, suggesting that cultivar exchange dwarfs selective forces that may act to create co-adaptive ant-cultivar combinations. Thus,

binding forces that appear to enforce host fidelity are relatively weak and pairwise associations between cultivar lineages and ant species have little opportunity for evolutionary persistence. This implicates that mechanisms other than partner fidelity feedback play important roles in stabilizing the leafcutter ant-fungus mutualism over evolutionary time.

4.1 INTRODUCTION

Different species engaged in a mutualistic interaction jointly occupy an ecological niche that allows for higher fitness than could be obtained by either symbiont acting alone. The persistence of an obligate mutualism through evolutionary time results from a positive balance between forces that hold the interaction together and other forces that promote selfish behaviors by the symbionts (Frank 1994, 1996). Traditionally, the interplay between selfish and cooperative forces has been viewed from a perspective of repeated interactions between a pair of mutualistic species (Hoeksema & Bruna 2000; Stanton 2003). However, recent data reveal that virtually all mutualisms involve more than two interacting species with at least occasional, and sometimes ubiquitous, horizontal transfer of symbionts, even when mechanisms ensure general vertical symbiont transfer (Herre *et al.* 1999). In a typical example, fig wasps were previously believed to form strict species pairs with their host figs, vertical co-transmission of symbiont genotypes being insured by the capture and death of the female wasp upon entering the fig in order to lay eggs. However, recent work revealed the existence of cryptic wasp species that may even produce mixed broods consisting of several wasp species within a single fig (Machado *et al.* 2005; Molbo *et al.* 2003). Similarly, although the most widespread moth pollinating

yucca was initially thought to be a single species, it is now recognized as a species complex with relatively narrow host niches (Pellmyr & Leebens-Mack 1999). Furthermore, the evolution of 'cheater' moths, which over-exploit the yucca seed resources, seems to be linked with host shifts (Pellmyr *et al.* 1996). However, patterns of species sharing, as observed through phylogenetic relationships, could be likewise due to continuous switching between several co-evolving partners, or to periods of long-term pairwise symbiont association, followed by an occasional symbiont switch. Thus, the rate at which symbiont switching happens, and thus, to a large extent, the nature of selection acting to maintain symbioses remains unclear. Only a detailed analysis of the genetic associations within a population of several interacting mutualist species can reveal the relative strength of forces acting to bind *vs*. dissolve pairings of symbiotic partners.

The symbiosis between fungus-gardening (attine) ants and their fungal cultivars is a well-studied obligate mutualism, dating back an estimated 60 million years (Mueller *et al.* 2001). Since the origin of this agricultural system, broad-scale coevolutionary interactions has involved both vertical symbiont transmission and horizontal sharing of lineages (Chapela *et al.* 1994; Mueller *et al.* 1998). Association of ant and fungal lineages through time appears to be insured both by the ant queens, which take bits of maternal fungus gardens on mating flights and then use them to start their own gardens (Mueller 2002; Weber 1972), and by the cultivar, which exhibits an aggression response to foreign strains, maintaining a garden monoculture (Bot *et al.* 2001; Poulsen *et al.* 2005. At the same time, population-level data indicate sharing of cultivar lineages (Bot *et al.* 2001; Green *et al.* (2002)), either through garden theft (Adams *et al.* 2000), through cultivar sexual reproduction and recombination (Mikheyev *et al.* 2006), or in the case of the

more primitive attine ants, through a free-living fungal intermediate (Green *et al.* 2002; Mueller *et al.* 1998, Vo et al, in press). Although earlier studies demonstrated the existence of at least occasional exchange of cultivar strains between different host ant species, we know little about the frequency of such exchanges or whether cultivar switching dominates specialization at the population level.

The most derived members of this agricultural symbiosis, the leaf-cutting ants in the sister genera *Atta* and *Acromyrmex*, along with other higher attines in the genera *Trachymyrmex* and *Sericomyrmex* (Schultz & Meier 1995; Wetterer *et al.* 1998b) cultivate specialized co-adapted fungi that apparently lack free-living relatives (Mueller *et al.* 1998, Vo *et al.*, in press). Although the two leaf-cutter genera consume similar types of vegetation (Weber 1972), they differ substantially in foraging ecology and nesting behavior (Hölldobler & Wilson 1990; Wetterer 1995). Mature *Atta* colonies have millions of workers and are housed in enormous subterranean nests that live for decades and occupy a defended territory, while destroying smaller nests of other leaf-cutting ants (; Borgmeier, 1922 #391; Fowler 1992; Hölldobler & Wilson 1990). By contrast, *Acromyrmex* constructs much smaller, sometimes arboreal, nests that have colony sizes ranging from thousands to hundreds of thousands of workers (Pereira-da-Silva *et al.* 1981; Wetterer 1993; Wetterer, 1995). Fowler (1983) noted that there is a negative correlation in the local abundance of the two genera, suggesting the existence of competition between them. Yet, cultivar sharing has been documented between congeneric species and, likely, even between ant genera (Bot *et al.* 2001; Mikheyev *et al.* 2006; Silva-Pinhati *et al.* 2004). Recent evidence further suggests that most, if not all, of

the fungi cultivated by leaf-cutting ants belong to a single biological species (Mikheyev *et al.* 2006; Silva-Pinhati *et al.* 2004; also see Stradling & Powell 1986).

Given the transmission of parental gardens by virgin queens, it seems plausible that some level of association between the ant and fungal lineages exists; certainly co-evolution at the deeper phylogenetic levels occurs (Chapela *et al.* 1994). Vertical transmission has been proposed as a dominant mechanism for the ants to control the movement and mixing of their cultivars (Bot *et al.* 2001), as well as an important force in aligning the reproductive interests of the ants and the cultivar (Mueller 2002). Alternatively, vertical transmission may enable a solitary queen to start a colony without wasting resources on finding a new garden during the critical colony founding stage. Later, the colony may well substitute its cultivar, either through the loss of the original garden or through acquisition of strains better suited to local environmental conditions (*e.g.*, better at processing locally available food sources). At least in the lab, *Atta* queens who lose their pellet or incipient garden can raise a brood of workers and potentially acquire a new garden (Fernandez-Marin & Wcislo 2005). Finally, sporulation and sexual reproduction by the cultivar may further facilitate genetic exchange between species (Mikheyev *et al.* 2006). Consequently, the ant-cultivar symbiosis could operate under two opposing co-evolutionary models – characterized either by long-term pairwise interaction between an ant species and its cultivar (as traditionally assumed), or by frequent garden exchanges between species.

Prolonged pairwise co-evolution predicts the existence of an association that substantially partitions cultivar genetic variance among the different ant species. By

contrast, frequent exchanges should result in relatively unstructured cultivar populations. The unique relationship between the single cultivar species and multiple sympatric species of ants allows for an explicit test of these predictions using powerful population genetic tools. To this end, we used microsatellite markers to survey the genetic structure of cultivars associated with all five species from both leaf-cutting genera (*Atta* and *Acromyrmex)* endemic to the Panama Canal Zone. Furthermore, taking advantage of the nested relationships between ant taxa (multiple ant species in two monophyletic genera), we evaluated the extent to which the genetic structure of the cultivar species tracks that of the ants.

4.2 MATERIALS AND METHODS

Samples and collecting. All material used in the study was collected in the Panama Canal Zone, home to three *Atta* species (*cephalotes*, *columbica* and *sexdens*) and two closely related species of *Acromyrmex* (*echinatior* and *octospinosus*) (Sumner *et al.* 2004). The distributions of all five species overlap in the Panama Canal Zone. Between 2000 and 2004, queenright *Ac. echinatior* and *Ac. octospinosus* colonies were collected around Gamboa (except one *octospinosus* colony that was collected on Ancon Hill) and were subsequently kept with their original fungus gardens in the laboratory at the University of Copenhagen. Thus, re-sampling of the same *Acromyrmex* colony in the field was impossible. Having lower population densities, *Atta* were sampled over a slightly larger range, from Pipeline road to Gamboa. However, all of the samples spanned an area with a diameter of only ~25 km, with the vast majority having been collected within 5 km of Gamboa. Except for ten live *At. columbica* colonies, which were also collected whole and

kept at the University of Copenhagen, most *Atta* samples were collected in 2001 and (2002)and stored in 95% ethanol. The final analysis involved 62 total gardens – 16 from each of the two *Acromyrmex* species and 30 *Atta* gardens (10 *cephalotes*, 15 *columbica* and 5 *sexdens*).

Molecular methods. DNA was extracted by incubating a single cluster of fungal gongylydia (structures used to feed ant larvae) in 150 µl of 5% aqueous Chelex resin for 1.5h at 60 ˚C and then at 99 ˚C for 10 minutes. Cultivars were genotyped using M13-tailed primers at 9 microsatellite loci: A0659B, A0815, B0150, B0312, B0358, B0430, B0447, C0625, D0115 (Scott *et al*, in prep). One µl of the extract was used as template and the reaction was carried out in a total volume of 10 µl. The PCR contained 1 x reaction buffer, 1 mM dNTPs, 0.01 µM M13-tailed primers, 0.15 µM other primer, 0.18 µM of fluorescently labeled M13 tail, 5 mM $MgCl_2$ and 0.1 U Taq polymerase. PCR reaction conditions involved an initial denaturing step of 94 ˚C for 2 min, followed by 20 cycles of 94 ˚C for 10 s, 60 ˚C for 20 s, and 72 ˚C for 10 s. Subsequently, 15 more cycles were performed with the annealing temperature at 53 ˚C in order to assure better annealing of the fluorescently labeled tail. Finally, the reaction was incubated at 72 ˚C for 1 h. The length of the amplified microsatellite fragments was read using an ABI PRISM® 377 genetic analyzer.

Statistical analyses. Some of the leaf-cutter cultivars posessed more than two microsatellite alleles (Kweskin 2003), likely a consequence of some polyploidy or genomic duplication. Although the cohabitation of multiple fungal strains in the same garden is also a possible explanation, it is unlikely, since extracts were made of localized

garden fragments less than one mm in diameter and since ants generally remove alien cultivar strains (Bot *et al.* 2001; Poulsen *et al.* 2005. In any case, the microsatellites resulted in a potentially not fully co-dominant data set, since the actual number of the alleles contributing to any one marker-peak could not be exactly determined. Thus, analogous to AFLP analysis, we conservatively scored only for the presence or absence of bands of a particular length for each microsatellite primer pair, generating a total of 64 polymorphic dominant markers. The overall data set contained only 3.4% missing data, permitting pairwise distance measures between cultivar genotypes to be carried out in good faith. Linear binary distance computed from the resulting data matrix was analyzed using non-metric multi-dimensional scaling (NMDS). Additionally, population genetic structure within the cultivars was analyzed using Structure 2.1 (Falush 2003). The analysis was carried out under a no admixture ancestry model (appropriate for dominant data) and correlated allele frequencies, with a burn in of 100,000 generations and a sampling interval of 500,000 additional generations. All other parameters were left as defaults. The most likely number of populations was estimated by increasing the proposed number of populations until the model likelihood peaked. To ensure repeatability of the analysis, each trial was repeated three or more times. Finally, an AMOVA analysis examining genetic differentiation between genera, and between species within genera, was carried out in GenAlEx (Peakall & Smouse 2006) using 9999 replicates to generate the null distribution. The probability of encountering genotypes identical at all loci was calculated by bootstrapping the alleles in the original data set and comparing allele profiles of any two randomly chosen individuals within each bootstrap pseudo-replicate. This analysis was carried out in Matlab using 10^6 pseudo-replicates.

4.3 RESULTS

General patterns and NMDS analysis. Although leaf-cutter cultivars do exhibit relatively little diversity at highly-variable ITS loci even across great geographic distances (Silva-Pinhati *et al*., 2004), microsatellite markers showed considerable variability at the population level (Table 4.1). Assuming unlinked markers, the probability of any two genotypes being identical by chance across all 64 dominant loci was only 0.0017. Out of the 62 fungus gardens, 56 had distinct multi-locus genotypes. Only two genotypes were detected more than once. One of the shared genotypes occurred in two gardens, each associated with different *Acromyrmex* species. The other shared genotype was found in four gardens, twice with *Ac. echinatior*, once with *Ac. octospinosus* and once with *At. sexdens*. Two NMDS components were able to adequately describe the data (stress1 = 0.11), although little ant host-related structure could be observed (Figure 4.2).

Bayesian analysis of population differentiation. The most likely model in the Structure analysis sub-divided cultivars into six populations. The structure result generally mirrored findings from the NMDS analysis, showing much unstructured variance, with perhaps some slight effect of ant host (Figure 4.3). Some members of most Structure-defined populations were shared between the two genera; sharing of the others cannot be ruled out due to the relatively small number of gardens sampled per species.

Nested analysis of molecular genetic variance. An AMOVA allowed an explicit evaluation of the extent to which different levels of ant phylogeny (genera and species nested within them) contributed to the structuring of fungal molecular variance. In fact,

confirming conclusions of exploratory NMDS and Bayesian data analysis methods, fungal molecular variance was largely unstructured with respect to ant phylogeny – garden to garden variation within ant species accounted for 90% of the total genetic variance (φ_{pt} = 0.098, P < 0.001). Differences between species accounted for 8% (φ_{pr} = 0.077, P < 0.001). Genus-level differences accounted for only 2% of the total variance and had only marginal significance (φ_{rt} = 0.022, P = 0.053) (Table 4.2). Thus, deeper levels of ant phylogeny contribute little, if anything, to the structure of cultivar genetic variance.

4.4 DISCUSSION

Our results are consistent with those of earlier studies of leaf-cutting ant cultivars, which found low genetic variability and evidence of genetic exchange between different leaf-cutting ant genera across large geographic scales (Mikheyev *et al.* 2006; Silva-Pinhati *et al.* 2004). Our analyses also show that such garden exchange likely happens continuously at the population level, as has previously been shown for congeneric species of both lower attines and leaf-cutting ants (Bot *et al.* 2001; Green *et al.* 2002). In total, these studies suggested ubiquitous cultivar sharing throughout the attine ant-fungus symbiosis, although the limits to such exchanges between different host ant lineages remain to a large degree unknown. It does appear that higher attines, such as *Trachymyrmex*, cultivate lower attine cultivars in nature on rare occasion (Mueller *et al.* 1998), while, at least in the lab, cultivars may be reciprocally exchanged between leaf-cutters and *Trachymyrmex* (Stradling & Powell 1986; Sánchez-Peña 2005; Seal & Tschinkel 2007). The existence of widespread exchange parallels that in the fungus-

gardening termites, where horizontal transmission through fungal fruiting fruiting normally is the default (Aanen *et al.* 2002; de Fine Licht *et al.* 2006). Thus, it seems that, in both the ants and the termites, coevolution occurs between suites of symbiotic species, rather than between species pairs.

The presence of the same genotype across different species and genera of ants indicates that fungus gardens routinely undergo replacement with strains acquired horizontally, as clones, from other species. We observed two repeatedly sampled genotypes that were shared between two or three species, indicating at least three horizontal interspecific transfers of clonal garden material. Thus, at least 5% (3/62) of colonies appeared to cultivate garden clones whose genotypes have originated in other species. Relatively limited sampling at the species level, which reduced the likelihood that a shared cultivar strain would also be collected in another species, likely made us underestimate the horizontal exchange of cultivars. Whatever the actual number of genotypes shared among species, these results argue against complex pairwise genetic integration between cultivar strains and particular ant species, because an identical genotype of the cultivar can service both leaf-cutter genera.

Despite apparent co-transmission of mutualist genotypes during colony reproduction (Weber 1972) and exclusion of alien garden lineages (Bot *et al.* 2001; Poulsen *et al.* 2005, cultivar switching dominates the symbiosis. Thus, associations between ant genotypes, be it at the level of species or genus, do not persist through evolutionary time. Yet, some significant differentiation of the cultivar population nonetheless exists at the species level. Several mechanisms, both adaptive and non-adaptive, may create such

structure in the cultivar population. Adaptive forces may associate particular ant-cultivar lineage combinations, either through differential fitness between host-symbiont pairings or through symbiont choice, whereby ants select fungi offering higher fitness (Mueller *et al.* 2004). Non-adaptive explanations include shared genealogy, fine-scale habitat partitioning between ant species (making intraspecific encounters and garden exchanges less frequent), and likely many others. The phylogenetic nesting of the five leaf-cutter species within two genera permits some discrimination between these two classes of explanations. If co-adaptation between ants and fungi occurs, substantial amounts of cultivar genetic variance should be partitioned with respect to ant genus, given the different evolutionary histories and ecological niches of *Acromyrmex* and *Atta* (Fowler 1983; Hölldobler & Wilson 1990; Wetterer 1995). Our data do not support this prediction, exhibiting marginally non-significant amounts of inter-generic structure; far less than even intraspecific genetic structure. Alternatively, selection could act on fungi with respect to host habitat preference. *At. cephalotes* and *Ac. octospinosus* both prefer relatively undisturbed forest habitats, whereas *At. sexdens* and *Ac. echinatior* often occur at relatively more disturbed, drier sites and *At. columbica* being an intermediate between these two extremes. However, our data do not support that expectation either, since both *Acromyrmex*-associated fungi being more similar to those cultivated by *At. sexdens*, as evidenced by clustering in the NMDS analysis in Figure 4.2, the φ_{pt} values in Table 4.2 and by the observation that *Acromyrmex* and *At. sexdens* may share identical cultivar genotypes.

The diffuse nature of the ant-fungus interaction may require adjustments in the conceptual model of coevolution used to explain many features of the attine symbiosis,

which is based on pairwise species interactions (Frank 1994). Our present results show that while avoidance of host exchange plays an important role in the mutualism (Bot *et al.* 2001; Mueller 2002; Poulsen *et al.* 2005), it does not ensure interaction specificity in the longer term. At that level, leaf-cutting ant-fungus interactions are characterized by (a) a widespread cultivar undergoing frequent exchange by a group of phylogenetically diverse ant hosts, and (b) independent dispersal by the cultivar, possibly much farther than its ant hosts (Mikheyev *et al.* 2006).

Prospects for co-adaptation. Frequent host switching may diminish the extent to which fungi are able to adapt to their ant hosts. The fate of a mutation arising in the fungus garden, and changing the fitness consequences of the cultivar's interaction with its current ant host, is less predictable than in a pairwise species interaction model. When a fungus becomes transferred to a different host, the fitness value of such a mutation depends both on the frequencies of other ant species to which a fungus may be transferred and the way this mutation affects pairwise interactions with these other hosts. For example, a mutation enhancing fungal fitness in one ant species may be detrimental in others, thus making its spread through the general leafcutter community unlikely. Furthermore, even if a mutation affects one ant host, with zero effect on the others, the fitness consequences of any mutation affecting a particular ant host, even while having no effect on the others, may still become attenuated due to the selection-drift balance if host shifts occur commonly enough.

Alternatively, reshuffling of the cultivar genome through recombination (Mikheyev *et al.* 2006) may create favorable gene-host combinations. In this case, a single gene mutation

in a fungus garden, although still vulnerable to selection-drift balance, may be maintained indefinitely in association with a particular ant host, though the non-specific remainder of the fungal genome continues to be freely exchanged among ant species. This scenario is likewise consistent with our data, based on presumably neutral microsatellite loci, which would not be expected to show any species-specific associations. However, this scenario appears unlikely, since genetically identical clones were observed shared between different species and genera of ants.

Although coevolution characterizes the attine ant-fungus symbiosis at the deepest phylogenetic levels (Chapela *et al.* 1994; Hinkle *et al.* 1994), its origins and maintenance appear mysterious in the face of frequent host switches observed at the global and population levels. However, in many other non-obligate mutualists, regionally co-adapted pairings may still exist if selection acts across a geographic mosaic of mutualist populations (Bell *et al.* 2005). Thus, a closer look at differences across populations of the same species may provide a key to elucidating the forces binding ant and cultivar lineages. Such investigations will be made easier by the realization that relatively frequent fungal cultivar switching occurs across genera of leaf-cutting ants, creating intriguing new possibilities for exploring such co-evolutionary trajectories.

Ant host	Locus name								
	A0659B	A0815	B0150	B0312	B0358	B0430	B0447	C0625	D0115
Atta cephalotes	4	4	8	2	6	7	2	5	4
Atta columbica	5	2	5	8	5	6	2	5	2
Atta sexdens	3	2	3	5	3	3	2	4	1
Acromyrmex echinatior	3	3	4	8	4	6	2	6	2
Acromyrmex octospinosus	3	3	4	8	4	4	1	5	2

Table 4.1. Number of alleles in each of the 9 microsatellite loci used in the study.

Atta			*Acromyrmex*			
cephalotes	***columbica***	***sexdens***	***echinatior***	***octospinosus***		
	0.158	*0.003*	*0.022*	*0.001*	***cephalotes***	***Atta***
0.022		*<0.001*	*0.006*	*<0.001*	***columbica***	
0.187	0.218		0.178	0.333	***sexdens***	
0.074	0.074	0.049		0.133	***echinatior***	***Acromyrmex***
0.108	0.161	0.013	0.029		***octospinosus***	

Table 4.2. Pairwise φ_{pt} distances between cultivar populations (lower triangular matrix) and their uncorrected significance values (upper triangular matrix). Statistically significant φ_{pt} distances are indicated in italics.

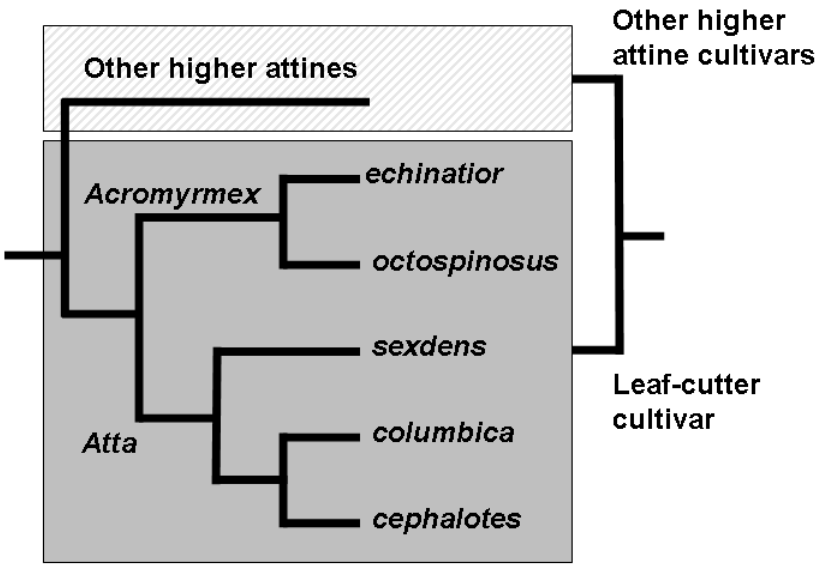

Figure 4.1. Phylogenetic relationship between leaf-cutting ants in this study and their cultivars (Borgmeier 1959; Schultz & Meier 1995; Chapela 1994; Schultz & Brady 2008). Two genera of leaf-cutting ants diffusely co-evolve with a widespread cultivar fungus (dark grey box). Most leaf-cutting ants cultivate one cultivar species, distinct from others associated with sister higher attine genera *Trachymyrmex* and *Sericomyrmex* (slashed light grey box). However, the association may be even more diffuse than presented here, since leaf-cutters can cultivate *Trachymyrmex* fungus and vice versa in the lab (Stradling & Powell 1986; Sánchez-Peña 2005; Seal & Tschinkel 2007)

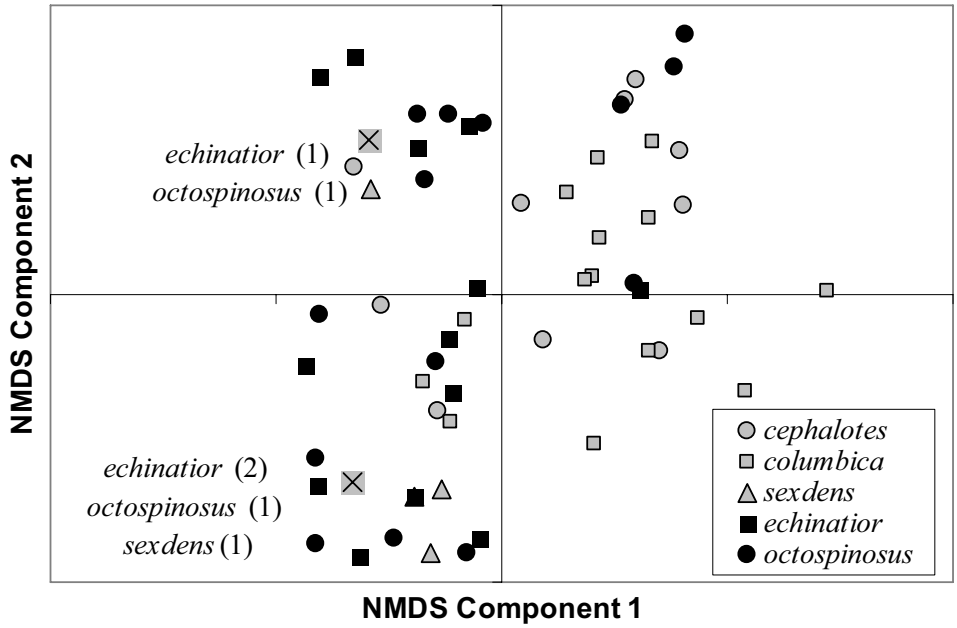

Figure 4.2. Plot of the first two components of the non-metric multidimensional scaling analysis of leaf-cutting ant cultivar genotypes. Cultivars associated with *Atta* and *Acromyrmex* genera are represented by grey and black symbols, respectively. Cultivar genotypes detected across species are indicated by crossed squares and annotations describing host ant species, as well as the numbers of gardens where the cultivar was collected in each species.

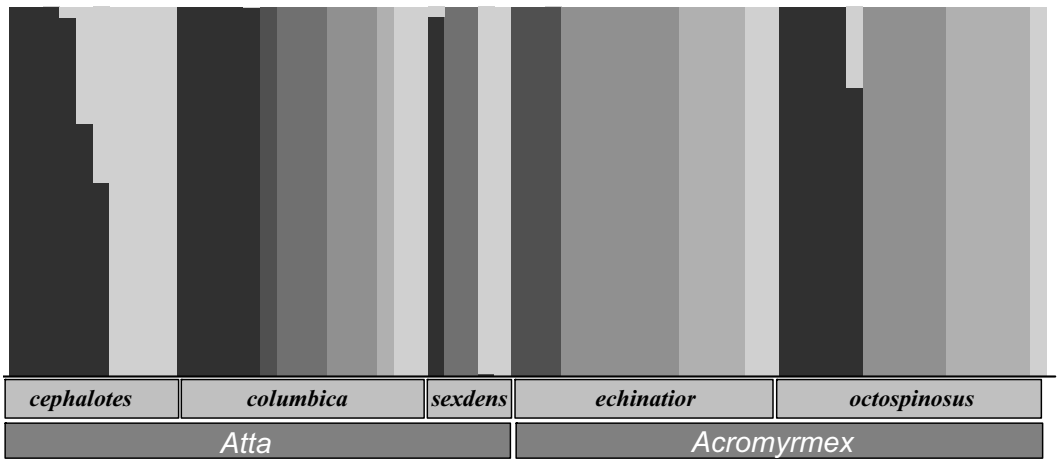

Figure 4.3. Genetic structure of cultivar populations with respect to ant host. Six estimated cultivar populations, represented by six shades of grey on a white background, are distributed among five leaf-cutter species

CHAPTER 5

Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists

Abstract: Although historical biogeographic forces, such as climate-driven range shifts, greatly influence the present day population genetic structure of animals and plants, the extent to which they affect microbial communities remains largely unknown. We examined the effect of post-glacial expansion on the population structure of the northern fungus-gardening ant *Trachymyrmex septentrionalis* and compared it with that of its two microbial mutualists: a community of lepiotaceous fungal cultivars and associated antibiotic-producing *Pseudonocardia* bacteria. The ant population genetic structure showed signs of population expansion and subdivision into eastern and western phylogroups that likely originated in the Pleistocene – a pattern shared by many other North American taxa found in the same region. Although dispersal limitation was present in all three symbionts, as suggested by genetic isolation increasing with distance, the host's east-west subdivision of population genetic structure was absent from the microbial mutualist populations. While neither the cultivar nor the *Pseudonocardia* genetic structure was correlated with that of the ants, they were significantly correlated with each other. These results show that biogeographic forces may act differently on macro- and microscopic organisms, even in the extreme case where microbial mutualists are vertically transmitted from generation to generation and share the same joint

ecological niche. It may be that historical climate change played a larger role in determining the population structure of the ant hosts, whereas present-day environmental forces, such as pathogen pressure, determine the structure of associated microbial populations.

5.1 INTRODUCTION

The study of animal and plant distribution patterns has occupied center stage in biology for centuries. Biogeographic regularities, which were extensively catalogued by naturalists, eventually formed the framework for major conceptual advances, such as the theory of evolution by natural selection, which was intuited by Darwin and Wallace from the patterns they observed in insular faunas (Darwin 1859; Wallace 1876). However, biogeographic observations have been limited to the realm of macroscopic organisms until relatively recently. In contrast to the extensive literature on animal and plant biogeography, much less is known about the nature of ecological and evolutionary forces shaping the geographic structure of microbial populations. The large effective populations size of many microbes together with the ability of some to form environmentally resistant spores, has lead to the commonly cited and controversial belief that "everything is everywhere, but the environment selects" (Baas Becking 1934).

Recent evidence suggests geographic isolation does indeed structure microbial communities (Papke & Ward 2004; Whitaker *et al.* 2003). At the same time, some of the prevalent phylogeographic patterns, such as latitudinal species gradients, appear absent in microbial communities, although they are common in many macroscopic taxa (Fierer & Jackson 2006). On the other hand, bacteria do appear to exhibit species-area

correlations similar to those of macroscopic animals (Bell *et al.* 2005; Green & Ostling 2003; Horner-Devine *et al.* 2004). Nonetheless, based on our limited understanding of processes driving bacterial community assembly, dynamics generating species-area curves of bacterial communities most likely differ from those operating on insular faunas, where stochastic events may play a larger role (Fenchel & Finlay 2005; Woodcock *et al.* 2006). Consequently, the extent to which the well-studied axioms of macroscopic animal phylogeography apply at the 'micro' scale remains unclear.

Extrapolation of well-known biogeographic processes to the microscopic scale is difficult because microbes have ecologies vastly different from those of macroscopic taxa. Extreme variations in bacterial abundance and community composition between microhabitats within any given locale further complicates comparisons between sites. However, the study of obligate mutualistic relationships between macroscopic animals and microbes, which occupy a joint ecological niche, allows us to circumvent the above problems, since the patterns of host distribution should influence microbial presence and community structure (*e.g.*, Fisher *et al.* (2001) and Linz *et al.* (2007)). Furthermore, the microbial mutualists should respond similarly to ecological forces important in phylogeographic structuring of host populations, such as range shifts and allopatric isolation, particularly in the case of vertically transmitted mutualists. Thus, the phylogeographic structure of the macroscopic host can serve as a hypothesis for the distribution of their microbial mutualists.

We used the well-studied fungus-gardening ant symbiosis (a 50 million year old obligate nutritional mutualism between ants and fungi) to explore phylogeographic concordance

between the macroscopic ant host and its microbial symbionts. The attine ants and their cultivar fungi show phylogenetic congruence at the basal levels, but cultivar sharing dominates at the population level (Chapela *et al.* 1994; Green *et al.* (2002); Hinkle *et al.* 1994; Mikheyev *et al.* 2007; Mueller *et al.* 2001). The most phylogenetically derived attine clades cultivate specialized fungi that have been found only in their nests (Mueller *et al.* 1998; Vo *et al.* in press). *Pseudonocardia* bacteria, which are housed in co-evolved crypts in the ants' cuticle, are thought to protect the garden from the specialized pathogen *Escovopsis* (Currie *et al.* 1999a; Currie *et al.* 2003a; Currie *et al.* 2003b ;Currie *et al.* 2006; Gerardo *et al.* 2006). Ant queens take both the *Pseudonocardia* and the fungal cultivar from their native gardens, assuring at least some vertical co-transmission (Currie *et al.* 1999a; Weber 1972). Despite this link, the association between the ants and their mutualists is inherently labile, and both inter- and intraspecific host switching has been documented for both cultivars and *Pseudonocardia* (Cafaro & Currie 2005; Green *et al.* 2002; Mikheyev *et al.* 2006; Mikheyev *et al.* 2007; Poulsen *et al.* 2005.

The northern fungus-gardening ant *Trachymyrmex septentrionalis*, which ranges through the Eastern United States, provides a particularly interesting case study. First, *T. septentrionalis* is largely allopatric with congenerics, except at the extreme edges of its range: in central Texas, where it co-occurs with *T. turrifex*, and in the south of Florida, where it may overlap with the extremely rare *T. jamaicensis* (Deyrup *et al.* 1988; Rabeling *et al.* 2007; Wheeler 1907). Although distributions of other fungus-gardener ants in the genera *Atta*, *Cyphomyrmex* and *Mycetosoritis* partially overlap with that of *T. septentrionalis* (Wheeler 1907), they all cultivate genetically distinct fungi (Chapela *et al.* 1994; Mueller *et al.* 1998). Thus, there should be no host-cultivar switching between

T. septentrionalis and other sympatric attines through most of its range. Second, *T. septentrionalis* has undergone a relatively recent population expansion after the retreat of Pleistocene glaciers that covered the north of its present range (Marshall *et al.* 2002). In general, we would expect its population structure to be similar to those of other temperate North American species, which often show signs of population differentiation in glacial refugia, followed by rapid population expansion during the inter-glacial periods (Pielou 1991). Thus, we predict that (a) *T. septentrionalis* populations would show signs of glacial retreat and post-glacial expansion and (b) if phylogeographic processes (*i.e.*, post-glacial range extension) act in the same manner on the microbes and on the ant hosts, the microbial mutualists should have congruent population structure.

5.2 MATERIALS AND METHODS

Sites and collection. Ant nests were collected in 2001-06 at the 16 sites listed in 5.1. Ants and fungi were preserved in 95% ethanol and stored at -80°C until DNA extraction. Only a single worker ant was analyzed per colony.

DNA extraction, amplification and sequencing. Protocols for PCR purification and sequencing conditions, as well as the procedure for fungal DNA extraction using the Chelex method can be found in Mikheyev *et al.* (2007). Ant and bacterial DNA was extracted simultaneously using DNeasy tissue kits according to the manufacturer's instructions (Qiagen). The elongation factor 1-α (*EF1α*) of the cultivar was amplified using primers by Mikheyev *et al.* (2006). The phylogenetic clusters identified using *EF1α* were confirmed by sequencing the ribosomal internal transcribed spacers 1 and 2 (*ITS*)

for a random subset of the cultivars in each of these groups using primers and reaction conditions by (White *et al.* 1990). The cytochrome oxidase 1 (*COI*) mitochondrial gene of the ants was amplified using the primer pair Ben and Jerry (Simon *et al.* 1994; Villesen *et al.* 2004). In addition, we amplified the *ITS* region of ant rDNA, using primers and conditions developed by Ji *et al.* (2003). The *Pseudonocardia* Elongation Factor-Tu (*EF-tu*) gene was amplified using a nested PCR procedure. The first reaction used the specific primer pair 1F and 920R from Poulsen *et al.* (2005). The second amplification, intended to improve sensitivity and specificity, was carried out using a 1:10 dilution of the first reaction as a template, and used a custom primer pair (ActinoNF 5'-GAC AAG GCG CCG GAA GAG-3' and ActinoNR 5'-CGT CCT CAC GCT GAT AAC-3'). One µl of the extract (or in the case of nested PCR, the diluted product from the previous reaction) was used as template in 10 µl reaction volumes. The PCR contained 1 x reaction buffer, 1 mM dNTPs, 0.5 µM primers, 5 mM $MgCl_2$ with 0.1 U of Bioline Taq polymerase. Reaction conditions involved an initial denaturing step of 94°C for 2 min, followed by 35 cycles of 94°C for 10 sec, 60°C for 20 sec, and 72°C for 30 sec. PCR purification and sequencing protocols were as published earlier by Mikheyev *et al.* (2006). We deposited our sequences in Genbank under accession numbers EU561361-EU561600.

In the ants, but not in the fungi, *ITS* often contained varying numbers of one and two-nucleotide repeats, making direct sequencing difficult and producing no readable sequence in some individuals at all. Although *ITS* and *COI* partitioned the ant genetic variance into the same phylogroups (see below), *ITS* data contained numerous nucleotide scoring ambiguities and was not used in the quantitative analysis.

Phylogeography of *T. septentrionalis*. A post-Pleistocene population expansion should result in a unimodal distribution of haplotype mismatches (Harpending *et al.* 1998). We calculated the haplotype mismatch distributions for a spatial expansion model implemented in Arlequin (v3.1) (Excoffier *et al.* 2005) and used Harpending's (1994) and SSD raggedness index to determine the goodness of fit of observed mismatch distributions to those predicted by the demographic expansion model.

Reconstruction of the cultivar and *Pseudonocardia* phylogenies. For phylogenetic analysis of the cultivar, DNA substitution models for each gene were estimated using Modeltest (Posada & Crandall 1998). Phylogenies for cultivars and *Pseudonocardia* were constructed in a manner similar to the those of the ants, using GTR+G for both fungal genes and F81+I+G model for the bacterial gene. Phylogenies were computed using MrBayes (v. 3.1) (Ronquist & Huelsenbeck 2003). In the case of the cultivars, the analysis was run with separate partitions for the *EF1α* and *ITS* genes, each partition with its own independently estimated set of parameters. The calculation was continued until the average standard deviation of split frequencies between the two runs dropped below 0.01. Afterwards, the first 75% of the generations were discarded as burn-in and a majority-rule consensus tree was computed to estimate posterior probabilities for each node. The MCMC simulation was performed several time to assure convergence to the same solution space. To confirm that genetic exchange through recombination does not occur between cultivar types, we conducted a partition homogeneity test on the same samples used to create the phylogeny, but with identical sequences removed, using PAUP's branch-and-bound search algorithm and 1000 pseudoreplicates (keeping 1 tree per pseudoreplicate) (Farris *et al.* 1995; Swofford 1993).

Comparative phylogeography. To separate long-term trends from short-term associations caused by kinship, comparisons between ant and fungal phylogeographic structure were carried out at the population level. Fst distances between *T. septentrionalis* populations were estimated in Arlequin using the Tajima-Nei nucleotide substitution model with a 0.009 Gamma rate heterogeneity value (Excoffier *et al.* 2005). The nucleotide substitution model and Gamma parameter were selected using a Modeltest block (Posada & Crandall 1998), which was reduced to include only models supported by Arlequin, which does not implement many of the models evaluated by Modeltest. Fungal community distances were measured using Jaccard's and Morisita's indexes. Both measures of community composition were highly correlated and gave qualitatively identical answers; only the results of computations using Jaccard's index are presented. Cultivar diversity was measured using Shannon's index. The much lower numbers of *Pseudonocardia* symbionts amplified prevented the computation of reliable population-level statistics. Instead, Mantel tests were performed directly on genetic distance matrixes. Because it is not clear how genetic distance between cultivar types should affect *Pseudonocardia* population structure, cultivars types were treated as discrete entities. Consequently, the cultivar distance matrix was coded as ones and zeros (membership in the same cultivar type represented by ones, zero otherwise). Genetic distance matrixes were computed in PAUP (v. 4.0 beta 10) (Swofford 1993) using nucleotide substitution models selected by Modeltest separately for each data set. Mantel tests of similarity matrixes were carried out using 10^6 permutations of the raw values in zt (Bonnet & Van de Peer 2002). In addition to Mantel tests, we tested the extent to which *Pseudonocardia* genetic variation was partitioned among cultivar types and ant

phylogroups using an AMOVA, carried out in Arlequin.

5.3 RESULTS

***T. septentrionalis* phylogeography.** Previously, Wheeler (1911) recognized two subspecies of *T. septentrionalis* based on morphological and demographic differences occurring between northern and southern populations, although a recent comprehensive revision of the U.S. *Trachymyrmex* by Rabeling *et al.* (2007), based on both morphological and molecular characters, did not support this distinction. Thus, geographic morphological variation noticed by Wheeler probably results from adaptive climate-driven intraspecific differences, such as those noted by Beshers (1994), and we treated *T. septentrionalis* as a single species. However, ant populations clustered into two phylogroups, approximately divided by the Mississippi river valley and having only a single mitochondrial haplotype in common (a typically western type found in one individual from North Florida) (Table 5.1, Figure 5.1). In both phylogroups, haplotype mismatch frequencies were consistent with a sudden population expansion (SSD and raggedness indexes $p > 0.20$ in both phylogroups).

Ant-cultivar interactions. *T. septentrionalis* cultivates several genetically distinct types of fungi (Figure 5.2). A partition homogeneity test using 181 parsimony-informative characters did not detect recombination of *ITS* and *EF1α* between cultivar types ($p = 0.35$), indicating that there is no gene flow between these types and suggesting that they are either genetically distinct clones or species. The relative frequencies of these types was unequal, approximately 39, 49 , 10 and 1 percent, for types A, B, C and D,

respectively (χ^2 = 44.5, d.f. = 3, p = $1.2*10^{-9}$). Based on our samples, the genetic diversity of the *Trachymyrmex* cultivars appears much greater than that of the leaf-cutting ant cultivars, and is comparable to the diversity present in the entire lower fungus-gardening ant symbiosis. Genetically divergent fungi often occur in each other's immediate vicinity, sometimes less than a meter apart.

The diversity of *T. septentrionalis*-associated cultivars markedly decreased northward and eastward (r = -0.78, p = 0.001 and r = -0.63, p = 0.011, respectively) from a center of diversity around Texas (Figure 5.2). As a result of this spatial structuring, the fungal community composition exhibited significant isolation by distance (r_{xy} = 0.23, p = 0.034). Likewise, the genetic relatedness between *T. septentrionalis* populations was geographically structured, largely due to the existence of two large phylogroups (r_{xy} = 0.38, p = 0.003; 5.2). However, there was no correlation between the ants' genetic and the fungal community similarity matrixes, even after the effects of geographic distance were factored out (r_{xyz} = 0.0043, p = 0.49).

Ant-cultivar-*Pseudonocardia* relationships. Despite low genetic variability (5.3), like the other two symbionts, *Pseudonocardia* showed slight, though significant, isolation by distance (N = 45, r_{xy} = 0.10, p = 0.041). There was no evidence for association between ant and *Pseudonocardia* lineages, as evidenced by the lack of a correlation of their genetic distance matrixes (N = 45, r_{xyz} = -0.08, p = 0.95). Likewise, the effect of ant phylogroup on *Pseudonocardia* genetic structure was not significant (p = 0.34) and explained only 0.5% of the molecular variance in *Pseudonocardia*

population structure. By contrast, there was a significant positive association between *Pseudonocardia* genotypes and fungal cultivar type (N = 43, r_{xyz} = 0.092, p = 0.029; cultivar type explained 19% of the molecular variance in *Pseudonocardia* population structure (p=0.003)).

5.4 DISCUSSION

The eastern and western phylogroups of *T. septentrionalis* were genetically differentiated at both nuclear and mitochondrial loci. Although the eastern and western populations share few mitochondrial haplotypes, suggesting a long-term differentiation, they are not separated by present-day geographical barriers. Given that *T. septentrionalis* species itself originated recently, approximately within the past million years (Schultz, Brady, 2008) it seems overwhelmingly likely that the two phylogroups arose at some point in the Pleistocene. The pattern of *T. septentrionalis* population subdivision parallels that of 'highland' fishes, amphibians, reptiles and mammals in central North America (Blair, 1958; Blair, 1965; Brant, Orti, 2003; Burbrink *et al.*, 2000; Mayden, 1988; Near *et al.*, 2001; Robinson, 1986; Walker *et al.*, 1998; Wiley, Mayden, 1985) (Figure 5.2). The congruence of phylogeographic patterns across such different taxa suggests shared historical biogeographic influences that are most commonly interpreted as genetic diversification in allopatric Pleistocene refugia, followed by expansion and secondary contact between diverged populations (Avise 2000).

The phylogeographic structure evident in *T. septentrionalis* was completely absent from the population genetic structure of the associated microbes. Since there was evidence of

population viscosity in both the fungal cultivars and the *Pseudonocardia*, the lack of congruence was not due to limitless dispersal by the microbes. However, previous work on leaf-cutting ant cultivars has shown that they are better dispersers than the ants, probably because of their ability to form easily dispersed spores (Mikheyev *et al.* 2006). A similar occasional de-coupling from vertical transmission appears to exist in the *T. septentrionalis* symbiosis. Thus, it is possible that the fungal communities were not fully isolated and had appreciable migration between Pleistocene refugia, while the relatively more viscous ant populations underwent genetic differentiation. Much less is known about the ecology of *Pseudonocardia*, but it seems likely that they are either capable of existence outside the symbiosis, or are better dispersers than the ants, which disperse much less than 500 meters/year during an annual mating flight, based on flight distances of the much larger and more powerful *Acromyrmex octospinosus* (Mikheyev, 2007).

The long-term existence of multiple species or clones occupying the same niche, in this case supposedly obligate fungal lineages associated with a single ant host, presents a puzzle. Extensive surveys of free-living lepiotaceous cultivars have thus far failed to find any free-living relative (Mueller *et al.* 1998; Vellinga 2004; Vo *et al.*, in prep). If a free-living existence is impossible for the cultivars, in the absence of other forces stochastic extinction or competition, this should lead to the fixation of a single cultivar species in the *T. septentrionalis* symbiosis. Conceivably, coexistence may be driven by migration of cultivars a the large diverse population farther south in Latin America, where they form complex associations with several ant species. This hypothesis is difficult to test, although it is supported by the observation that cultivar diversity decreases northward,

away from the presumed center of diversity (Figure 5.1). Alternatively, diversity may be maintained by frequency-dependent selection. For example, low-frequency cultivars may be less likely to encounter specialized pathogens, such as locally adapted strains of *Escovopsis* (Currie *et al.* 1999a).

Although showing no correlation with ant population structure, *Pseudonocardia* populations were structured with respect to the cultivar types. These results are consistent with our knowledge of *Pseudonocardia* biology, since they play a role in controlling the garden pathogen *Escovopsis* (Currie *et al.* 1999a) that itself tracks cultivar genotypes (Currie *et al.* 2003b; Gerardo *et al.* 2006. Like cultivars, they are exchanged between sympatric species of ants (Poulsen *et al.* 2005). While ant-*Pseudonocardia* genotype pairings may be vertically propagated by dispersing ant queens (Currie *et al.* 1999b; Weber 1972), these associations appear ephemeral. The existence of cultivar-specific population differentiation in the *Pseudonocardia* predicts that (a) pathogen strains differ between cultivar species and (b) different *Pseudonocardia* strains differ in their efficacy against pathogens. Thus, it appears likely that cultivar diversity is maintained by frequency-dependent selection as a result of *Escovopsis* (or some other) disease pressure.

Although attine cultivars were initially believed to be ancient asexual clones (Chapela *et al.* 1994, more recent work has documented the existence of a connection to free-living cultivars in lower attines, and the presence of recombination in leaf-cutting ants (Mueller *et al.* 1998; Mikheyev *et al.* 2006; Vo *et al.*, in press). Although no data direct on recombination exists on *Trachymyrmex*-associated fungi, basidiocarp production

was observed in lab colonies (Mueller 2002) and there is evidence that purifying selection acts on meiosis-specific genes (Mikheyev *et al.* 2006). So, it seems reasonable to believe that the genetically distinct types found associated with *T. septentrionalis* represent different reproductively isolated species, although additional sampling may reveal evidence of genetic exchange.

Recognizing the possible existence of several fungal species allows us to make direct comparisons with other well-studied attine systems. For example, earlier it has been suggested that apparent intraspecific cultivar diversity may actually be a case of cryptic ant speciation, with each species specializing on its own cultivar (Mueller *et al.* 1998). Our data show that this is not a general rule and that multiple cultivars can be involved with the same ant species (although see Schultz *et al.* (2002) for a possible exception). The many-to-one association of the *T. septentrionalis*-fungus symbiosis contrasts with that of the leaf-cutting ants, which cultivate largely one fungal species (Mikheyev *et al.* 2006). If the cultivars have no free-living state, there must be intense competition between the *T. septentrionalis* cultivar types for their ant hosts. Given that *T. septentrionalis* fitness remains the same even when experimentally raised on distantly related (Figure 5.1) leaf-cutter cultivar (Seal & Tschinkel 2007), it would seem unlikely that the different *Trachymyrmex* cultivars would have substantially different effects on ant fitness. Thus, in the presence of cultivar exchange, a single 'super cultivar' may potentially replace all others if it can markedly increase ant fitness or be preferred by the ants through symbionts choice (Mueller 2002). This may have been the case at some point in leaf-cutting ant evolution, because their sole cultivar has low genetic variation, consistent with a sudden expansion of a single species (Silva-Pinhati *et al.* 2004).

Conclusion. The discordance between the population genetic structure of the ant host and its microbial mutualists illustrates the different ecological forces acting on macro- and microscopic organisms. The Pleistocene climatic changes, which have shaped present-day population genetic structure of the ants, have had little effect on the current structure of their microbial mutualists. This could be due either to the ability of microbes to travel between Pleistocene refugia, which prevented differentiation, or to the more rapid equilibration of their population genetic structure following the retreat of the glaciers. In either case, although microbial populations are not free of geographic structure, the connectivity between their populations is far greater than that that of the ants, as has been noted earlier for leaf-cutting ant fungal cultivars (Mikheyev *et al.* 2006). Our data illustrate how fundamental differences between macro- and microscopic organisms, such as differences in dispersal rates, result in markedly different phylogeographic patterns. Historical, rather than present-day coevolutionary forces, appear to have a greater effect on macroscopic organisms than even on their immediate microbial associates. Thus, perhaps in contrast to macroscopic taxa, knowledge of microbial biogeography may benefit more from the study ecological interactions than from accumulation of geographic patterns.

5.5 TABLES AND FIGURES

State	Locality Name	Ants	Fungi	*Pseudonocardia*	Lat	Long
AL	Prattville	5	5	3	32.44	86.47
AR	Devil's Den	5	5	5	35.79	94.25
FL	Tallahassee	5	5	3	30.44	84.48
GA	Waycross	5	4	3	31.16	82.20
IL	Sand Ridge	5	5	0	40.43	89.91
IL	Dixon Springs	5	5	3	37.38	88.66
LA	Reeves	5	4	3	30.52	93.05
MS	Starkville	5	5	1	33.51	88.74
NC	Hoffman	5	5	4	35.02	79.62
NC	Buxton	4	4	2	35.24	75.53
NJ	Lebanon	5	2	3	39.88	74.56
NY	Centereach*	5	10	3	40.86	73.08
TX	Angelina	8	8	7	30.87	94.18
TX	Smithville	5	5	3	30.08	97.17
VA	Pamplin City	2	2	2	37.29	78.69
	Total:	**74**	**74**	**45**		

5.1. Collection localities and the number of successfully PCR-amplified specimens. Only one ant was sampled per colony. Samples from the Western phylogroup of *T. septentrionalis* are in highlighted in grey; all other samples are from the Eastern phylogroup.

* Population was sampled twice, in 2001 and in 2006, 5 colonies each time, to confirm the stability of cultivar community composition through time.

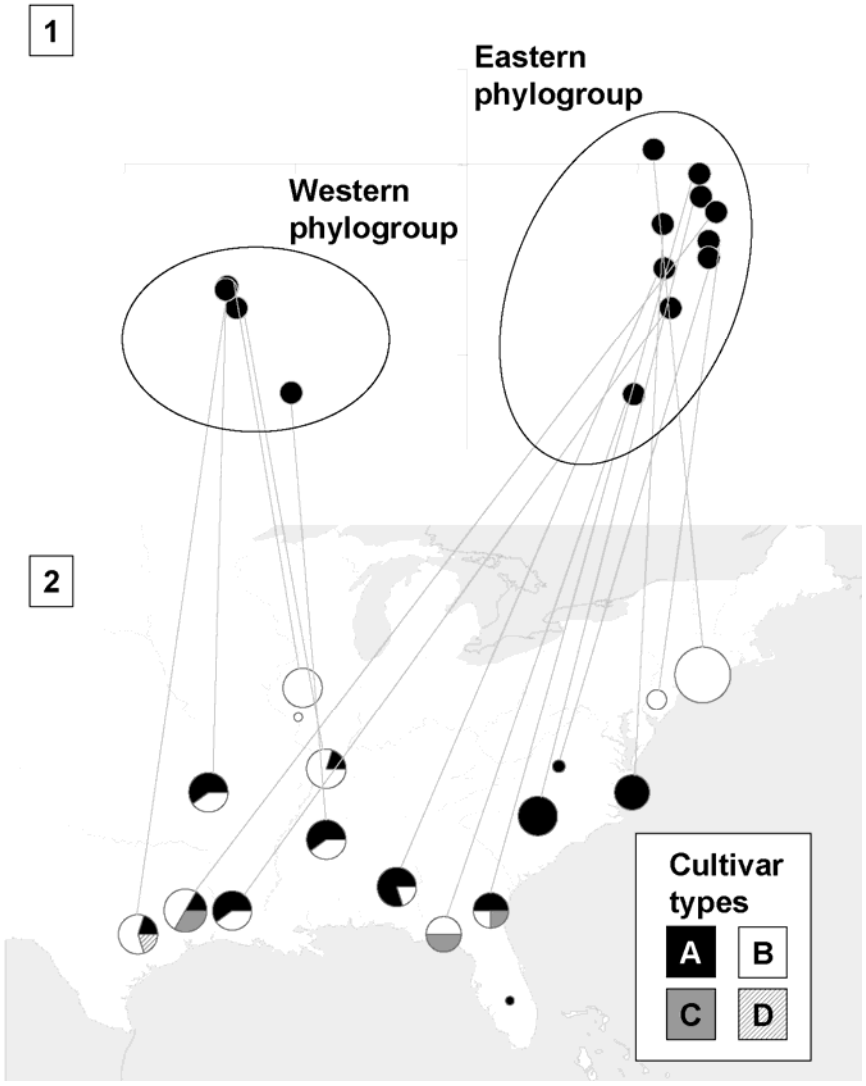

Figure 5.1. Phylogeographic structure of ants and their cultivars. (1) Non-metric multidimensional scaling analysis (NMDS) of the ant populations. The first and second components are plotted on the x- and y-axis, respectively. (2) Cultivar community structure. At every sampling site cultivar community structure is represented by a pie chart depicting relative proportions of the four cultivar types (area proportional to sample size, ranging from 1 to 10). Lines connect cultivar communities structure to the NMDS plot of ant genetic structure for all samples with more than 5 collections.

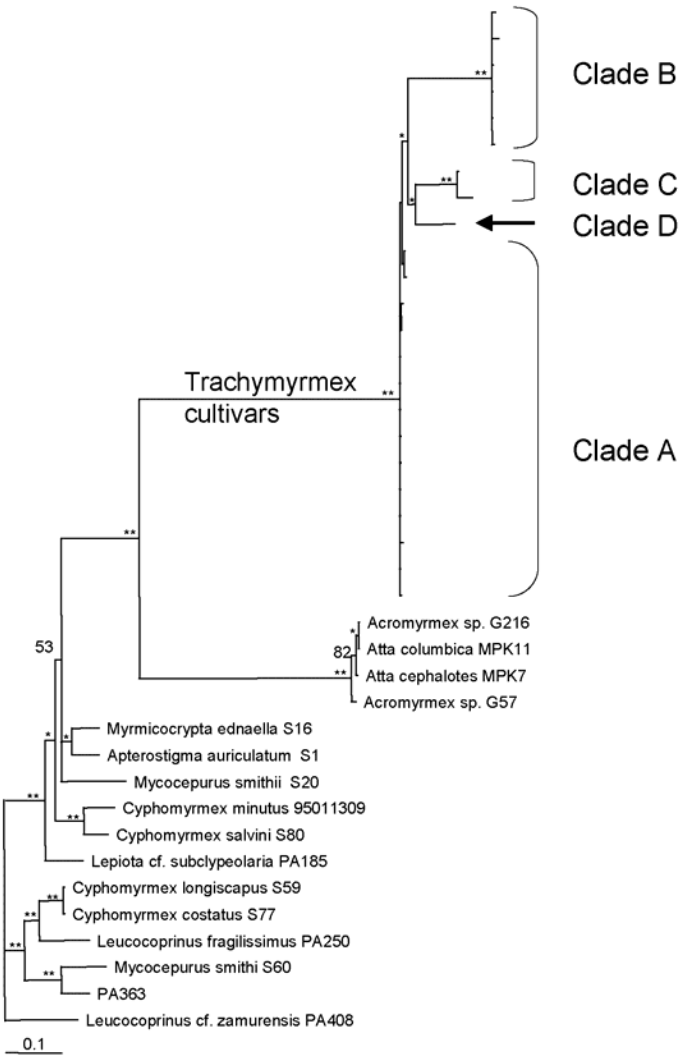

Figure 5.2. Bayesian phylogeny of the cultivar fungi based on a combined analysis of *ITS* and *EF1α*. Outgroups are labeled according host ant species and collection code specified in the Genbank title field (codes of free-living relatives begin with the letters "PA"). Nodes labeled with * and ** have >90 and 100 percent posterior probability respectively. The tree includes 9, 18, 2 and 1 representatives of types A, B, C and D, respectively. The scale bar corresponds to 0.1 substitutions per site.

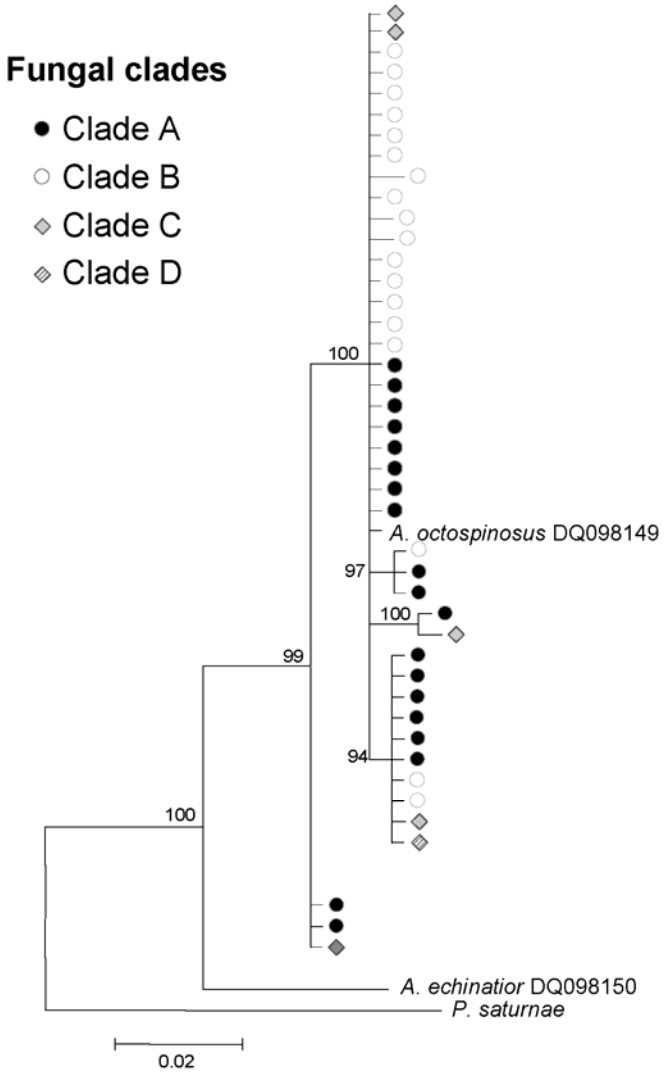

Figure 5.3. Phylogeny of *Pseudonocardia* symbionts with outgroups are from Poulsen *et al.* (2005). *Pseudonocardia* population structure was uncorrelated with that of the ants. Although there was no clear phylogenetic mapping of *Psedonocardia* phylogeny on cultivar type, there was nonetheless a slight, significant association between the two, suggesting differential association due to environmental factors, rather than by vertical co-transmission with the ants. The scale bar corresponds to 0.02 substitutions per site.

Bibliography

Aanen DK, Eggleton P, Rouland-Lefevre C*, et al.* (2002) The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14887-14892.

Abomo-Ndongo S, Tourvieille J, Guillaumin JJ (2002) The Buller phenomenon in *Armillaria heimii* Pegler, a bipolar diploid basidiomycete. *Cryptogamie Mycologie* **23**, 335-347.

Adams RMM, Mueller UG, Holloway AK, Green AM, Narozniak J (2000) Garden sharing and garden stealing in fungus-growing ants. *Naturwissenschaften* **87**, 491-493.

Anderson JB, Kohn LM (1998) Genotyping gene genealogies and genomics bring fungal population genetics above ground. *Trends in Ecology & Evolution* **13**, 444-449.

Avise JC (2000) *Phylogeography*. Harvard University Press, Cambridge, MA.

Baas Becking LGM (1934) *Geobiologie; of inleiding tot de milieukunde*. W.P. Van Stockum & Zoon, The Hague.

Bascompte J, Jordano P (2007) Plant-animal mutualistic networks: The architecture of biodiversity. *Annual Review of Ecology Evolution and Systematics* **38**, 567-593.

Bascompte J, Jordano P, Olesen JM (2006) Asymmetric coevolutionary networks facilitate biodiversity maintenance. *Science* **312**, 431-433.

Becerra JX (2003) Synchronous coadaptation in an ancient case of herbivory. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12804-12807.

Bell T, Ager D, Song JI*, et al.* (2005) Larger islands house more bacterial taxa. *Science* **308**, 1884-1884.

Beshers SN, Traniello JFA (1994) The adaptiveness of worker demography in the attine ant *Trachymyrmex septentrionalis*. *Ecology* **75**, 763-775.

Bishop DK, Park D, Xu LZ, Kleckner N (1992) DMC1 - a meiosis-specific yeast homolog of *Escherichia coli* RecA required for recombination, synaptonemal complex-formation, and cell-cycle progression. *Cell* **69**, 439-456.

Blair WF (1958) Distributional patterns of vertebrates in the southern United States in relation to past environments. In: *Zoogeography* (ed. Hubbs CL), pp. 433–468. American Association for the Advancement of Science, Washington, DC.

Blair WF (1965) Amphibian speciation. In: *The Quaternary of the United States: a Review of Volume for the VII Congress of the International Association for Quaternary Research* (eds. Wright HEJ, Frey DG), pp. 543–556. Princeton University Press, Princeton, NJ.

Blanche D (1954) Découverte d'un foyer de fourmis champignonnistes a la Guadeloupe. *Bulletin de la Société Française d'Histoire Naturelle des Antilles* **4**.

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

Borgmeier T (1922) Combates entre sauvas. *Chacares e Quintais, Sao Paulo* **26**, 51.

Borgmeier T (1959) Revision der Gattung Atta Fabricius (Hymenoptera, Formicidae). *Studia Entomologica* **2**, 321-390.

Bot ANM, Rehner SA, Boomsma JJ (2001) Partial incompatibility between ants and symbiotic fungi in two sympatric species of Acromyrmex leaf-cutting ants. *Evolution* **55**, 1980-1991.

Brant SV, Orti G (2003) Phylogeography of the Northern short-tailed shrew, *Blarina brevicauda* (Insectivora : Soricidae): Past fragmentation and postglacial

recolonization. *Molecular Ecology* **12**, 1435-1449.

Brooks DR, McLennan DA (1991) *Phylogeny, ecology, and behavior : a research program in comparative biology*. University of Chicago Press, Chicago, IL.

Burbrink FT, Lawson R, Slowinski JB (2000) Mitochondrial DNA phylogeography of the polytypic North American rat snake (*Elaphe obsoleta*): A critique of the subspecies concept. *Evolution* **54**, 2107-2118.

Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Canadian Journal of Microbiology* **51**, 441-446.

Carvalho DB, Smith ML, Anderson JB (1995) Genetic exchange between diploid and haploid mycelia of *Armillaria gallica*. *Mycological Research* **99**, 641-647.

Chapela IH, Rehner SA, Schultz TR, Mueller UG (1994) Evolutionary history of the symbiosis between fungus-growing ants and their fungi. *Science* **266**, 1691-1694.

Cherrett JM (1968) Some aspects of the distribution of pest species of leafcutting ants in the Caribbean. *American Society for Horticultural Science. Tropical Region*. **12**, 295-310.

Christian CE (2001) Consequences of a biological invasion reveal the importance of mutualism for plant communities. *Nature (London)* **413**, 635-639.

Clark MA, Moran NA, Baumann P, Wernegreen JJ (2000) Cospeciation between bacterial endosymbionts (Buchnera) and a recent radiation of aphids (Uroleucon) and pitfalls of testing for phylogenetic congruence. *Evolution* **54**, 517-525.

Currie CR, Mueller UG, Malloch D (1999a) The agricultural pathology of ant fungus gardens. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7998-8002.

Currie CR, Scott JA, Summerbell RC, Malloch D (1999b) Fungus-growing ants use

antibiotic-producing bacteria to control garden parasites. *Nature* **398**, 701-704.

Currie CR, Stuart AE (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 CR, Bot ANM, Boomsma JJ (2003a) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* **101**, 91-102.

Currie CR, Wong B, Stuart AE*, et al.* (2003b) Ancient tripartite coevolution in the attine ant-microbe symbiosis. *Science* **299**, 386-388.

Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* **101**, 91-102.

Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* **311**, 81-83.

Darwin C (1859) *On the origin of species by natural selection*. Murray: London.

de Fine Licht HH, Boomsma JJ, Aanen DK (2006) Presumptive horizontal symbiont transmission in the fungus-growing termite *Macrotermes natalensis*. *Molecular Ecolology* **15**, 3131-3138.

De Vienne DM, Giraud T, Shykoff JA (2007) When can host shifts produce congruent host and parasite phylogenies? A simulation approach. *Journal of Evolutionary Biology* **20**, 1428-1438.

DePriest PT (2004) Early molecular investigations of lichen-forming symbionts. *Annual Review of Microbiology* **58**, 273-301.

Deyrup MA, Carlin N, Trager J, Umphrey G (1988) A review of the ants of the Florida Keys. *Florida Entomologist* **71**, 163-176.

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

Farris JS, Kallersjo M, Kluge AG, Bult C (1995) Constructing a significance test for incongruence. *Systematic Biology* **44**, 570-572.

Febvay G, Hostachy B, Rahbe Y, Kermarrec A (1988) Principes d'une nouvelle strategie de lutte contre la fourmi-manioc, *Acromyrmex octospinosus* Reich, en Guadeloupe. *Bulletin Agronomique (Petit-Bourg)* **7**, 10-15.

Feinsinger P, Futuyma DJ, Slatkin M (1983) Coevolution. Sinauer Associates.

Fenchel T, Finlay BJ (2005) Bacteria and island biogeography. *Science* **309**, 1997-1997.

Fernandez-Marin H, Wcislo WT (2005) Production of minima workers by gynes of *Atta colombica* Guerin-Meneville (Hymenoptera : Formicidae : Attini) that lack a fungal pellet. *Journal of the Kansas Entomological Society* **78**, 290-292.

Fierer N, Jackson RB (2006) The diversity and biogeography of soil bacterial communities. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 626-631.

Fisher MC, Koenig GL, White TJ*, et al.* (2001) Biogeographic range expansion into South America by *Coccidioides immitis* mirrors New World patterns of human migration. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 4558-4562.

Fontenla Rizo JL (1993) Ant fauna from Isla de la Juventud and from some keys of the Cuban Archipelago. *Poeyana Instituto de Ecologia y Sistematica Academia de*

Ciencias de Cuba **0**, 1-7.

Fowler HG (1983) Distribution patterns of Paraguayan leaf-cutting ants (Atta and Acromyrmex) (Formicidae: Attini). *Studies on Neotropical Fauna and Environment***18**, 121-138.

Fowler HG (1992) Patterns of colonization and incipient nest survival in *Acromyrmex niger* and *Acromyrmex balzani* (Hymenoptera: Formicidae). *Insectes Sociaux* **39**, 347-350.

Frank SA (1994) Genetics of mutualism - The evolution of altruism between species. *Journal of Theoretical Biology* **170**, 393-400.

Frank SA (1996) Models of parasite virulence. *Quarterly Review of Biology* **71**, 37-78.

Gerardo NM, Jacobs SR, Currie CR, Mueller UG (2006) Ancient host-pathogen associations maintained by specificity of chemotaxis and antibiosis. *PLOS Biology* **4**, 1358-1363.

Gomel L, Febvay G, Madignier Ml, Kermarrec A (1989) Une situation préoccupante en Guadeloupe: la fourmi-manioc met les bouchees doubles. *Phytoma* **410**, 10-15.

Green AM, Mueller UG, Adams RMM (2002) Extensive exchange of fungal cultivars between sympatric species of fungus-growing ants. *Molecular Ecology* **11**, 191-195.

Green JL, Ostling A (2003) Endemics-area relationships: The influence of species dominance and spatial aggregation. *Ecology* **84**, 3090-3097.

Guimaraes PR, Rico-Gray V, Oliveira PS*, et al.* (2007) Interaction intimacy affects structure and coevolutionary dynamics in mutualistic networks. *Current Biology* **17**, 1797-1803.

Harpending HC (1994) Signature of ancient population growth in a low-resolution

mitochondrial DNA mismatch distribution. *Human Biology* **66**, 591-600.

Harpending HC, Batzer MA, Gurven M*, et al.* (1998) Genetic traces of ancient demography. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 1961-1967.

Herre EA, Knowlton N, Mueller UG, Rehner SA (1999) The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution* **14**, 49-53.

Hinkle G, Wetterer JK, Schultz TR, Sogin ML (1994) Phylogeny of the attine ant fungi based on analysis of small subunit ribosomal RNA gene sequences. *Science* **266**, 1695-1697.

Hoeksema JD, Bruna EM (2000) Pursuing the big questions about interspecific mutualism: a review of theoretical approaches. *Oecologia* **125**, 321-330.

Holway DA, Suarez AV, Case TJ (1998) Loss of intraspecific aggression in the success of a widespread invasive social insect. *Science* **282**, 949-952.

Horner-Devine MC, Lage M, Hughes JB, Bohannan BJM (2004) A taxa-area relationship for bacteria. *Nature* **432**, 750-753.

Hölldobler B, Wilson EO (1990) *The Ants*. Harvard University Press, Cambridge, MA.

Ji YJ, Zhang DX, He LJ (2003) Evolutionary conservation and versatility of a new set of primers for amplifying the ribosomal internal transcribed spacer regions in insects and other invertebrates. *Molecular Ecology Notes* **3**, 581-585.

Johannesson H, Stenlid J (2004) Nuclear reassortment between vegetative mycelia in natural populations of the basidiomycete Heterobasidion annosum. *Fungal Genetics and Biology* **41**, 563-570.

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis.

Trends in Ecology & Evolution **17**, 164-170.

Kermarrec A, Mauleon H (1990) Psychotropic substances imparing the vigilance of Acromyrmex octospinosus Reich (Attini, Formicidae). In: *Applied myrmecology, a world perspective* (eds. Vander Meer RK, Jaffe K, Cedeno A), pp. 645-662. Westview Press, Boulder, CO.

Kosakovsky Pond SL, Frost SDW, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. *Bioinformatics* **21**, 676-679.

Krieger MJB, Keller L (2000) Mating frequency and genetic structure of the Argentine ant Linepithema humile. *Molecular Ecology* **9**, 119-126.

Kweskin M (2003) *Molecular and behavioral ecology of fungus-growing ants and their fungi*, MA Thesis, University of Texas at Austin.

Linz B, Balloux F, Moodley Y, *et al.* (2007) An African origin for the intimate association between humans and *Helicobacter pylori*. *Nature* **445**, 915-918.

Machado CA, Robbins N, Gilbert MTP, Herre EA (2005) Critical review of host specificity and its coevolutionary implications in the fig/fig-wasp mutualism. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 6558-6565.

Maddison WP (1997) Gene trees in species trees *Systematic Biology* **46,** 523-536.

Malato G, Kermarrec A, Troup JM (1977) Donnes nouvelles sur l'invasion de la Guadeloupe par *Acromyrmex octospinosus* Reich (Formicidae, Attini). I. Aspects biogeografiques et perspectives de controle chimique. *Nouvelles Agronomiques Antilles-Guyane* **3**, 473-484.

Marshall SJ, James TS, Clarke GKC (2002) North American ice sheet reconstructions at the last glacial maximum. *Quaternary Science Reviews* **21**, 175-192.

Mayden RL (1988) Vicariance biogeography, parsimony, and evolution in North American fresh water fishes. *Systematic Zoology* **37**, 329-355.

McGlynn TP (1999a) Non-native ants are smaller than related native ants. *American Naturalist* **154**, 690-699.

McGlynn TP (1999b) The worldwide transfer of ants: geographical distribution and ecological invasions. *Journal of Biogeography* **26**, 535-548.

Mehdiabadi NJ, Hughes B, Mueller UG (2006) Cooperation, conflict, and coevolution in the attine ant-fungus symbiosis. *Behavioral Ecology* **17**, 291-296.

Mikheyev AS, Mueller UG, Abbot P (2006) Cryptic sex and many-to-one coevolution in the fungus-growing ant symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, 10702–10706.

Mikheyev AS, Mueller UG, Boomsma JJ (2007) Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. *Molecular Ecology* **16**, 209-216.

Mikheyev AS, Vo T, Mueller UG (2008) Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists. *Molecular Ecology* **17**, 4480-4488.

Mikosch TSP, Sonnenberg ASM, Van Griensven L (2001) Isolation, characterization, and expression patterns of a DMC1 homolog from the basidiomycete *Pleurotus ostreatus*. *Fungal Genetics and Biology* **33**, 59-66.

Milne RI, Abbott RJ (2004) Geographic origin and taxonomic status of the invasive Privet, *Ligustrum robustum* (Oleaceae), in the Mascarene Islands, determined by chloroplast DNA and RAPDs. *Heredity* **92**, 78-87.

Mitchell CE, Power AG (2003) Release of invasive plants from fungal and viral

pathogens. *Nature (London)* **421**, 625 - 627.

Molbo D, Machado CA, Sevenster JG, Keller L, Herre EA (2003) Cryptic species of fig-pollinating wasps: Implications for the evolution of the fig-wasp mutualism, sex allocation, and precision of adaptation. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 5867-5872.

Möller A (1893) Die Pilzgerten einiger suedamerikanischer Ameisen. *Botanische Mittheilung aus den Tropen* **6**: 1-127.

Montoya JM, Pimm SL, Sole RV (2006) Ecological networks and their fragility. *Nature* **442**, 259-264.

Mueller UG (2002) Ant versus fungus versus mutualism: Ant-cultivar conflict and the deconstruction of the attine ant-fungus symbiosis. *American Naturalist* **160**, S67-S98.

Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR (2005) The evolution of agriculture in insects. *Annual Review of Ecology Evolution and Systematics* **36**, 563-595.

Mueller UG, Poulin J, Adams RMM (2004) Symbiont choice in a fungus-growing ant (Attini, Formicidae). *Behavioral Ecology* **15**, 357-364.

Mueller UG, Rehner SA, Schultz TR (1998) The evolution of agriculture in ants. *Science* **281**, 2034-2038.

Mueller UG, Schultz TR, Currie CR, Adams RMM, Malloch D (2001) The origin of the attine ant-fungus mutualism. *Quarterly Review of Biology* **76**, 169-197.

Munkacsi AB, Pan JJ, Villesen P*, et al.* (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.

Murakami T, Higashi S, Windsor D (2000) Mating frequency, colony size, polyethism and sex ratio in fungus-growing ants (Attini). *Behavioral Ecology and Sociobiology* **48**, 276-284.

Nara T, Saka T, Sawado T*, et al.* (1999) Isolation of a LIM15/DMC1 homolog from the basidiomycete *Coprinus cinereus* and its expression in relation to meiotic chromosome pairing. *Molecular and General Genetics* **262**, 781-789.

Near TJ, Page LM, Mayden RL (2001) Intraspecific phylogeography of *Percina evides* (Percidae : Etheostomatinae): an additional test of the Central Highlands pre-Pleistocene vicariance hypothesis. *Molecular Ecology* **10**, 2235-2240.

Normark BB, Judson OP, Moran NA (2003) Genomic signatures of ancient asexual lineages *Biological Journal of the Linnean Society* **79**, 69 - 84.

Ortius-Lechner D, Gertsch PJ, Boomsma JJ (2002) Variable microsatellite loci for the leafcutter ant Acromyrmex echinatior and their applicability to related species. *Molecular Ecology* **9**, 114-116.

Pagnocca FC, Bacci M, Fungaro MH*, et al.* (2001) RAPD analysis of the sexual state and sterile mycelium of the fungus cultivated by the leaf-cutting ant Acromyrmex hispidus fallax. *Mycological Research* **105**, 173-176.

Paoletti M, Rydholm C, Schwier EU*, et al.* (2005) Evidence for sexuality in the opportunistic fungal pathogen Aspergillus fumigatus. *Current Biology* **15**, 1242-1248.

Papke RT, Ward DM (2004) The importance of physical isolation to microbial diversification. *FEMS Microbiology Ecology* **48**, 293-303.

Peakall R, Smouse PE (2006a) genalex6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **8**, 288-295.

Pellmyr O, Leebens-Mack J (1999) Forty million years of mutualism: Evidence for Eocene origin of the yucca-yucca moth association. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 9178-9183.

Pellmyr O, LeebensMack J, Huth CJ (1996) Non-mutualistic yucca moths and their evolutionary consequences. *Nature* **380**, 155-156.

Pereira-da-Silva V, Forti LC, Cardoso LG (1981) Dinámica populacional e caracterização dos ninhos de *Acromyrmex coronatus* (Fabricius, 1804) (Hymenoptera, Formicidae). *Revista Brasileira de Entomologia* **25**, 87-93.

Pielou EC (1991) *After the Ice Age: the return of life to glaciated North America* University of Chicago Press, Chicago, IL.

Polak E, Hermann R, Kues U, Aebi M (1997) Asexual sporulation in Coprinus cinereus: Structure and development of oidiophores and oidia in an *Amut Bmut* homokaryon. *Fungal Genetics and Biology* **22**, 112-126.

Polis GA, Winemiller KO (1996) *Food webs: integration of patterns and dynamics* Chapman and Hall, New York, NY.

Pontecorvo G (1956) The parasexual cycle in fungi. *Annual Review Microbiology* **10**, 393-400.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* **14**, 817-818.

Poulsen M, Bot ANM, Currie CR, Nielsen MG, Boomsma JJ (2003) Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant Acromyrmex octospinosus. *Functional Ecology* **17**, 260-269.

Poulsen M, Cafaro M, Boomsma JJ, Currie CR (2005) Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of

Acromyrmex leaf-cutting ants. *Molecular Ecology* **14**, 3597-3604.

Quinlan RJ, Cherrett JM (1979) The role of fungus in the diet of the leaf-cutting ant Atta cephalotes (L.). *Ecological Entomology* **4**, 151-160.

Rabeling C, Cover SP, Johnson RA, Mueller UG (2007) A review of the North American species of the fungus-gardening ant genus Trachymyrmex (Hymenoptera : Formicidae). *Zootaxa*, 1-53.

Ramesh MA, Malik SB, Logsdon JM (2005) A phylogenomic inventory of meiotic genes: Evidence for sex in Giardia and an early eukaryotic origin of meiosis. *Current Biology* **15**, 185-191.

Rezende EL, Lavabre JE, Guimaraes PR, Jordano P, Bascompte J (2007) Non-random coextinctions in phylogenetically structured mutualistic networks. *Nature* **448**, 925-U926.

Robinson HW (1986) Zoogeographic implications of the Mississippi River Basin. In: *The Zoogeography of North American Freshwater Fishes* (eds. Hocutt CH, Wiley EO), pp. 267– 285. John Wiley & Sons, New York, NY.

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

Ross KG, Fletcher DJC (1985) Genetic origin of male diploidy in the fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), and its evolutionary significance. *Evolution* **39**, 888-903.

Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 5435-5440.

Schultz TR, Meier R (1995) A phylogenetic analysis of the fungus-growing ants

(Hymenoptera: Formicidae: Attini) based on morphological characters of the larvae. *Systematic Entomology* **20**, 337-370.

Schultz TR, Solomons SA, Mueller UG*, et al.* (2002) Cryptic speciation in the fungus-growing ants *Cyphomyrmex longiscapus* Weber and *Cyphomyrmex muelleri* Schultz and Solomon, new species (Formicidae, Attini). *Insectes Sociaux* **49**, 331-343.

Seal JN, Tschinkel WR (2007) Co-evolution and the superorganism: switching cultivars does not alter the performance of fungus-gardening ant colonies. *Functional Ecology* **21**, 988-997.

Selosse MA (2001) Adding pieces to fungal mosaics. *New Phytologist* **149**, 159-162.

Shinohara A, Ogawa H, Ogawa T (1992) RAD51 protein involved in repair and recombination in *Saccharomyces cerevisiae* is a RecA-like protein. *Cell* **69**, 457-470.

Shoemaker DDW, Deheer CJ, Krieger MJB, Ross KG (2006) Population genetics of the invasive fire ant *Solenopsis invicta* (Hymenoptera: Formicidae) in the United States. *Annals of the Entomological Society of America* **99**, 1213-1233.

Silva-Pinhati ACO, Bacci M, Hinkle G*, et al.* (2004) Low variation in ribosomal DNA and internal transcribed spacers of the symbiotic fungi of leaf-cutting ants (Attini : Formicidae). *Brazilian Journal of Medical and Biological Research* **37**, 1463-1472.

Smith JM, Smith NH (1998) Detecting recombination from gene trees. *Molecular Biology and Evolution* **15**, 590-599.

Stanton ML (2003a) Interacting guilds: Moving beyond the pairwise perspective on mutualisms. *American Naturalist* **162**, S10-S23.

Stradling DJ, Powell RJ (1986) The cloning of more highly productive fungal strains - a factor in the speciation of fungus-growing ants. *Experientia* **42**, 962-964.

Strauss SY, Irwin RE (2004) Ecological and evolutionary consequences of multispecies plant-animal interactions. *Annual Review of Ecology Evolution and Systematics* **35**, 435-466.

Suarez AV, Case TJ (2002) Bottom-up effects on persistence of a specialist predator: Ant invasions and horned lizards. *Ecological Applications* **12**, 291-298.

Suarez AV, Holway DA, Case TJ (2001) Patterns of spread in biological invasions dominated by long-distance jump dispersal: Insights from Argentine ants. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 1095-1100.

Sumner S, Aanen DK, Delabie JC, Boomsma JJ (2004) The evolution of social parasitism in Acromyrmex leaf-cutting ants: a test of Emery's rule. *Insectes Sociaux* **51**, 37-42.

Swofford DL (1993) PAUP - A computer program for phylogenetic inference using maximum parsimony. *Journal of General Physiology* **102**, A9-A9.

Sánchez-Peña S (2005) *Essays on organismal aspects of the fungus-growing ant symbiosis : ecology, experimental symbiont switches and fitness of Atta, and a new theory on the origin of ant fungiculture*, Ph.D. dissertation, Univrsity of Texas at Austin.

Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: Reproduction, speciation and classification. *Annual Review of Phytopathology* **37**, 197-246.

Therrien P (1986) *Behavioral ecology of the leaf-cutting ant, Acromyrmex octospinosus*

(Reich), in Guadeloupe, Ph.D. dissert., University of British Columbia, Canada.

Vellinga EC (2004) Ecology and distribution of lepiotaceous fungi (Agaricaceae) - A review..*Nova Hedwigia* **78**, 273-299.

Villesen P, Gertsch PJ, Frydenberg J, Mueller UG, Boomsma JJ (1999) Evolutionary transition from single to multiple mating in fungus-growing ants. *Molecular Ecology* **8**, 1819-1825.

Villesen P, Mueller UG, Schultz TR, Adams RMM, Bouck AC (2004) Evolution of ant-cultivar specialization and cultivar switching in Apterostigma fungus-growing ants. *Evolution* **58**, 2252-2265.

Villesen P, Murakami T, Schultz TR, Boomsma JJ (2002) Identifying the transition between single and multiple mating of queens in fungus-growing ants. *Proceedings of the Royal Society of London Series B-Biological Sciences* **269**, 1541-1548.

Vo T, Mueller UG, Mikheyev AS (in press) Free-living fungal symbionts (Lepiotaceae) of fungus-growing ants (Attini: Formicidae). *Mycologia*.

Wallace AR (1876) *The geographical distribution of animals: With a study of the relations of living and extinct faunas as elucidating the past changes of the Earth's surface*. Harper & Brothers, London.

Walker D, Moler PE, Buhlmann KA, Avise JC (1998) Phylogeographic patterns in *Kinosternon subrubrum* and *K. baurii* based on mitochondrial DNA restriction analyses. *Herpetologica* **54**, 174-184.

Weber NA (1972) *Gardening ants, the attines* Memoirs of the American Philosophical Society, Philadelphia, PA.

West-Eberhard MJ (2003) *Developmental plasticity and evolution* Oxford University

Press, Oxford.

Wetterer JK (1993) Foraging and nesting ecology of a Costa Rican leaf-cutting ant, *Acromyrmex volcanus*. *Psyche* **100**, 65-76.

Wetterer JK (1995) Forager size and ecology of Acromyrmex coronatus and other leaf-cutting ants in Costa Rica. *Oecologia, Berlin* **104**, 409-415.

Wetterer JK, Gruner DS, Lopez JE (1998a) Foraging and nesting ecology of *Acromyrmex octospinosus* (Hymenoptera : Formicidae) in a costa Rican tropical dry forest. *Florida Entomologist* **81**, 61-67.

Wetterer JK, Schultz TR, Meier R (1998b) Phylogeny of fungus-growing ants (Tribe Attini) based on mtDNA sequence and morphology. *Molecular Phylogenetics and Evolution* **9**, 42-47.

Wheeler WM (1907) The fungus-growing ants of North America. *Bull. Am. Mus. Nat. Hist.* **23**, 669-807.

Wheeler WM (1911) Descriptions of some new fungus growing ants from Texas, with Mr. CG Hartman's observations on their habits, pp. 245-255.

Whitaker RJ, Grogan DW, Taylor JW (2003) Geographic barriers isolate endemic populations of hyperthermophilic archaea. *Science* **301**, 976-978.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *Innis, M. a., Et al. (Ed.). Pcr Protocols: a Guide to Methods and Applications*. Academic Press, Inc.: San Diego, CA.

Wiley EO, Mayden RL (1985) Species and speciation in phylogenetic systematics, with examples from the North American fish fauna. *Annals of the Missouri Botanical Garden* **72**, 596-635.

Wirth R, Beyschlag W, Ryel R, Herz H, Hölldobler B (2003) *The herbivory of leaf-*

cutting ants. A case study on Atta colombica in the tropical rainforest of Panama Springer Verlag, Berlin, Heidelberg, New York.

Woodcock S, Curtis TP, Head IM, Lunn M, Sloan WT (2006) Taxa-area relationships for microbes: the unsampled and the unseen. *Ecology Letters* **9**, 805-812.

Wootton JT (2002) Indirect effects in complex ecosystems: recent progress and future challenges. *Journal of Sea Research* **48**, 157-172.

Xu JP, Horgen PA, Anderson JB (1996) Somatic recombination in the cultivated mushroom *Agaricus bisporus*. *Mycological Research* **100**, 188-192.

VITA

Alexander Mikheyev attended the Bronx High School of Science (1992-1996) and then Cornell University (1996-2000). He started his graduate studies at the University of Texas in 2002, after completing a MS in Biology at Florida State University.

Permanent Address: 1510 W North Loop Blvd., #1038, Austin, TX 78756.

This dissertation was typed by the author.