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Chemical and molecular ecology of the North American slave-making ant *Polyergus*  
(Hymenoptera, Formicidae) and its closely related host (*Formica* spp.)

By

Candice Wong Torres

A dissertation submitted in partial satisfaction of the

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in

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Committee in charge:

Professor Neil D. Tsutsui, Chair

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Professor Craig Moritz

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## ABSTRACT

Chemical and molecular ecology of the North American slave-making ant *Polyergus* (Hymenoptera, Formicidae) and its closely related host (*Formica* spp.)

by

Candice Wong Torres

Doctor of Philosophy in Environmental Science, Policy and Management

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Parasites contribute greatly to the generation of the planet's biodiversity, exploiting all levels of the biological hierarchy. Examples range from selfish DNA elements within genomes to social parasites that invade whole societies. Slave-making ants in the genus *Polyergus* are obligate social parasites that rely exclusively on ants in the genus *Formica* for colony founding, foraging, nest maintenance, brood care, and colony defense. To acquire their slave labor, *Polyergus* workers raid neighboring *Formica* colonies for brood to bring back to their colony. In this dissertation, I explore the interaction between North American *Polyergus* slave-making ants and their slaves in the genus *Formica* to gain a better understanding of the possible coevolutionary dynamics between them.

In the first dissertation chapter, I investigate how enslavement by *Polyergus breviceps* affects the nestmate recognitions system of its hosts, *Formica altipetens*. To do this I compared the chemical, genetic and behavioral characteristics of enslaved and free-living *Formica* colonies. I found that enslaved *Formica* colonies were both more genetically and chemically diverse than their free-living counterparts. Enslaved *Formica* workers were also less aggressive towards non-nestmates compared to free-living *Formica*. These results suggest that parasitism by *Polyergus* dramatically alters both the chemical and genetic context in which their kidnapped hosts develop such that it may affect how they recognize nestmates.

In the second chapter of my dissertation, I study how the presence of multiple host species in sympatry shapes the chemical and genetic structure of a single population of *Polyergus breviceps*. To successfully adapt to a particular host, slave-making ants may mimic or camouflage themselves with the species-specific chemical cues that their slaves use to recognize and accept nestmates. If such host specialization should continue through several generations, genetic structuring according to host may occur. I collected both chemical and genetic data from *Polyergus* colonies from the same locality parasitizing three different species of *Formica*. I concluded that the *Polyergus* from this location can be distinguished

chemically according to host species and there is sufficient genetic evidence from both maternally and biparentally inherited markers to propose that host-races have formed in this species of slave-maker.

My final chapter examines the phylogeography and population structure of two currently recognized species of North American *Polyergus* (*P. breviceps* and *P. lucidus*) and the hosts that they enslave (*Formica* spp). I used sequence from one mitochondrial gene (*cytochrome oxidase I*) and three nuclear genes (*28S*, *elongation factor 1-alpha*, *arginine kinase*) to reconstruct the ancestor-descendent relationships between several populations of *Polyergus* and the different species of *Formica* they enslave. Additionally, I subjected the DNA sequence data to a Bayesian method of species delimitation to explore North American *Polyergus* species boundaries. Lastly, I use the mitochondrial DNA sequence data to consider the relative effects of geography and host species on the population genetic structure of *P. breviceps* and *P. lucidus*. On the whole, North American *Polyergus* populations generally follow a broad west-east phylogeographic pattern, and can be divided into three distinct species: *P. breviceps* as two species and *P. lucidus* as it is currently recognized. Although population structuring of *P. breviceps* can be primarily explained by geography and to some extent host species, *P. lucidus* population structure is more strongly dictated by host association and not by geographic distribution.

Overall, the results from my dissertation provide insight into the interaction between a social parasite and its host at both ecological and evolutionary timescales. Because of their ecological diversity, widespread distribution and unique evolutionary trajectory, *Polyergus* slave-makers and their *Formica* hosts present a unique case study of possible host-parasite coevolution.

## **DEDICATION**

To my dearest husband, Kent, who has selflessly provided me endless support throughout my entire graduate school career and to my children who have always served as my ultimate source of motivation.

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## INTRODUCTION

Parasites encompass an enormous fraction of the earth's biodiversity and affect nearly all living species (Thompson 1994). As exploiters of host resources, parasites can be found at all levels of the biological hierarchy, as hitchhiking genes in genomes and cell-invading viruses to blood-sucking deer ticks and organisms that invade whole societies. The latter example includes the social parasites, organisms that infiltrate social groups and take advantage of the helping behaviors of their hosts. Many social parasites belong to an ecologically important order of insects, the Hymenoptera (Wilson 1971) and examples of socially parasitic species can be found in ants (Hölldobler and Wilson 1990), bees (ex: Smith et al. 2007), and wasps (see Gamboa 2004). Despite the prevalence of this type of interaction in Hymenoptera, social parasites have received relatively little attention compared to the parasites of solitary organisms (D'Ettorre and Heinze 2001). Unlike many disease-causing parasites, social parasites are often closely related to their hosts (Emery's rule; Emery 1909). This close relationship could have arisen either because they evolved directly from their hosts (Buschinger 1986) and/or because it is easier for the needs of a social parasite to be met by a related species with the same ecology (Wilson 1971). Consequently, social parasites present interesting and apt model systems for illuminating novel evolutionary pathways to the parasitic lifestyle. Moreover, social parasites offer a unique opportunity to study the ecology and evolution of social systems, particularly with respect to the mechanisms by which the cohesion of societies are maintained.

Found exclusively in the Hymenopteran family, Formicidae, slave-making ants are social parasites that rely on their hosts for colony establishment, brood care, nest maintenance, foraging and colony defense (Hölldobler and Wilson 1990). Incapable of founding a colony of their own, slave-making ant queens must expel or kill an existing host queen and take-over that colony to acquire the first generation of slaves that will help raise her worker brood (Wilson 1971). Once fully matured, slave-making ant workers conduct raids on neighboring host colonies for additional worker pupa to replenish their supply of slaves (Buschinger 2009). When the kidnapped hosts emerge from their cocoons, they imprint on the odors found within the slave-making ant colony (Le Moli and Mori 1985). By doing so, they learn to accept all individuals encountered as nestmates and go about performing tasks for the slave-making ant colony.

The evolution and ecology of slave-makers have been contemplated over by biologists as far back as Darwin (1859) and have continued to spark research in the fields of chemical ecology, social insect behavior, and host-parasite coevolution (D'Ettorre and Heinze 2001; Lenoir et al. 2001; Brandt et al. 2005; Buschinger 2009; Bagnères and Lorenzi 2010). Slave-making ants are estimated to have evolved independently at least 10 times in ants, with a large concentration of species in the subfamilies Myrmicinae and Formicinae (D'Ettorre and Heinze 2001). Within the latter subfamily is the Holarctic genus, *Polyergus* Latreille, consisting of five currently recognized species of obligate slave-making ant that enslave ants in the closely related and highly diverse genus, *Formica* Linnaeus (Hölldobler and Wilson

1990; Bolton 1995). These five species include: *P. rufescens* from Europe, *P. nigerrimus* from Russia, *P. samurai* from Japan, and *P. breviceps* and *P. lucidus* from North America. In my dissertation, I examine the chemical ecology, population genetics and phylogeography of *Polyergus* and their *Formica* slaves species from North America to gain a better understanding of the possible coevolutionary dynamics between them. Previous research on other slave-making ant species has shown their potential to engage in a coevolutionary arms race with their hosts (Brandt et al. 2005). This is not surprising considering that slave-making ants depend heavily on their slaves for survival and reproduction and that these social parasites have been found to have substantial negative effects on their hosts' ecology with regard to raiding and colony take-over by queens (D'Ettorre and Heinze 2001).

To date, most of the research on *Polyergus* has focused primarily on their natural history, the raiding behavior of slave-making ant workers and the chemical strategies by which *Polyergus* queens and workers use to avoid host rejection (e.g. Topoff et al. 1985; Goodloe et al. 1987; Topoff et al. 1988; Bonavita-Cougourdan et al. 1996; Liu et al. 2003; Bono et al. 2006; King and Trager 2007). Currently, no population genetic or phylogeography studies on *Polyergus* have been performed despite their comparatively widespread distribution and the diversity of host species they enslave. Additionally, the chemical ecology of North American *Polyergus* workers and their slaves as it relates to the parasite's ability to achieve colony integration has not yet been examined. Addressing these gaps in the *Polyergus* literature will give us insights into the ecological and evolutionary interactions between *Polyergus* and their hosts in the genus *Formica*.

In the first chapter of my dissertation, I examine the effects of slave-making by *Polyergus breviceps* on the nestmate recognition system of its host, *Formica altipetens*. Ants, like other eusocial insects, have evolved advanced recognition systems that allow them to determine which individuals should be accepted and which should be rejected from their colony. They achieve this by using their antenna to access whether chemical cues on another ant matches a neuronal-based template of acceptable cues. A mismatch between these cues and the template often results in aggressive attack on ants recognized as non-nestmates (Vander Meer and Morel 1998). A large body of research supports that the cues used in ant nestmate recognition are cuticular hydrocarbons (CHCs) and that these cues can be both species and colony specific (see van Zweden and d'Ettorre 2010 for review). Slave-making ants, however, have managed to exploit the recognition system of their hosts. Not only do slave-making ant colonies consist of ants from two different genera, they also contain slaves raided from different colonies that would typically not be nestmates. To determine how such colony integration is achieved and how this may alter nestmate recognition of parasitized *Formica*, I compared the following attributes between enslaved and free-living *Formica* colonies: chemical cue composition, genetic variation, and behavior towards non-nestmates. This chapter represents the first study of this particular population of *Polyergus breviceps* and its host and gives us insight into how the act of slave-making affects the structure and function of ant recognition systems.

In chapter 2, I examine whether there is chemical and genetic evidence for host specificity in a population of *Polyergus breviceps* that parasitizes three different species of *Formica* in sympatry. Though rare, a few populations of North American *Polyergus* may occur in areas where multiple species of host are available for enslavement. However, no single colony has been observed to enslave more than one species at a time (Bono et al. 2007; King and Trager 2007). Additionally, there appears to be evidence for host specificity for obligate slave-making ants with regard to queen host fidelity during colony foundation and raiding behavior (Goodloe and Sanwald 1985; Goodloe et al. 1987; Bono et al. 2006). A possible adaptation that may allow host specialization to occur is the adjustment of *Polyergus* worker chemical cues to closely match the chemical profiles of their hosts. This allows *Polyergus* to avoid host detection and become well integrated into a colony. Since different species of hosts may be expected to have differing chemical profiles, I expected that *Polyergus* specializing on such hosts should become chemically distinguishable from one another. Long-term host specialization of *Polyergus* may then lead to genetic differentiation according to host, assuming some level of restricted gene flow. To test these assumptions, I collected chemical, nuclear microsatellite, and mitochondrial DNA data from colonies of *Polyergus breviceps* enslaving *F. argentea*, *F. accreta* and *F. fusca* to test for genetic clustering according to host. The results from this chapter may provide an interesting example of host race formation in sympatry.

The last chapter of my dissertation (Chapter 3) explores the population genetics and phylogeographic patterns of North American *Polyergus* and their hosts, *Formica*. Populations isolated by great distance or geographic barriers typically experience reduced gene flow, causing populations to genetically diverge. Like other social parasites, *Polyergus* populations follow disjunct distribution patterns and tend to only be in abundance when there is a high enough density of *Formica* to sustain them. Extensive gene flow between populations of *Polyergus* may be restricted due to limited dispersal of founding queens (Topoff 1999; Ward 2005). As such, both host use and geography could have an effect on the ancestor-descendent relationships among populations of *Polyergus*. In this final chapter, I use mitochondrial sequence data from the gene cytochrome oxidase I (COI) and nuclear sequence data from three slower evolving genes (28S, elongation factor 1 alpha, and arginine kinase) to reconstruct the phylogenetic relationships among populations of *P. breviceps* and *P. lucidus* along with their *Formica* hosts. Because the genus *Polyergus* is currently undergoing a taxonomic revision, due in part to suspicions that *P. breviceps* and *P. lucidus* may be species complexes, I also subjected the DNA sequence data to a Bayesian method of species delimitation to estimate the number of North American *Polyergus* species. Lastly, I use the COI data to examine the possible effects of host and geography on the population structure of *P. breviceps* and *P. lucidus*. Together, these analyses provide a broader view of how *Polyergus* populations may have evolved along with their hosts.

In sum, the three chapters of my dissertation address fundamental questions about how social parasites and their hosts may interact at both ecological and evolutionary scales. The first chapter explores this ecological interaction from the

hosts' point of view through a study of how slave-making by *Polyergus* affects the hosts' chemical and genetic colony structure and their ability to recognize nestmates. In the second chapter, we examine this ecological interaction from the parasite's perspective by looking at how the presence of multiple hosts in sympatry may affect the chemical and genetic structure of a single *Polyergus* population. In the final dissertation chapter, I expand to a broader scale by studying the phylogeography of North American *Polyergus* populations and their hosts, viewing this host-parasite interaction through evolutionary time. Slave-making ants in the genus *Polyergus* and their hosts *Formica* provide a unique and fascinating example of how a rare but relatively widespread parasite may coevolve with their host.

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## **CHAPTER 1**

The effect of social parasitism by *Polyergus breviceps* on the nestmate recognition system of its host, *Formica altipetens*

## INTRODUCTION

Eusocial insects display highly cooperative, altruistic behavior in which sterile or effectively sterile workers collectively contribute to brood care, nest maintenance, foraging and colony defense. To maintain colony integrity, social insects have evolved advanced recognition systems that allow them to distinguish nestmates (often kin) from non-nestmates (Michener and Smith 1987; Tsutsui 2004). Individuals recognized as foreign are excluded from the colony, frequently via aggressive encounters (Waldman 1988). This discriminatory ability is achieved through the olfactory (antennal) detection of chemical cues, or "labels", expressed on the cuticle of insects (Ozaki et al. 2005). These chemical cues are compared to a learned, neuronal-based template of referential cues to determine whether an individual accepted into the colony (Lacy and Sherman 1983; Errard et al. 2006a). Currently, the most widely accepted model of nestmate recognition asserts that if social insects encounter individuals whose cues mismatch their internalized template, that individual is rejected from the colony (see: van Zweden and d'Ettorre 2010 for review).

Cuticular hydrocarbons (CHCs) have long been implicated as the chemical cues used for nestmate recognition (Smith and Breed 1995) and recent literature strongly supports this in ants (Bonavita-Cougourdan et al. 1987; Lahav et al. 1999; Thomas et al. 1999; Wagner et al. 2000; Akino et al. 2004; Lucas et al. 2005; Torres et al. 2007; Martin et al. 2008b; Guerrieri et al. 2009; Lalzar et al. 2010). Cuticular hydrocarbons (CHCs) are waxy, exoskeletal compounds that originally evolved for desiccation and microbial resistance but have since taken on secondary functions as nestmate recognition cues (Smith and Breed 1995; Singer 1998; Howard and Blomquist 2005). In many insects, particularly ants, cuticular hydrocarbon profiles tend to be species-specific (Howard 1993; Martin et al. 2008a). Often, however, the relative proportions of CHCs also vary intraspecifically, as different colonies express distinctive mixtures of these CHCs (Lorenzi 2003; Martin et al. 2008a; Lenoir et al. 2009; van Zweden and d'Ettorre 2010). Although CHCs likely have a strong genetic component to them (Crozier and Dix 1979; van Zweden et al. 2009), the complete chemical profile that occurs on an individual ant may be modified to include CHCs acquired from colony mates through social interactions including grooming and food exchange, (Soroker et al. 1994; Soroker et al. 1995), environmental influences (Heinze et al. 1996; Katzerke et al. 2006), or diet (Liang and Silverman 2000).

Social parasites have evolved the ability to evade the recognition systems of their hosts, and can gain access to the resources of an entire colony. They do this by mimicking or camouflaging themselves with some of the same chemicals (often CHCs) their hosts use to recognize each other (Dettner and Liepert 1994; Lenoir et al. 2001; D'Ettorre et al. 2002). Slave-making ants in the genus *Polyergus* are obligate social parasites that depend entirely on their host (genus *Formica*) for nest maintenance, brood care, and foraging. A colony is founded when a newly mated *Polyergus* queen infiltrates a potential host nest, kills the *Formica* queen, obtains the queen's chemical scent, and takes her place as the new queen of the colony (Johnson et al. 2001). After usurpation, the host workers rear the *Polyergus* queen's offspring:

the slave-making workers. With the host queen eliminated, *Polyergus* workers must replenish their supply of slaves by kidnapping pupae from neighboring *Formica* colonies (Buschinger et al. 1980; Topoff and LaMon 1982; Hölldobler and Wilson 1990; Mori et al. 1991). Previous studies on *P. breviceps* show they raid from several different *Formica* colonies annually (Topoff et al. 1985; Bono et al. 2006a). As a result, ants from different *Formica* colonies (all of the same species) coexist within the same nest as the *Polyergus* workers and queen.

Slave-making ants appear to take advantage of the fact that their kidnapped host workers imprint on the chemical cues of individuals they encounter on eclosion and such behavior facilitates the integration of enslaved workers into the slave-maker colony (D'Ettorre and Heinze 2001; Bagnères and Lorenzi 2010). Additionally, all colony members may share and recognize a common odor or set of cues known as a gestalt odor (Crozier and Pamilo 1996) and such a phenomenon may also explain how *Polyergus* colonies maintains cohesiveness across members of different genera and nest origins.

In this study, we explore the effect of social parasitism by the slave-making ant, *Polyergus breviceps*, on the nestmate recognition of its host, *Formica altipetens*, in eastern-central Arizona. *F. altipetens* are mound-building ants that are primarily single-nested (monodomous) and have nest distributions characteristic of strong intraspecific competition among colonies (Cushman et al. 1988). Therefore, *F. altipetens* are expected to maintain distinct colonies boundaries, presumably via recognition of genetically and chemically similar colony mates and rejection of dissimilar, foreign individuals. However, if a *Formica* colony is enslaved by *Polyergus*, we expect that the genetic and chemical composition of *Formica* within enslaved colonies to be altered as a consequence of *Polyergus* raiding behavior. These two differences in colony characteristics may then affect the nestmate recognition of enslaved *Formica*. When *Formica* workers are stolen as pupae and reared in a *Polyergus* colony, they are exposed to an unusually wide breadth of chemical labels (from enslaved *Formica* originating from multiple neighboring colonies and from the slave-makers). To avoid rejecting their chemically diverse nestmates, enslaved *Formica* may be more permissive of variations in CHC cues they encounter. Given what we know about the biology of this system, we expect that the social environment, developmental context, and resulting behaviors will be dramatically different for free-living versus enslaved *Formica*.

Here we test three hypotheses for how the nestmate recognition system of enslaved *Formica* differs from free-living *Formica* 1) colonies with enslaved *Formica* will be more genetically diverse than free-living colonies of *Formica* 2) the composition of chemical cues among enslaved *Formica* within colonies will be more diverse than within free-living *Formica* colonies and 3) enslaved *Formica* will be less aggressive towards non-nestmates compared to their free-living counterparts.



## METHODS

### Collection and field site information

We collected individuals used in the behavioral portion of this study in July 2008 from the alpine region of Williams Valley, Arizona (elevation: 2650m) where mound-building *Formica altipetens* are enslaved by *Polyergus breviceps*. Based on behavioral assays between free-living *Formica* workers and observations in the field, we ascertained that these colonies appear monodomous (single-nested) and have no apparent satellite colonies associated with them (as found in Cushman et al. 1988). This allows us to be confident of nest membership during collection.

In June 2009, we returned to our field site to collect *Formica* and *Polyergus* for chemical and genetic analysis. We performed an additional collection for these two analyses as we suspected that those collected in 2008 had degraded chemical profiles due to improper storage and we also wanted to increase the number of colonies we sampled. Additionally, we observed several slave-raids during June 2009 that we did not observe in July 2008 when *Polyergus* colonies were apparently inactive. This suggests that raiding behavior likely occurs earlier and possibly within a shorter time span at our field site compared to what has been recorded at other locations throughout Arizona and in the western United States (typically July-August; see Topoff et al. 1985; Bono et al. 2006b). To our knowledge this is the first published record of raiding behavior for this population of *Polyergus breviceps*.

### Genetic analysis

Whole genomic DNA was extracted from the heads of all chemically analyzed individuals (190 enslaved and 190 free-living *Formica*) using the Qiagen DNeasy Blood and Tissue kit, following the manufacture's protocol.

To determine whether there was higher genetic variation within nests containing enslaved *Formica* compared to free-living *Formica*, we genotyped individuals at 11 microsatellite loci developed from three different species of *Formica*: Fy4, Fy5, Fy7, Fy13 (*F. yessensis*; Hasegawa and Imai 2004) FL12, FL20, FL21, FL29 (*F. lugubris*; Chapuisat 1996), and FE16, FE21, FE37 (*F. exsecta*; Gyllenstrand et al. 2002). Preliminary analysis suggested that these loci amplified well and produced at least two alleles per locus. For all loci, we performed 10ul Polymerase Chain Reactions (PCR) using 1.0ul of DNA template, 1X reaction buffer (provided with the TAQ polymerase), 300μM of each dNTP, 0.8μM of each primer and 0.04-0.075 units of Promega GoTaq Flexi DNA Polymerase (San Luis Obispo, CA, USA). We used the following temperature program: initial denaturation of 5 minutes at 95°C, 36 cycles of 30 secs at 30°C, 30 secs at the appropriate annealing temperature, and a 30 second extension at 72°C, and ending with a final extension step for 5 minutes at 72°C. Details of the PCR reaction conditions used for each set of loci (Fy, FL and FE) can be found in Table 1. All primers were fluorescently labeled using Applied Biosystems (ABI; Carlsbad, CA, USA) dyes VIC, 6FAM, PET and NED and the size standard LIZ was added to the resulting PCR products. We estimated

allele sizes using an ABI 96 capillary 3730xl DNA Analyze ad visualized and scored allele sizes with Peak Scanner v 1.0 (ABI).

We used the program MicroChecker (v 2.2.3, Van Oosterhout et al. 2004) to identify scoring errors due to stuttering, null alleles, and large allele dropout across all individuals analyzed. We used the program GENALEX (v 6.41; Peakall and Smouse 2006) to test whether loci were in Hardy-Weinberg equilibrium at the colony and whole population levels and to obtain allele frequency statistics and standard diversity measurements. The program Arlequin v 3.1 (Excoffier et al. 2005) was used to test for linkage disequilibrium (LD) across all pairs of loci used in this study. Since LD can often occur because of undetected population structuring (as we might expect given this population could be structured by colonies), we also tested for evidence of LD within each colony and compared the amount of LD found in enslaved versus free-living colonies. We tested for differences in the genetic composition within colonies (number of alleles, frequency of loci in HWE, frequency of LD, etc.) found within enslaved and free-living colonies using t-tests accounting for unequal variance between samples if necessary or using Mann-Whitney U tests if the data was non-normally distributed.

## **Chemical data collection and analysis**

### *Chemical extraction*

Nineteen *Polyergus* and *Formica* workers were collected in 2009 from each of the 10 *Polyergus* colonies and 10 free-living *Formica* colonies sampled (190 *Polyergus* workers, 190 enslaved *Formica*, and 190 free-living *Formica*). We performed whole body extracts from individual worker ants by soaking each ant for 10 minutes in 200ul of hexane, after which the ant was removed. Upon evaporating the hexane, the body was placed in 95% ethanol for subsequent genetic analysis (see above). We kept hexane extracts in 9mm diameter gas chromatography vials (Varian, Palo Alto, CA) at -18°C at the field site. Chemical extracts were kept on dry ice and brought back to a lab in Arizona where the hexane was completely evaporated under nitrogen.

### *GC/MS analysis*

We analyzed ant cuticular hydrocarbon (CHC) profiles using an Agilent 7890A Gas Chromatograph (GC) interfaced with a 5975C Mass Spectrometer (MS) with triple access detector. We used an Agilent DB-5, 30m x 320µm x 0.24µm capillary column for separation of chemical compounds in the GC. To help characterize, compare, and identify the possible components of individual worker profiles, we first analyzed pooled extracts from enslaved *Formica*, free-living *Formica*, and *Polyergus* workers. To see if individual profiles needed to be filtered of polar compounds before GC/MS analysis, we ran half the volume of the pooled samples through a silica gel column constructed from a glass pipette stoppered with Pyrex fiber glass wool (8 microns, Sigma, MO) and filled with approximately 1ml of silica gel (column chromatography grade; Sigma, MO). The other half of the unfiltered extract was run on the GC/MS for comparison. We did not detect any differences between the profiles of filtered or unfiltered extracts and thus conducted GC/MS analysis on unfiltered individual extracts.

For analysis of individual ant CHC profiles, dried chemical extracts were re-eluted in 60ul of hexane, transferred into a vial insert (Varian #392611594) and 2ul of the extract was injected into the GC/MS by autosampler (Agilent 7683 Series) and run in splitless mode using the following temperature program: 70°C for 2min, 30°C/min to 200°C, 3°C/min to 300°C with a 5 minute hold at the final temperature. We set the MS to perform a full scan from 45-500 amu. A 10% solution of alkane standard mix (Fluka Analytical, MO, USA) was also run every 20 samples to monitor any changes in retention times and to provide another method for identifying chemical peaks across sample batches.

#### *Determination of chemical composition and variability*

We used the software MSD Productivity ChemStation (rev E.02.00) to detect, identify, and integrate individual and pooled chemical profiles. To determine whether there was higher variation in the chemical profiles of colonies with enslaved *Formica* compared to those of free-living *Formica*, we examined individual chemical profiles using two different methods. For our first method, we identified and compared the relative abundance of 14 chemical peaks that represented a subset of the peaks found across all *Formica* profiles (Table 3). These peaks could be clearly identified by their mass spectra for all *Formica* samples analyzed and, therefore consistently compared. Second, we counted the total number of peaks in the CHC profiles detected by the ChemStation software that met particular cut off criteria (see below). This other method allowed us to incorporate analysis of minor peaks that contributed less than 3% of the total composition of the individual 's chemical profile. Though these minor peaks were not clearly identifiable by mass spectrum, they may still include cues important for nestmate recognition. For both types of chemical variation analyses, we only analyzed chemical profiles that had sufficient concentrations of the 14 aforementioned chemical peaks to increase accuracy of detection of chemical peaks in individual CHC profiles.

For our first method of chemical analysis, we choose 14 chemical peaks that we could consistently detect, identify, and integrate across all individual samples (Table 3). We used pooled samples of enslaved *Formica*, and free-living *Formica* to choose these 14 chemical peaks because the concentrations of chemical peaks found in individual samples are lower and, therefore, the mass spectra are often difficult to interpret and use for proper identification of each peak. For analysis using these peaks, we used the RTE integrator and library search function in ChemStation. Using the pooled sample data, we custom-built a library of chemical peaks that each represented 3% or more of the total chemical profile after integration (this percentage was chosen based on the ability to reliably detect and identify chemical peaks present in individual profiles. Below this threshold, peaks could not be reliably detected, even if present). Each library entry represented a peak based on full mass spectrum data that could be matched to the chemical peaks found in individual GC/MS profiles. Preliminary library match searches were performed on a select group of individual samples to ensure reliability of library matches. Default parameters were used in the RTE integrator except that the minimum peak area detected was set to 0.5% of the largest peak. After integration, chemical peaks were subject to a library search parameters using the aforementioned customized library

including 14 peaks found across all *Formica* workers (enslaved and free-living) to verify the same chemical peaks were being analyzed across all samples. Default parameters were used for the library search strategy with the exception of U+A (set to 3) and the flag threshold (set to 2). We used the percentage peak area out of the 14 peaks counted to look at the variation in the relative amounts of these chemical peaks across enslaved and free-living *Formica*.

For our second variation measurement, we set the ChemStation software to detect peaks using the ChemStation integrator function that allowed us to count peaks within a specified retention time window of 10-30 minutes. By doing so we eliminated counting of peaks due to small amounts of impurities found naturally in the hexane or peaks that show up inconsistently at higher retention times due to issues of column bleed. We set up the ChemStation Integrator parameters using defaults for all parameters except that the initial threshold was set to 15 to maximize counting of clearly defined but minor peaks. Since the ChemStation integrator may be picking up peaks that may not be properly identified as CHCs, we also ran library match analysis (with default parameters) using a library of custom built chemical peaks found both in *Polyergus* and *Formica* that we believe only include CHCs. We used one-tailed t-tests to determine whether the average number of peaks detected within colonies was higher for enslaved compared to free-living *Formica*.

We also pooled together samples of *Polyergus* workers to qualitatively compare the CHC profiles of the social parasite and its hosts. We analyzed the pooled *Polyergus* chemical profiles using the same RTE integrator parameters mentioned above, custom built a library of identified chemical peaks based off of this pooled profile, and ran a library match search of the *Polyergus* specific library against one that contained *Formica* specific compounds to identify matching chemical peaks between the social parasite and its host. These matches were confirmed by visual comparison of the mass spectra for each chemical peak identified by ChemStation as having a match of 99% (the highest match score).

We used the packages *vegan* and *ecodist* in the program R to run a Nonmetric Multi-Dimensional Scaling (NMDS) analysis on the 14 peak chemical data set ( see first method of chemical variation analysis). We plotted the first and second factors of the NMDS to determine if enslaved and free-living *Formica* could be distinguished from one another based on differences in the relative proportions of the 14 chemical peaks. To determine whether there was a difference in the variation of individual worker chemical profiles of enslaved versus free-living individuals, we performed an analysis of similarity (ANOSIM) using the package *vegan* in R and the function *anosim*. ANOSIM uses a rank order dissimilarity matrix (such as one derived from a NMDS analysis) to determine whether there is a significant difference between two or more sample groups based on whether there are greater differences between these groups compared to within groups. Lastly, we compared the average distances from colony centroid points calculated from a chemical distance matrix derived in the R package *vegan*, using the program *betadisper*. Again, only the 14 specified chemical peaks was used to build the matrix and perform this analysis. This analysis addresses our second hypothesis using the 14 chemical peaks by comparing the

average distance of individuals from the colony centroid point of plotted chemical profiles. If our second hypothesis is supported, the average distances from the centroid point of enslaved *Formica* colonies will be greater than that of free-living colonies, indicating higher variation of the 14 chemical peaks amongst enslaved individuals within a colony compared to free-living individuals

### **Behavioral assays and analysis**

To test for differences in behavior of enslaved and free-living *Formica* towards non-nestmates, we performed behavioral assays in which we paired three workers from a designated focal colony with three individuals from a foreign colony (referred to hereafter as “invaders” or “invading colony”). Focal *Formica* workers were either enslaved *Formica* collected from a *Polyergus* colony (n=6) or from neighboring free-living *Formica* colonies (n=6) of approximately the same nest diameter. This avoids possible variations in behavior due to colony size since nest diameter may serve as a proxy for it (Cushman et al. 1988). Focal workers were paired with workers from invading colonies originating from nests close to the focal nest (less than 40m away and assumed to be within the raiding distance of *Polyergus* as we observed in 2009, n=12) or far from the same focal nest (+150m and known to be outside the raiding distance, see Topoff et al. 1985; Bono et al. 2006b, n=12), n=12). For each behavioral assay, we paired workers from the aforementioned focal and invading colonies and categorized them into four treatment types (six colony pairings of 10 trials each were performed per treatment type): enslaved versus invading free-living *Formica* from close nests, enslaved versus free-living *Formica* from far nests, free-living *Formica* versus free-living from close nests, and free-living *Formica* versus free-living from far nests.

For each assay trial, three *Formica* workers from the same focal colony were marked on their gasters with a small dot of acrylic paint, placed in a neutral arena lined with Fluon, and allowed to acclimate. Next, three unmarked workers from an invading colony were introduced to the neutral arena and their behaviors and which individuals initiated them (focal or invader) were recorded for three minutes. If the initiator of aggression could not be determined, the aggressive act was counted as mutually aggressive (neither focal or invader was counted as an aggressor). We recorded the following as aggressive acts: mandible flaring (threat display), leg and antenna pulling, mandible grappling, and gaster flexing (includes attempts to apply formic acid). All behavioral trials were conducted blind so that the observer did not know whether pairings involved negative controls or treatments. Negative controls consisted of 10 trials pairing together nestmates from all involved focal colonies (n=12).

We analyzed behavioral trials based on the presence or absence of aggression in the focal worker (enslaved or free-living) once we first observed an aggressive act. In other words, if the invading individual initiated aggression, the focal individual was counted as having an absence of aggression. We tested the hypothesis that enslaved *Formica* would be less aggressive towards non-nestmates compared to free-living *Formica* by running a generalized linear mixed model (GLMM) in the program *R* (<http://cran.r-project.org>) using the package *lme4* and

the function *lmer*. As a response variable, we used presence or absence of aggression of focal worker and the identity of the focal worker (enslaved or free-living) and the distance of the invading worker (originating from a nest close or far from the focal nest) as fixed-factors in the model. To avoid issues of pseudo-replication, we chose worker nest identity as a random factor in the model. We tested for the interaction between focal worker identity and distance of the invading nest and also examined several reductive models where we looked at the effect of distance alone, the effect of worker identity alone, and both added together in the model (not interacting). A model incorporating the identity of the focal worker and distance as interacting terms yielded the best-fit model based on the AIC scores produced from the models involving the factors mentioned above and conducting ANOVA tests on those models.

## RESULTS

### **Genetic composition and variation in enslaved vs. free-living *Formica***

#### *Summary across all colonies (treated as one population)*

We genotyped 371 *Formica* workers (189 enslaved, 182 free-living) at 11 loci, yielding 78 alleles. The average number of alleles per locus was  $7.091 \pm 1.676$  (SE) and the average effective number of alleles was  $3.473 \pm 0.653$ . Across the whole population the expected heterozygosity ( $H_e$ , unbiased) was  $0.589 \pm 0.075$  (SE) and the average fixation index ( $F_{st}$ ) was  $0.071 \pm 0.044$  (SE).

The test for Hardy-Weinberg Equilibrium (HWE) for the whole population showed only three loci with non-significant departures: Fy5, FL12, and FE21. Analysis using MicroChecker suggested no evidence of large allele drop out for any of the loci. However, possible null alleles were suggested for Fy4, Fy7, FE16, FL20, and FL29.

Tests for pairwise linkage disequilibrium between all pairs of loci across all *Formica* individuals revealed that 80% of the possible loci pairs were in linkage disequilibrium.

#### *Comparison of genetic composition of enslaved and free-living colonies*

Since the formation of colonies may affect how population of ants are structured, we also tested for departures from HWE on a per colony basis. For each colony, we found no loci that were consistently in or out of HWE. However, the average number of loci in HWE for enslaved *Formica* colonies was  $7.8 \pm 1.87$  (mean  $\pm$  SD) while for free-living colonies the average number of loci in HWE was  $4.3 \pm 1.49$  and the difference between these were significant ( $t = 4.6179$ ,  $p=0.0001$ ).

Although the average LD on a per colony basis ( $27.9 \pm 12.8\%$ ) was lower than if all individuals were treated as one population, we found no significant difference between the percentage of pair-wise LD found in enslaved and free-living *Formica* colonies ( $t = -0.7728$ ,  $p = 0.45$ ).

We found that the average number of alleles ( $N_a$ ), number of effective alleles ( $N_e$ ), number of polymorphic loci, heterozygosity ( $H_e$ ) and unbiased heterozygosity

were higher in colonies of enslaved *Formica* compared to free-living *Formica* (Table 4).

### **Chemical composition and variation in enslaved and free-living *Formica* and their social parasites, *Polyergus breviceps***

Our comparison of *Polyergus* and *Formica* chemical profiles revealed that *Polyergus* have at least twice the number of chemical peaks within their profiles compared to *Formica* profiles (see Table 2). We found pooled samples of *Polyergus* had 39 chemical peaks that were either absent from or below 0.5% of the total peak areas found in the pooled *Formica* profiles (72.2% of the peaks identified in Table 2). The pooled *Formica* profiles shared 16 chemical peaks with those of the *Polyergus* profiles (Figure 2, 29.6% of the peaks identified in Table 2). Ten of the 14 chemical peaks used in the chemical analysis of individual *Formica* profiles (see below for details), matched those found in *Polyergus* (Figure 3; Table 3). Overall, it appears that the chemical peaks shared between *Polyergus* and *Formica* are primarily saturated alkanes and alkenes (as opposed to branched alkanes or co-eluting chemical peaks).

After removal of some individual chemical profiles based on poor quality, we analyzed chemical profiles of 326 *Formica* workers (146 enslaved, 180 free-living) and looked for differences between enslaved and free-living workers in the quantities of the 14 major peaks present (see Table 3) and the total number of major and minor peaks (less than 3% of the total composition of profile peaks) present as detected by the ChemStation software.

Qualitatively, there appears to be no difference in presence of the 14 major peaks for enslaved *Formica* compared to free-living *Formica* profiles. Quantitatively, however there are differences in the relative proportions of these 14 peaks for enslaved and free-living *Formica* profiles analyzed collectively as groups (as opposed to a per colony basis). The NMDS plot revealed a subtle distinction between enslaved and free-living *Formica* individuals (Figure 4). The ANOSIM showed that the differences among enslaved *Formica* individuals were greater than that of free-living *Formica* and that, overall, there is a significant difference between the relative proportions of the 14 chemical peaks analyzed for both groups ( $R=0.114$ ,  $p=0.001$ ; Figure 5). These differences do not appear to be driven by those peaks that are shared with *Polyergus* but likely from those peaks unique to *Formica* (data not shown). The average ( $\pm$ SD) distance of individual chemical profiles from the centroid of their colony (plotted data not shown) was  $0.091\pm 0.032$  for enslaved *Formica* colonies and  $0.76\pm 0.30$  for free-living colonies but the difference between them was not significant ( $t = 1.119$ ,  $p=0.139$ ).

The total number of peaks detected by ChemStation was significantly higher in colonies of enslaved *Formica* compared to free-living colonies (mean  $\pm$  S.D. of enslaved= $53.29\pm 6.1$ , mean for free-living= $39.69\pm 4.0$ ,  $t=5.91$ ,  $p<0.001$ ). The same was also true for the number of peaks detected after using the ChemStation's library match and produced slightly smaller but similar mean values for both groups (mean of enslaved= $47.61$ , mean of free= $34.81$ ,  $t=5.89$ ,  $p<0.001$ ).

### **Behavior of enslaved and free-living *Formica* towards free-living non-nestmates**

Across all four treatment pairings (n=24), the average proportion of trials resulting in aggression was  $0.795 \pm 0.163$ . Aggression was not observed in any of the negative controls.

Overall, enslaved *Formica* workers were significantly less aggressive towards free-living non-nestmates compared to workers from free-living focal colonies (Figure 1, z value=3.61, p=0.0003). When taking into account distance of the nest of the invading non-nestmate from the focal worker (close versus far), we see that enslaved *Formica* show a lower frequency of aggression towards non-nestmates from nearby compared to those far away. In contrast, free-living *Formica* appear less aggressive towards non-nestmates from distant colonies compared those from neighboring ones. However, when we tested the interaction between identity of the focal worker (enslaved versus free-living) and distance of the invading colony (close versus far from the focal nest), we found it was significant for a p value of less than 0.1 but not less than 0.05 (z value=-1.89, p=0.057). Additionally, distance alone does not appear to determine whether the focal ant is aggressive towards non-nestmates (z value=1.733, p=0.08).

### **DISCUSSION**

Results from this study support all three of our hypotheses: enslavement by *Polyergus* appears to increase the genetic and chemical diversity of *Formica* within parasitized colonies compared to *Formica* within free-living colonies (Hypothesis 1 and 2), and parasitized *Formica* behave less aggressively towards non-nestmates compared to free-living *Formica* (Hypothesis 3).

In support of our first hypothesis, we saw significantly higher genetic variation of *Formica* within enslaved colonies compared to free-living *Formica* colonies. The higher measurements of genetic diversity we found for colonies of enslaved *Formica* is likely a signature of *Polyergus* raiding *Formica* pupae from neighboring *Formica* colonies that presumably contribute different alleles to the enslaved colony. These results are not surprising given personal observations of raiding behavior in this population of *Polyergus* and other previously studied populations (Topoff et al. 1985; Bono et al. 2006b) in which slaves were collected from multiple different *Formica* colonies.

A possible consequence of having a more genetically diverse pool of individuals living within one colony is that there may also be increased chemical diversity of *Formica* living within the *Polyergus* colony. This may be expected if we assume chemical cues are controlled by a strong underlying genetic component and, indeed, there have been recent studies that support this idea (van Zweden et al. 2009; Soro et al. 2011). Additionally, *Formica* may also pick up chemical cues from their slave-makers through social grooming and food exchange, creating an even



more diverse set of cues that may be shared among enslaved *Formica* within a *Polyergus* colony. These should cause colonies of enslaved *Formica* to be more chemically diverse than free-living *Formica*.

Analysis of the cuticular hydrocarbon (CHC) profiles of *Polyergus* and enslaved *Formica* workers provides insights into the chemical cues that may shape the nestmate recognition behavior of enslaved *Formica*. Despite sharing some chemical peaks with their slaves, *Polyergus* appeared to maintain their own, genus-specific CHC profile: we found 39 chemical components unique to the slave-maker. These results are in line with previous research showing that slave-making ants tend to maintain a species-specific profile, despite some matching of cues with their host (Habersetzer and Bonavita-Courgourdan 1993; Liu et al. 2003; but see Errard et al. 2006b). *Formica* also had a few chemical peaks that did not appear in the *Polyergus* profile. These unique differences between slave-maker and slave suggest that enslaved *Formica* are, indeed, exposed to a diversity of different of chemical cues within the nest (whereas free-living *Formica* are not). Because a complete sharing of all available CHC cues between *Formica* and *Polyergus* workers within the same colony does not occur, a common gestalt odor (all individuals sharing common chemical cues through social interactions) does not appear to apply to our study system.

However, there is still the possibility that parasitism by *Polyergus* influences the chemical profiles of enslaved *Formica* such that parasitized individuals may have profiles that differ from their free-living counterparts. In particular, *Formica* raided from several different colonies could contribute a mix of chemical cues amongst enslaved *Formica*, if each raided colony possessed different chemical signatures. Comparison of enslaved and free-living *Formica* CHC profiles shows that parasitism by *Polyergus* does alter the chemical composition of *Formica* ant profiles, both qualitatively and quantitatively. Although both groups of *Formica* shared 14 of the most prominent and identifiable compounds (eg. peaks featured in Figure 3; henceforth termed “major peaks”), individually ants differed quantitatively in the relative proportions of these peaks. More specifically, in the NMDS and ANOSIM analyses, individual enslaved *Formica* were quantitatively distinguishable in the relative proportions of major peaks from individual free-living *Formica*. Still, these analyses were done by lumping together all enslaved or free-living *Formica* individuals, without regard to colony membership (individuals were the units of analysis). Therefore, the results of the NMDS and ANOSIM analyses only tell us that there is a quantitative difference in major peaks between individual *Formica* that are enslaved compared to free-living ones, but they do not address our second hypothesis (that individuals within enslaved *Formica* colonies will be more chemically variable than free-living ones).

To test our second hypothesis, we considered the aforementioned major peaks on a per colony basis, and found no difference in the variation of the relative proportion of the major peaks within enslaved colonies versus free-living *Formica* colonies. In contrast, the analyses that included minor chemical peaks (those with concentrations too low to confirm identification by mass spectrum) within colonies revealed that enslaved *Formica* colonies had a larger total number of chemical peaks

than free-living *Formica* colonies, suggesting greater variation in the presence or absence of such peaks. It may be that some of these minor peaks include the cues that are important for *Formica* nestmate recognition. When these minor peaks were included in our analyses, we saw support for our second hypothesis of higher chemical diversity within enslaved *Formica* ant colonies. These results also emphasize the fact that, although certain chemical peaks may comprise a large proportion of a chemical profile, their presence or relative abundance may not be solely responsible for nestmate recognition.

Because colonies of enslaved *Formica* are both more chemically and genetically diverse than free-living *Formica*, we expected that there may be differences in how the two groups behave towards non-nestmates. Our behavioral data support the hypothesis that enslaved *Formica* workers are less aggressive towards non-nestmates compared to free-living *Formica*. To date, only one study by Habersetzer (1993) has explored colony-mate recognition behavior in a slave-making ant system. This study showed that free-living *Formica rufibarbis* were always the initiators of aggression when paired with *F. rufibarbis* enslaved by *Polyergus rufescens*. Enslaved *F. rufibarbis* showed no aggression towards enslaved non-nestmate *F. rufibarbis* as well as non-nestmate *P. rufescens*. In line with our study, Habersetzer's findings suggest that parasitism by *Polyergus* affects the nestmate recognition of their host by reducing aggression towards non-nestmates. However, due to sampling and experimental design, the Haberstzter study could not explicitly test the same behavioral hypothesis we test here (a direct comparison of nestmate recognition between enslaved and free-living *Formica*).

Differences in aggression between enslaved and free-living *Formica* could arise from differences in the chemical environment of the two types of colonies. In particular, enslaved *Formica* are exposed to *Polyergus*' diverse chemical cues as well as a diversity of cues from enslaved *Formica* from several different colonies. In contrast, free-living *Formica* are only exposed to a lower variety of cues from genetically less diverse (and likely closely related) nestmates. Since chemical cue exposure is key to forming the nestmate recognition template used by ants to distinguish friend from foe (Errard et al. 2006a), template expansion could contribute to the observed reduction in aggression of enslaved *Formica*. Under a template-chemical cue matching model (Lacy and Sherman 1983), a template that expands to incorporate a larger diversity of cues is expected to produce an overall higher rate of acceptance of encountered individuals. Indeed, a previous study using artificially mixed species colonies showed that increased chemical dissimilarity among nestmates leads to lower aggression toward ants from other colonies (Errard et al. 2006a). More specifically, we expected to find lower aggression towards ants from colonies in close proximity to enslaved *Formica* colonies, as they are likely to have been raided and/or be closely related to previously raided nests. As such, enslaved *Formica* are more likely to be familiar with the chemical cues of neighbors within raiding distance and have these cues in their templates.

Such a model of template broadening also implies that a template would have to be updated frequently in enslaved *Formica* as new members are regularly added to the colony through seasonal raids. Indeed, the idea templates can be updated in

response to ecological changes has been previously proposed (Errard and Hefetz 1997; Leonhardt et al. 2007).

A study performed by Lorenzi (2003) on the socially parasitized wasp *Polistes atrimandibularis* showed that workers in parasitized colonies were more tolerant of non-nestmates compared to unparasitized ones. However, unlike our finding of no aggression between pairs of nestmates, Lorenzi found that wasp nestmates were also more often rejected in parasitized colonies. Lorenzi reasoned that template broadening in the parasitized wasps might have made it more difficult for them to compare their template to the cues of other wasps. He concluded that changes in the parasitized wasps' template resulted in a general impairment of their recognition system. While this may also be the case in our system, we feel there may be another explanation for the patterns of behavior we saw in parasitized *Formica* other than template broadening.

Although enslaved *Formica* displayed less frequent aggression toward free-living *Formica* from nearby colonies (consistent with template broadening, discussed above), we found that they were also less often aggressive toward non-nestmates from distant nests. Because these non-nestmates originated from colonies well outside the raiding distance, the reduced aggression toward them cannot be explained by template broadening (since enslaved *Formica* would be unlikely to have encountered and incorporated the cues of such distant non-nestmates). Instead, this observation suggests that enslaved *Formica* exhibit an overall lower frequency of aggression towards non-nestmates, whether or not they have encountered them before. Lowered aggression may arise if a so-called shift in the "acceptance threshold" of an individual has occurred so that the cost of rejecting nestmates with diverse cues is minimized (see Liebert and Starks 2004 for review). When such a shift happens, mismatches between the template and encountered chemical cues may be tolerated as long as the level of incongruence between the two does not exceed this threshold. Reeve's response threshold model makes certain predictions about whether such a threshold will shift, and in what direction, depending on what is the most adaptive situation (Reeve 1989). In our study system, enslaved *Formica* may shift their acceptance threshold such that the rules of matching the template and the label are less stringent. This would allow enslaved *Formica* to be more permissive toward deviations between their template and the cues they are evaluating, thereby decreasing overall aggression towards non-nestmates.

Taken as a whole, our genetic, chemical and behavioral data reveal that social parasitism by *Polyergus breviceps* has affected the nestmate recognition system of its host, *Formica*. Here we find that enslaved colonies of *Formica* show more chemical and genetic diversity than free-living *Formica*. Behaviorally, enslaved *Formica* show a lower frequency of aggression towards non-nestmates compared to their free-living counterparts, even if these non-nestmates are likely to have never been encountered before. This difference in behavior may be explained by the fact that raiding behavior by the slave-maker clearly increases chemical variation within the enslaved colony, thus producing a situation in which nestmate recognition in enslaved *Formica* may be altered to ensure cohesiveness of the mixed colony.

This is the first study that gives us insight into how nestmate recognition functions in ants in the face of increased genetic and chemical variation caused by the annual addition of new colony members by a slave-making ant. In this way, we can begin to explore the mechanisms of template broadening and shifts in the threshold of acceptance as they relate to the behavioral response of an organism. These components are important for understanding the perception and action aspects of nestmate recognition in ants (how individuals evaluate, interpret, and act upon differences in the template of chemical cues encountered). Finally, this study provides insight into the ecological consequences of slave-making for *Formica* which is important for understanding the possible co-evolutionary dynamics between this social parasite and its hosts.

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**Table 1.** Variations in PCR conditions microsatellite loci using 10ul reaction volumes.

Species of origin and loci	MgCl <sub>2</sub>	BSA <sup>1</sup>	Taq	T <sub>a</sub> <sup>2</sup>
<i>F. yessensis</i> (Fy4, Fy5, Fy7, Fy13)	1.25 mM	0 mg/ml	0.04 units	48°C
<i>F. lugubris</i> (FL12, FL20, FL21, FL29)	1.25 mM	0 mg/ml	0.075 units	54°C
<i>F. exsecta</i> (Fe16, Fe21, Fe37)	1.5 mM	0.2mg/ml	0.075 units	56°C

<sup>1</sup> Bovine Serum Albumin (New England Biolabs), added as a reaction enhancer

<sup>2</sup> Annealing temperature

**Table 2.** 55 chemical peaks found in pooled samples of *Polyergus breviceps* colonies with peak numbers presented in the order of their retention times. This table continues onto the next page.

Peak Number	Suspected chemical class	Major Diagnostic ions (minor ion peaks <sup>3</sup> )	In <i>Formica</i> ?
1	C21 monoene	294	
2	C21 monoene	294	
3	C21 alkane	296	X
4	9 and 11-Me C21 alkanes	140, 168, 196, 295 (310)	
5	C22 alkane	310	X
6	C23 diene	181, 196, 222, 320	
7	C23 monoene	322	X
8	C23 monoene	322	
9	C23 alkane	324	X
10	9 and 11-Me C23	140, 168, 196, 224, 309, 323 (338)	
11	3-Me C23 and C24 monoene	281, 309, 336	
12	C24 alkane	338	X
13	Multiple co-eluting multi-methyls (unknown)	168, 211, 295, 323, 337	
14	C25 diene	(250) 348	
15	C25 monoene	(322) 350	X
16	C25 monoene	350	
17	C25 alkane	352	X
18	C25 diene	138, 208, 281, 348	X
19	11 and 13-Me C25	168, 196, (224), 351	
20	7 Me C25 + unknown	112, 252, 281, 351 (365)	
21	5-Me C25	149, 280, 309, 351	
22	C25 diene with methyl group alkanes (unknown)	124, 168, 211, 239, 323, 348 (365)	
23	4-Me C26? And C26 monoene	309, 357, 364	
24	C26 alkane	366	X
25	13-Me C26 +unknown	168, 182, 196, 323, 351, 365	
26	13-Me C26 + unknown	168, 182, 196, 210, 351, 365	
27	C27 diene	180, 250, 264, 278, 292, 376	
28	C27 monoene	111, 125, 350, 378	X
29	C27 monoene	108, 222, 250, 318, 345, 378	
30	C27 diene	138, 222, 250, 309, 376	X

<sup>3</sup> These peaks, though diagnostic for proper identification of peaks used in this study, were not taken into account when determining the chemical class indicated above.

31	11 and 13 Me- C27	168, 196, 224, 379	X
32	4, 23 dimethyl C26	337, 365, (392)	X
33	Pentamethyl C25?	155, 196, 239, 280, 323, 351, 393	
34	C28 alkane	394	
35	9, 23 dimethyl C27	296, 224, 281, 351, 379 (393)	
36	C28 muliti-methyl (unknown)	168, 182, 196, 210, 224, 238, 393	
37	C29 monoene	406	X
38	C29 alkane	408	X
39	11 and 13 and 15-Me C29	141, 168, 196, 224, 252, 280, 407	
40	C29 unknown	112, 169, 206, 253, 281, 309, 337, 365, 407	
41	5 and 7 Me-C29	336, 365, 379, 393, 407	
42	15, 19 diMe-C29 and 12 and 15-Me C30	168, 196, 224, 239, 267, 295, 407, 421	
43	5, 25 dimethyl C28	365, 393, 421	
44	C27 trimethyl (unknown)	155, 196, 224, 280, 308, 351, 379, 393, 407, 421	
45	C30 unknown	183, 224, 252, 281, 309, 379, 407, 421	
46	14 and 15 Me C30	182, 196, 210, 224, 238, 379, 393, 407, 421	
47	4 and 12 Me C30	207, 281, 351, 393, 407, 421	
48	C31 monoene	407, 434	
49	C31 alkane	436	X
50	11, 13, 15 and 17-Me C31 (co-eluting)	168, 196, 224, 252, 280, 309, 435	
51	Unknown multi-methyl	168, 196, 224, 239, 267, 295, 323, 449	
52	Unknown	183, 157, 211, 252, 280, 309, 435, 489	
53	11, 12, 15 and 17-Me C33 (co-eluting)	168, 196, 224, 252, 281, 308, 337, 463	
54	Unknown multi-methyl	168, 196, 239, 323, 351, 477	
55	Unknown C34	168, 239, 281, 309, 365, 477	

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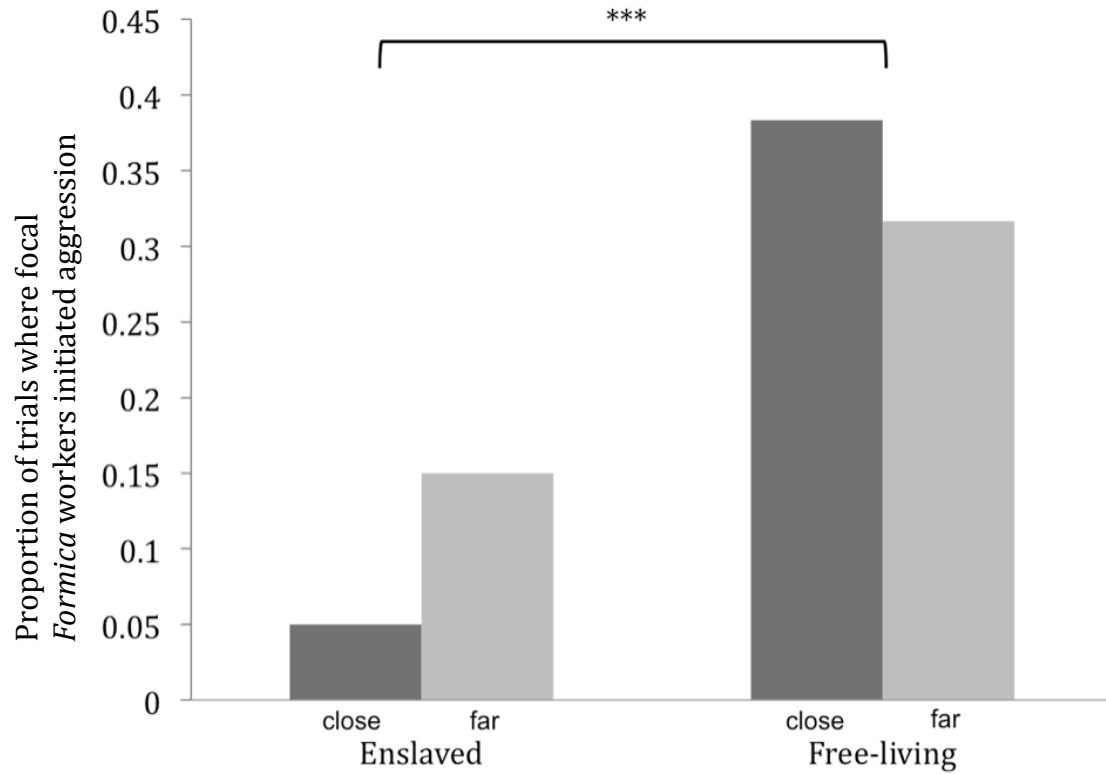
**Table 3.** A subset of fourteen major peaks present in enslaved and free-living *Formica altipetens* chemical profiles and used in the NMDS and ANOSIM analyses.

Peak Number	Retention time (min)	Suspected chemical class	Diagnostic ions (additional ion <sup>4</sup> )	In <i>Polyergus</i> ?
1	10.77	C21 alkane	296	X
2	13.2	C23 monoene	(294) 322	X
3	13.6	C23 alkane	324	X
4	15.3	C24 alkane	338	X
5	16.58	C25 dialkene	(250) 348	
6	16.66	C25 monoene	350	
7	16.73	C25 monoene	350	X
8	16.8	C25 alkane	352	X
9	17.1	C25 dialkene	348	X
10	17.4	C26 monoene	337, 364	
11	18.57	C27 monoene	(350) 378	X
12	20.6	C27 alkane	380	
13	21.1	C29 monoene	(380) 406	X
14	24.6	C29 alkane	408	X

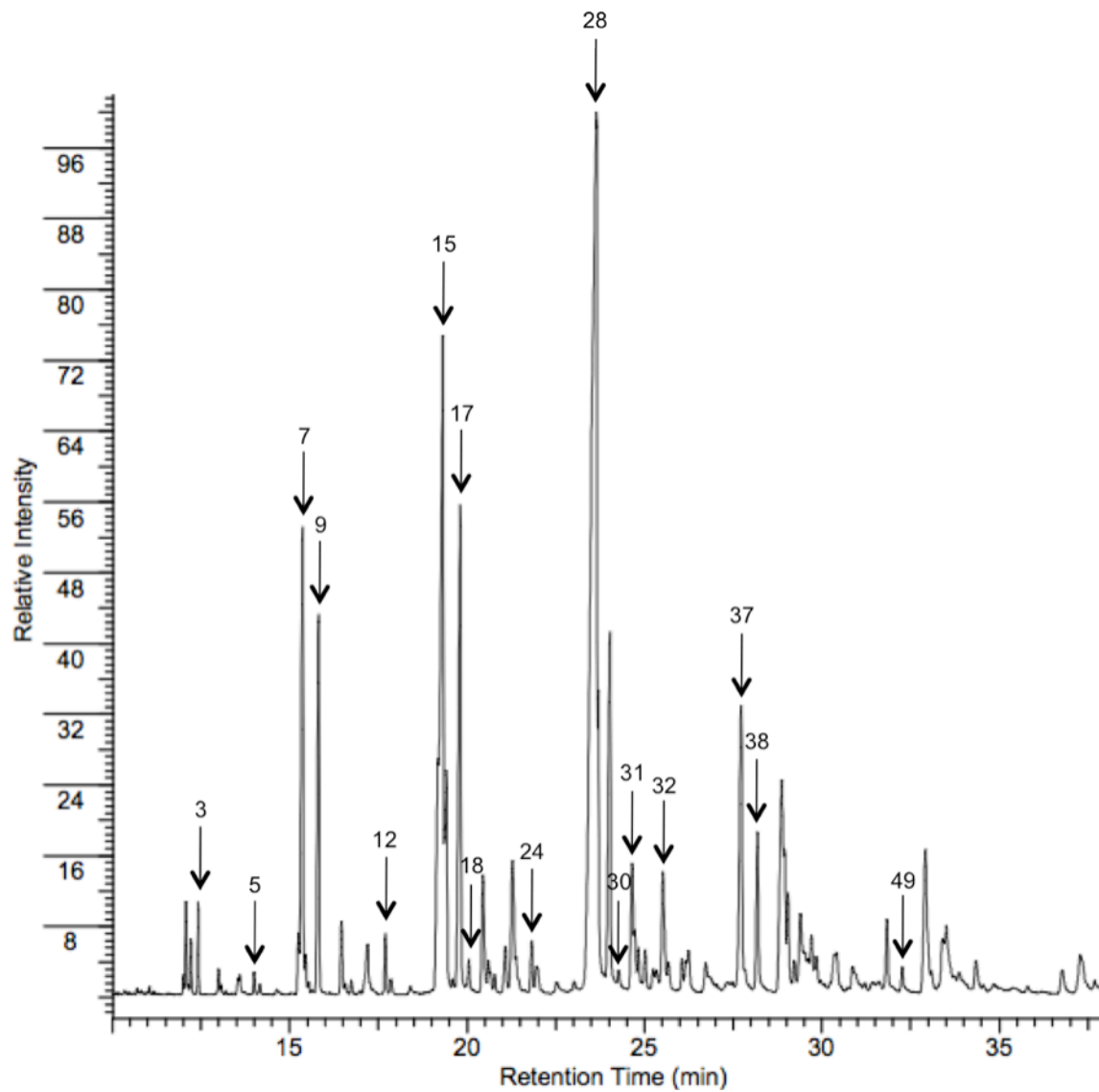
<sup>4</sup> These peaks, though diagnostic for proper identification of peaks used in this study, were not taken into account when determining the chemical class indicated above.

**Table 4.** Comparison of average ( $\pm$ SD) genetic composition found within colonies of enslaved (N=10) and free-living *Formica* (N=10). Na=number of alleles, Ne=effective number of alleles, and He=expected heterozygosity and all other statistics listed below are averaged over the 11 microsatellite loci used in this study.

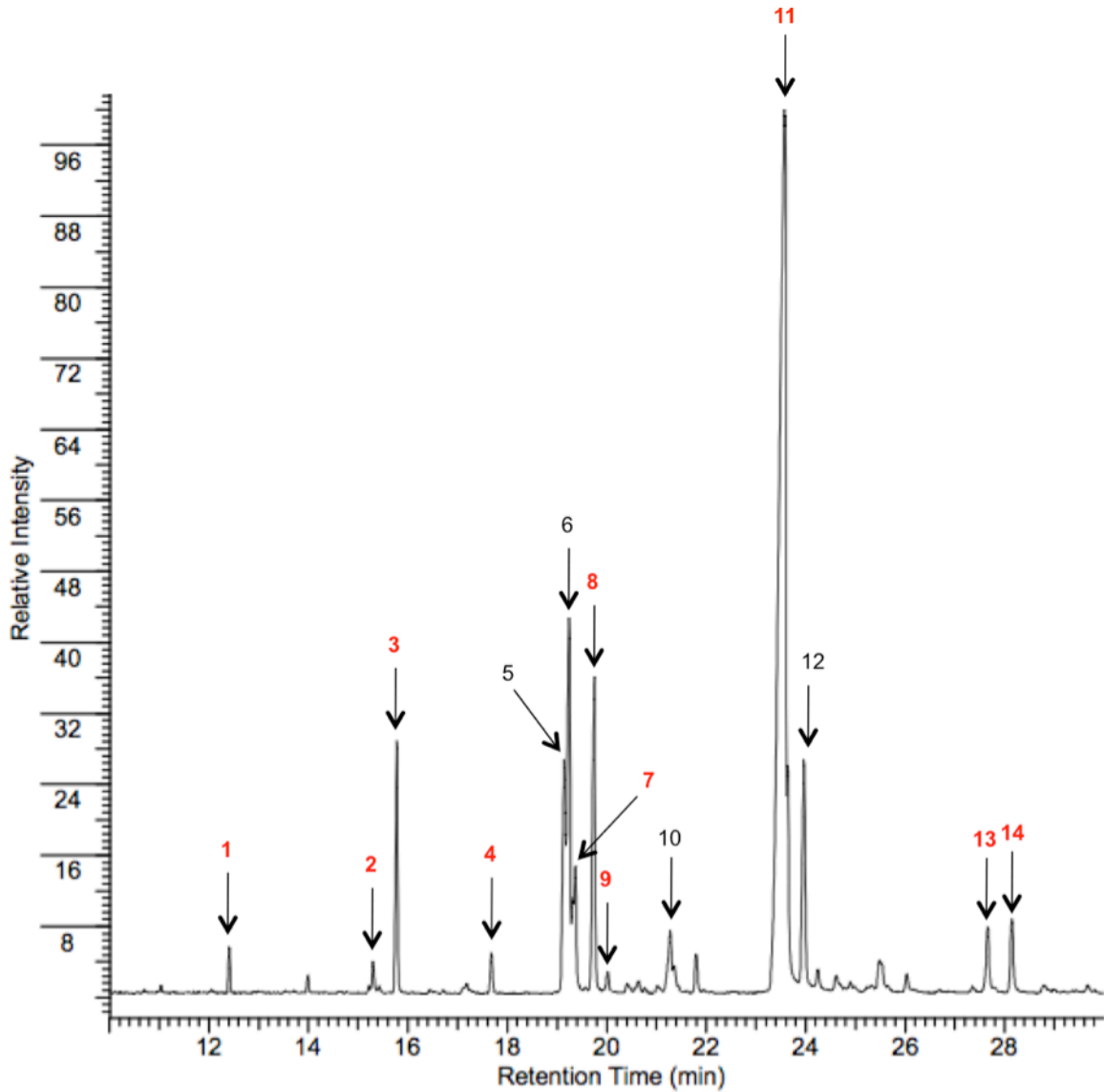
	Na	Ne	Polymorphic loci (=>5% frequency)	He	Unbiased He
Enslaved <i>Formica</i>	4.52 $\pm$ 0.46	3.04 $\pm$ 0.37	3.95 $\pm$ 0.39	0.551 $\pm$ 0.31	0.5661 $\pm$ 0.32
Free-living <i>Formica</i>	2.46 $\pm$ 0.41	1.94 $\pm$ 0.24	2.30 $\pm$ 0.33	0.404 $\pm$ 0.76	0.4157 $\pm$ 0.78
p-values <sup>5</sup>	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001



**Figure 1.** Comparison of the proportion behavioral trials which focal workers (enslaved or free-living) initiated aggression towards non-nestmates from colonies close by (<40m from focal nest) or far away (+.150m from focal nest). Negative controls between paired nestmates of focal colonies are not shown here as none of these trials resulted in aggression. \*\*\* indicates a p-value of <.0005 when comparing enslaved and free-living categories as a whole (lumping close and far variables within).

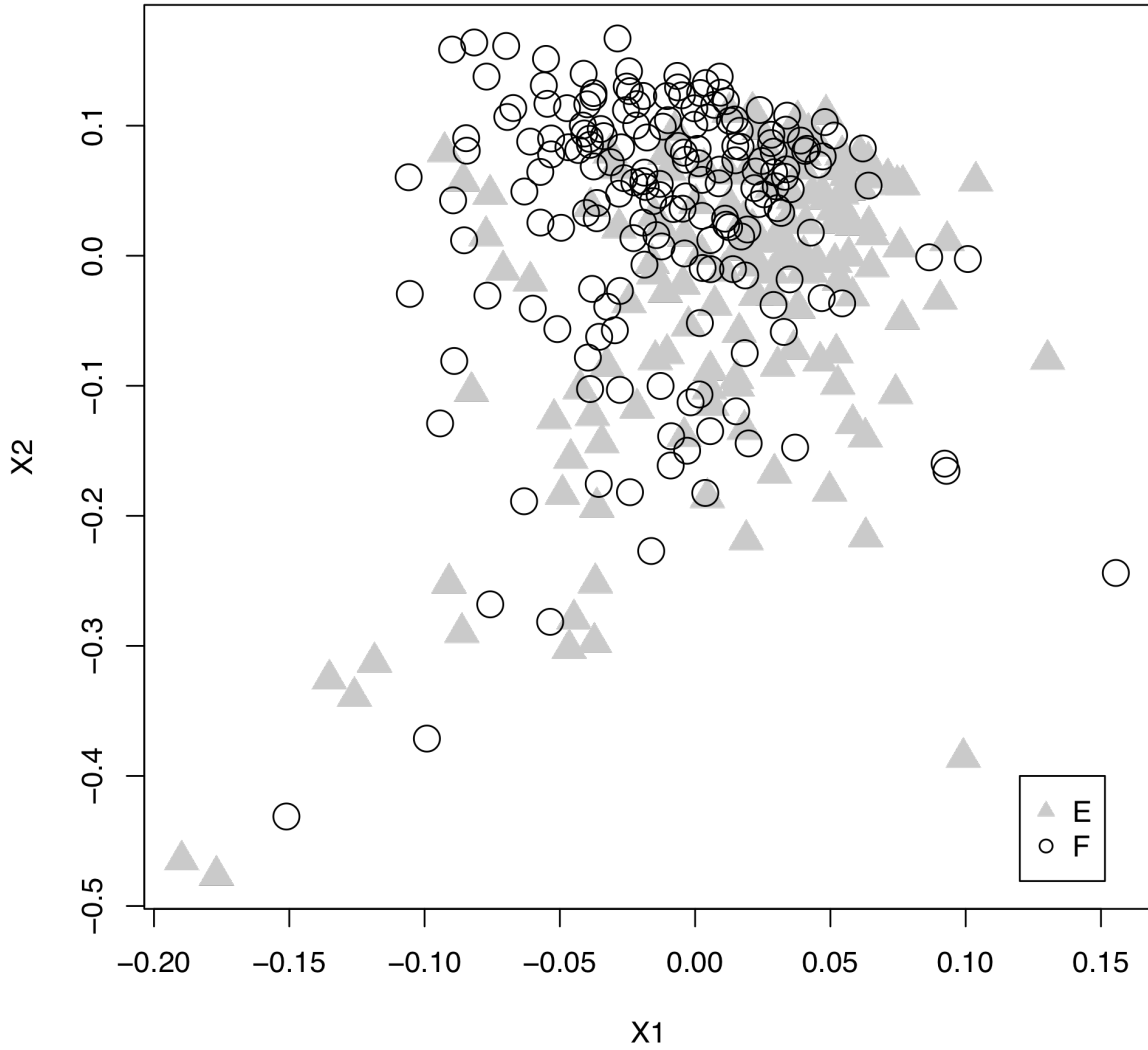


**Figure 2.** Pooled chemical profile of 20 *Polyergus breviceps* workers from 10 different colonies. Numbered peaks correspond to the chemical components found in Table 2 that are also present in the host, *Formica altipetens*.

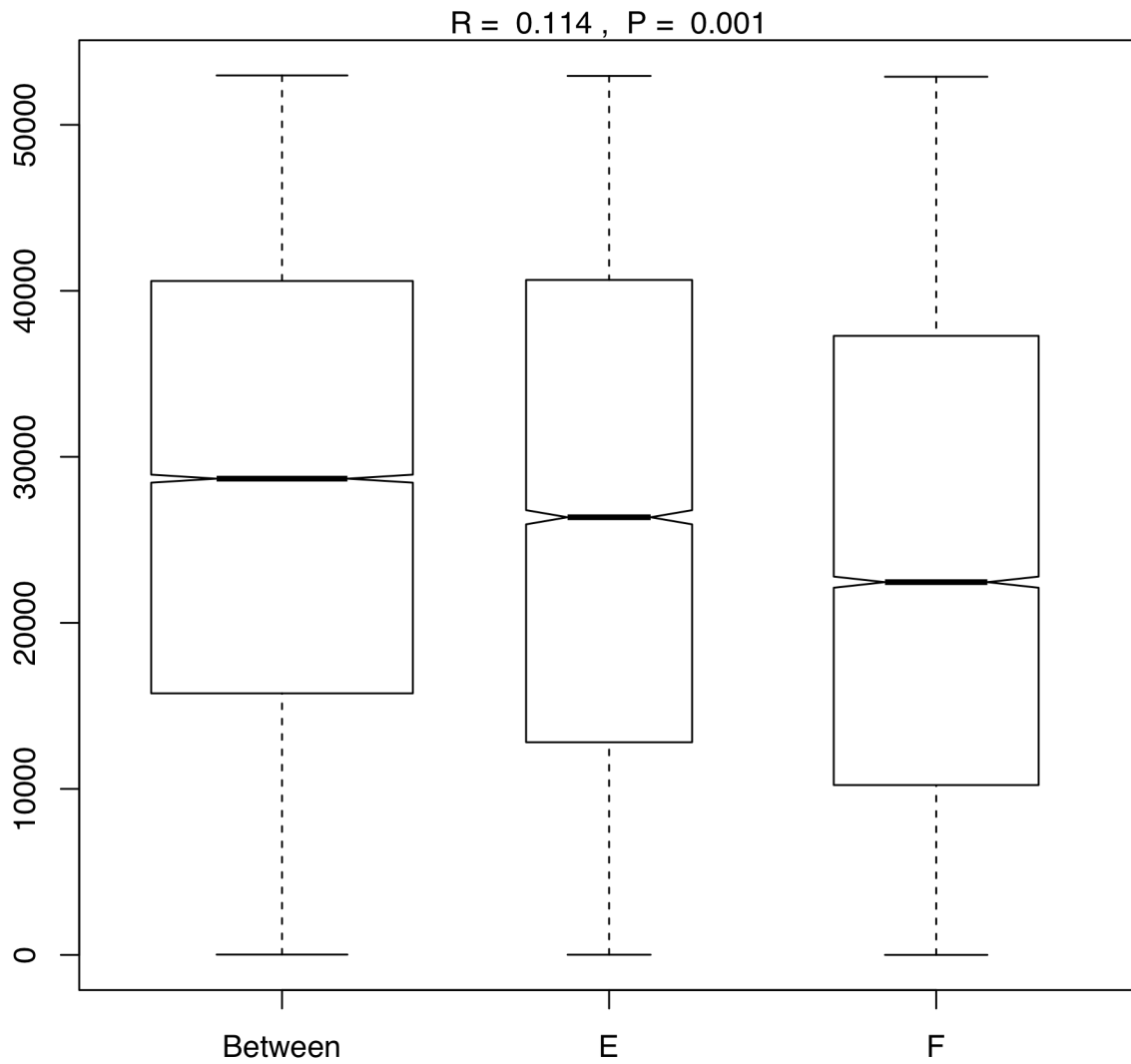


**Figure 3.** Pooled chemical profile of 20 enslaved *Formica* workers from 10 different colonies. Numbers correspond to the 14 chemical peaks present in both enslaved and free-living *Formica* that were used in one method of chemical analysis (see Table 3). Numbers in red represent chemical peaks also present in *Polyergus breviceps*.





**Figure 4.** NMS (Nonmetric multidimensional scaling) plot of individual *Formica* workers from enslaved colonies (E; triangles) and free-living colonies (F; circles) based on the relative proportions of 14 chemical peaks detected in their cuticular hydrocarbon profiles.



**Figure 5.** ANOSIM (Analysis of Similarity) results showing degree of dissimilarity within and between enslaved (E) and free-living (F) *Formica* workers grouped together. Y axis shows dissimilarity ranks. The R value indicated above is the test statistic for ANOSIM and ranges from -1 to 1 with 0 indicating complete similarity between the two groups being compared.

## CHAPTER 2

Chemical ecology and population genetics of the slave-making ant, *Polyergus breviceps*, parasitizing multiple sympatric species of ant in the genus *Formica*

## INTRODUCTION

The availability of multiple species of host in sympatry may lead to the diversification among parasites that specialize on them (Criscione et al. 2005). When host specificity is followed by selection on particular host-adaptive traits, new lineages of parasites may form and reproductive barriers may arise (e.g. Filchak et al. 2000; Sorenson et al. 2003; Forbes et al. 2009; Malenke et al. 2009). Despite phenotypic and ecological evidence for host specificity and formation of host races, complete speciation may not occur if gene flow reconnects the genetic lineages that have specialized on different hosts (e.g. Spottiswoode et al. ; Feder et al. 1988; Marchetti et al. 1998). Examination of these ecological and population-level processes in parasite-host systems allow us to gain a better understanding of the evolutionary consequences of specialization on hosts in sympatry.

Brood parasites and social parasites are interesting cases for studying host-race formation and the possibility of sympatric speciation, when more than one host is available. Unlike conventional ecto and endoparasites, these parasites take full advantage of the nurturing behaviors of their host. Avian brood parasites, those exploit the rearing behavior of other birds by laying eggs in their hosts' nests, provide convincing examples of host-race formation (i.e. *gentes*; Marchetti et al. 1998; Gibbs et al. 2000) and even speciation in sympatry (Sorenson et al. 2003). Less well studied are social parasites, those that exploit the vast resources of entire societies of ants, bees, wasps or termites.

Evasion of host recognition systems through mimicry or camouflage is critical to the survival of social parasites, including avian brood parasites and those of social insects. For avian brood parasites, matching of host egg morphology (Brooke and Davies 1988; Starling et al. 2006) and mimicking of host offspring begging behavior (Langmore et al. 2008) and mouth markings (Payne 1982; Payne 2005) increases the likelihood of the parasite's success. In the case of social insects, matching of the chemical cues used in nestmate recognition is important for acceptance of parasites into a host colony (Lenoir et al. 2001).

Slave-making ants in the genus *Polyergus* are obligate social parasites that rely on their closely related ant hosts, *Formica* spp., to perform all colony tasks including brood care, nest maintenance, defense, and foraging. Unlike other social parasites, slave-making ants have a two-fold impact on their hosts (Brandt et al. 2005a). First, slave-making ant queens take over an existing host colony by killing and replacing the host queen (Topoff et al. 1988). The usurped *Formica* colony members then assist the new *Polyergus* queen in raising her offspring: the slave-making ant workers. These workers impose the second effect of slave-making: frequent raiding of neighboring *Formica* host nests for brood to replenish the supply of slave workers (Topoff et al. 1985; Bono et al. 2006).

Because of their obligatory relationship with their hosts, slave-making ants must avoid rejection by using some form of chemical adaptation to achieve colony integration. Previous research on the chemical ecology of *Polyergus* queens has shown that they achieve colony-take over by acquiring the chemical cues of the

murdered host queen, so that the *Polyergus* queen is accepted by the resident *Formica* workers (Johnson et al. 2001). However, *Polyergus* workers are also expected to be chemically adapted, by either mimicking or camouflaging themselves with the same nestmate recognition cues as their hosts (see Lenoir et al. 2001). This allows slave-maker and slave to be well integrated into a single colony, and may also ease opposition against slave-making workers when they break into host colonies to raid brood (Bagnères and Lorenzi 2010). In ants, the chemical cues that are likely important for recognition and acceptance of colony mates are cuticular hydrocarbons (CHCs) that occur on the exoskeleton (Bonavita-Cougourdan et al. 1987; Lahav et al. 1999; Thomas et al. 1999; Wagner et al. 2000; Akino et al. 2004; D'Etterre and Heinze 2005; Lucas et al. 2005; Torres et al. 2007; Martin et al. 2008b; Guerrieri et al. 2009; Lalzar et al. 2010). CHC chemical profiles are often species-specific in their composition (Howard 1993; Martin et al. 2008a). There is substantial evidence for a strong genetic component underlying CHC diversity (Crozier and Dix 1979; Beye et al. 1997; Beye et al. 1998; van Zweden et al. 2009; Soro et al. 2011) but, in some cases, environmental influences may also contribute (Heinze et al. 1996; Liang and Silverman 2000; Katzerke et al. 2006). Therefore, the CHC profiles of slave-making ants likely experience strong selection to be closely adapted to a particular host species.

Here we examine the chemical and molecular ecology of the slave-making ant, *Polyergus breviceps*, which primarily parasitizes three morphologically recognized species of *Formica* (*F. accreta*, *F. argentea* and *F. fusca*) in sympatry. The main goal of this study is to test for host specificity, which may suggest host-race formation in a currently recognized, single population of social parasite. Behavioral studies of slave-making ants in the genus *Polyergus* strongly suggest a high level of ecological specialization on different host species, in areas where more than one species may be available. Newly mated slave-making ant queens display host fidelity when infiltrating a new *Formica* colony (Goodloe and Sanwald 1985) and it has been suggested that imprinting on the chemical odors of the host within their natal nest leads the new slave-making queens to choose the same host species to parasitize in the next generation (Schumann and Buschinger 1994). In the case of raiding behavior, studies have shown that *Polyergus* workers only raid colonies belonging to one species of host, even when other host species are available (Goodloe et al. 1987; Bono et al. 2006; King and Trager 2007).

Given the ecological evidence for host specificity in queen establishment and raiding behavior, we predict that the *Polyergus breviceps* at our study site will exhibit both chemical and genetic signatures of host specialization. First we predict that the chemical profiles (which include CHCs) of *Polyergus* will be distinguishable according to host, reflecting chemical adaptation of the slave-maker to its slaves. Second, assuming long-term host specialization and restricted gene flow between colonies parasitizing different hosts, we expect *Polyergus* will be genetically differentiated according to slave species. Here, we use maternally inherited mitochondrial DNA to test for long-term host fidelity on the part of slave-making ant queens and biparentally inherited microsatellite (nuclear) DNA to examine possible assortative mating with males according to host.

## METHODS

### Field site and collection information

We conducted this study at Sagehen Creek Field Station, a University of California-Berkeley natural reserve located 8.4 miles north of Truckee, CA in Nevada County. At this site, *Polyergus breviceps* enslaves at least four different species of *Formica* including: *F. fusca*, *F. argentea*, *F. accreta* and, more rarely, *F. neorufibarbus* (P.S. Ward, personal communication). In July 2010, we collected 20 *Polyergus* and 20 *Formica* from each of 10 different *Polyergus* colonies (400 ants total, 200 from each genus). For five of these colonies, the host was identified as *F. argentea*, three of these colonies were identified as *F. accreta*, and two of the colonies had *F. fusca* as the host. Host species was identified by microscope using the morphological key for the *Formica* fusca group developed by Francoeur (1973). Ants were collected as they were exiting their nest or from within 0.3 meters of the nest entrance to ensure nest identity.

### Chemical data collection and analysis

After collection of *Polyergus* and *Formica* workers from the field, we froze the specimens and soaked them for 10 minutes in 200ul of chromatography grade hexane in 9mm glass GC vials (Agilent Technologies, Santa Clara, CA). Individual ants were then removed from the solvent, allowed to air dry, and transferred into 95% ethanol for later genetic analysis. The hexane extracts were stored at -20°C and kept on dry ice for transport back to the laboratory. We evaporated the hexane from each sample under nitrogen, re-eluted the extract in 40ul of hexane, and transferred it to a small glass insert with polymer spring (Varian, Palo Alto, CA).

To analyze the cuticular hydrocarbon extracts, 2ul of the extract was injected in splitless mode into an Agilent 7890A Gas Chromatograph (GC) coupled with a 5975C Mass Spectrometer (MS) with triple access detector. We used a DB-5, 30m x 320µm x 0.24µm capillary column from Agilent to separate out the components of individual CHC profiles and ran the following temperature program with a 5 minute solvent delay: 70°C for 2min, 30°C/min to 200°C and then 3°C/min to 325°C for 10 min. The MS was set to scan from 40-600amu.

We viewed and analyzed the components of all chemical profiles using the software MSD ChemStation (v. E.02.00.493, Agilent Technologies, Santa Clara, CA). From each of the 10 colonies, we sampled two or more chromatograms from each genus (*Polyergus* and *Formica*) that had the highest concentration of total chemical peaks to custom-build a library of chemical components. With this library, we assessed the presence or absence of 105 chemical peaks in the CHC profiles of all individuals sampled by creating a library search report in ChemStation. This procedure allowed us to match the mass spectrum of each chemical peak with a mass spectrum from the library. We used default settings for the library match options with the following exceptions: U+A (4), Flag (1), Min Est Purity (50). We used ChemStation integrator and the "autointegrate" function to detect and calculate the amounts of each chemical peak present in the profile in units of peak area, and only those peaks that matched up to one of the 105 library entries were

counted as present. Relative amounts of each chemical peak were determined by calculating the percentage area contribution of library-matched peaks identified by the ChemStation integrator on a per individual basis.

To test the hypothesis that *Polyergus breviceps* individuals would display a host-specific chemical profile, we conducted a Nonmetric Multi-Dimensional Scaling (NMDS) analysis on the relative peak areas of *Polyergus* profiles using the program R and the packages *vegan* and *ecodist*. Chemical peaks that were not present or that could not be detected by the ChemStation integrator were counted as zero. We used the percentage peak area to calculate a Bray-Curtis pairwise distance matrix. We performed an analysis of similarity (ANOSIM) on this matrix to test for differences in the chemical profiles between individuals based on host using the function *anosim* in R. Next we performed the same NMDS and ANOSIM analyses but included the individual chemical profiles of the hosts (*Formica* spp.) enslaved by the *Polyergus* sampled to see whether *Polyergus* workers clustered more closely with the hosts that inhabit their colony than with other available host species.

In total, we selected 105 chemical peaks to analyze for the presence in relative abundance or absence across all *Polyergus* and *Formica* individuals. To determine which of these 105 peaks most likely contributed to any separation of *Polyergus* by hosts, we performed a SIMPER (similarity percentage) analysis using PRIMER 6 (Clarke 1993).

### **Genetic analysis**

We extracted whole genomic DNA from the heads of *Polyergus* and *Formica* workers using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), following the recommended protocol and eluting the DNA in 200ul of buffer AE. All *Polyergus breviceps* individuals were genotyped using six microsatellite primer pairs developed for *Polyergus breviceps* (pol1, pol2, pol3, pol4, pol5, pol12; Bono et al. 2007) and two primer pairs developed for *Formica yessensis* (Fy4 and Fy13; Hasegawa and Imai 2004). We amplified each locus in 10ul PCR reactions consisting of: 1X reaction buffer, 1.25-2mM of MgCl<sub>2</sub>, 300μM of each dNTP, 0.8μM of each primer and 0.04 -0.075 units of GoTaq Flexi DNA Polymerase (Promega, San Luis Obispo, CA). For amplification, we used the following temperature program: initial denaturation for 5 minutes at 95°C, 36 cycles of 30 secs at 30°C, 30 secs at the appropriate annealing temperature, and a 30 second extension at 72°C and ending with a final extension step for 5 minutes at 72°C. Variations in reagent concentrations and annealing temperatures can be found in Table 1. We labeled all forward primers with fluorescent labels (VIC, 6FAM, PET and NED) provided by Applied Biosystems (ABI; Carlsbad, CA, USA) and added LIZ size standard to the resulting PCR products to estimate allele sizes. PCR products suspended in formamide were separated on an ABI 96 capillary 3730xl DNA Analyzer. We visualized and scored allele sizes using Peak Scanner v 1.0 software (ABI).

Using the program Microchecker (v 2.2.3, Van Oosterhout et al. 2004), we looked for evidence of possible scoring errors in microsatellite genotyping due to the presence of stutter, null alleles, and/or large allele dropout for all *Polyergus* individuals analyzed. We tested for deviations from Hardy-Weinberg equilibrium

(HWE) and calculated allele frequency measurements and fixation indices (F) using GenAEx v6.41 (Peakall and Smouse 2006). The presence of linkage disequilibrium (LD) was detected using Arlequin v 3.1 (Excoffier et al. 2005).

To test the hypothesis that *Polyergus* colonies may separate out genetically according to host according to microsatellite data, we performed two types of analyses for detecting population clustering: one in the program STRUCTURE (v 2.3.3, Falush et al. 2003) and the other a discriminate analysis of principle components (DAPC) in the program R. STRUCTURE uses Bayesian methods to detect population structuring by forming clusters within which the assumptions of HWE and linkage equilibrium are maximized. In STRUCTURE, we explored a range of possible numbers of population clusters (K) from 2 to 10 (the total number of colonies sampled) using a burn-in length of 50,000 followed by 100,000 Markov Chain Monte Carlo (MCMC) repetitions. We used both the admixture and the correlated allele frequency model under default settings. STRUCTURE has two options that allow for the user to set up prior populations of origin using “popflag” and “popinfo.” During initial runs with and without these options, we determined there were no detectable differences between them. Here, we present results from runs with both population options turned on and the species of host enslaved by the genotyped *Polyergus* individual set as prior “populations.” Since we were particularly interested in whether *Polyergus* at our site were genetically grouped according to host, we performed an additional 10 runs each of K=3 (for the three species of hosts identified morphologically) and K=4 (an additional host was indicated to be present according to mtDNA results, see below) at 100,000 burn-in length and 1,000,000 MCMC repetitions. We summarized the clustering patterns found in our runs of K=3 and K=4 using CLUMPP (v 1.1.2 ; Jakobsson and Rosenberg 2007) and visualized them using DISTRUCT (v 1.1 ; Rosenberg 2004). We estimated the “true” number K using the Structure Harvester which summarizes likelihood values outputted from STRUCTURE and utilizes the Evanno et al. (2005) method to calculate delta K (Earl and vonHoldt 2012).

If the assumptions of STRUCTURE are not met (i.e. departures from HWE and LD are not associated with population structure but instead with inbreeding or scoring errors), STRUCTURE may oversplit a population (see: Pritchard et al. 2010). Therefore, we also performed a DAPC analysis in the program R using the function *dapc*. DAPC is a multivariate analysis method that combines the advantages of PCA and discriminate analysis to determine assignment of individuals to genetic clusters without prior knowledge of population structure (Jombart et al. 2010). Because this method transforms genetic data using PCA, the assumptions of HWE and no LD do not need to be met to determine genetic clustering.

To test the hypothesis of genetic separation by host using maternally inherited DNA, we also amplified and sequenced a fragment of the mitochondrial gene, cytochrome oxidase I (COI), from 1-2 individuals of each genus (*Polyergus* and *Formica*) for all ten colonies sampled. To amplify COI for *Polyergus* individuals, we designed a forward primer, CI13Pbrev-5' CACTGCAATTTTACTTCTTT 3', and paired it with a reverse primer designed for *Polyergus samurai*, CI24 (Hasegawa et al. 2002). We amplified all *Formica* using a primer we developed from the COI gene of



*Formica wheeleri*, COIFwhF -5' TTCCTTTGCTTGTATGATCAATTT 3' and paired it with CI24. In 10ul PCR reactions we added 1X reaction buffer, 3mM of MgCl<sub>2</sub>, 300μM of each dNTP, 0.8μM of each primer and 0.04 units of GoTaq Flexi DNA Polymerase (Promega, San Luis Obispo, CA). All COI mtDNA fragments were amplified using the following temperature program: a 2 min initial denaturation 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 49°C and 1 min at 72°C, and ending with a 10 min extension step at 72°C. We verified the amplification of PCR products on 1% agarose gels and performed PCR clean up using a mixture of Exo Nuclease I and Shrimp Alkaline Phosphatase. For the sequencing reaction, approximately 33ng of DNA from the purified PCR product, and 0.46 pmol of primer were added to 4ul of BigDye Terminator (ABI) and the following temperature program was used: 96° for 1 min then 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Reaction cleanup was performed using Agentcourt cleanSEQ (Beckman Coulter genomics, Danvers, MA). We used a 96 capillary 3730xl DNA analyzer for sequencing and edited all sequences and aligned them in the program MEGA 5.05 (Tamura et al. 2011).

To determine whether the COI gene fragments of *Polyergus* workers from each colony separated out by host, we used the program MEGA to build maximum parsimony (MP) and maximum likelihood (ML) trees using one representative host from each species of *Formica* to root these trees and producing a bootstrap consensus trees inferred from 500 replicates. For the MP tree, we used the Close-Neighbor-Interchange algorithm and the ML tree was inferred based on the Tamura-Nei model (Tamura and Nei 1993).

### **Correlation between chemical and genetic distances**

Using the GC-MS chemical profiles and genetic data, we tested whether the genetic distance between individuals is correlated with the chemical distance between them. We constructed pairwise distance matrices of 183 individual chemical profiles using Bray-Curtis distances in R, and microsatellite genotypes for these same individuals using the genetic distance option in GenAlEx v6.41. Another set of pairwise distance matrices was made using 17 individual mtDNA (COI) sequences with uncorrected p distances and their Bray-Curtis chemical distances. To test for the significance of correlation between the genetic and chemical distance, we performed two Mantel tests on each data set (microsatellites and mtDNA) using the function *mantel* in the R package *vegan* and 10,000 permutations.

## **RESULTS**

### **Comparison of chemical profiles from three species of host (*Formica*) and *Polyergus* chemical profiles parasitizing those hosts**

Qualitatively, the chemical profiles of the three species of *Formica* enslaved by *P. breviceps* (*F. accreta*, *F. argentea*, and *F. fusca*) showed species specific differences (Figure 1). ANOSIM and NMDS analyses of these enslaved *Formica* confirmed separation according to species (Figures S1 and S2, respectively; ANOSIM

results:  $R=0.47$ ,  $p=0.001$ ). There also appeared to be qualitative differences among the chemical profiles of *P. breviceps* that enslaved different species of host (Figure 2). The NMDS plot of individual *P. breviceps* profiles showed that the majority *Polyergus* individuals grouped according to the three different hosts (Figure 3) and the results from the ANOSIM analysis confirmed these host-specific differences to be significant ( $R= 0.575$ ,  $p=0.001$ ; Figure S3). *Polyergus breviceps* colonies that were parasitizing the same species tended to overlap more with each other than they did with *Polyergus* parasitizing other species, but did not always form tightly clustered groups by colonies alone (Figure S6). Interestingly, both the *Polyergus* and the *Formica* from one colony (P9) possessed chemical profiles that were outliers compared to their conspecifics from other colonies (Figures S4, S5).

When all enslaved *Formica* were included with their slave-makers in the NMDS analysis, *Polyergus* individuals tended to group with their host species, with the exception of *Polyergus* parasitizing *F. argentea* (Figure 4).

The SIMPER analysis revealed that 15 of the 105 peaks analyzed by GC/MS contributed to over 50% of the chemical differences found between *F. accreta* and *F. argentea*. Twelve out of the 105 peaks analyzed distinguished *F. fusca* and *F. accreta* by over 50%. *F. fusca* and *F. argentea* had 11 of 105 peaks that differentiated them by over 50%. Five of these distinguishing peaks were found across all three comparisons mentioned above. A table of the retention times of the SIMPER identified peaks are shown in S8.

## **Genetic analysis of *Polyergus breviceps* parasitizing one of three hosts**

### *Microsatellite data*

We genotyped a total of 193 individual *Polyergus breviceps* (59 *P. breviceps* on *F. accreta* from 3 colonies, 95 *P. breviceps* on *F. argentea* from 5 colonies, 39 *P. breviceps* on *F. fusca* from 2 colonies). Results from MICROCHECKER revealed that, while there was no sign of large allele dropout, five out of the eight loci were flagged as possibility having null alleles. MICROCHECKER assumes the presence of null alleles due to excess homozygotes for most alleles at a single locus but not across all loci (see Van Oosterhout et al. 2004). The assumption of MICROCHECKER is that homozygote excess detected across all loci indicates population-level effects including assortative mating and subpopulation structuring but these assumptions may not be met if loci are statistically linked, as we found here (see LD results below). Taking all *Polyergus* as a single population, only one out of the eight loci (Fy4) was in HWE. When testing for HWE at the level of colony and host, we found no loci that were consistently in equilibrium. For the entire population of *Polyergus*, the average number of alleles per locus per colony was  $2.56 \pm 0.48$  (SD) and the average expected heterozygosity was  $0.39 \pm 0.08$ . The average number of private alleles per colony was  $0.20 \pm 0.21$ . The average fixation index (F) found across all loci for the whole population was  $0.181 \pm 0.16$ . When analyzing *Polyergus* using the species of host as a population unit, the average fixation indices per locus were:  $0.023 \pm 0.34$  (*F. accreta*),  $0.027 \pm 0.21$  (*F. argentea*), and  $0.060 \pm 0.34$  (*F. fusca*). We found an 87.5% occurrence of LD across all possible locus pairings when considering all sampled *Polyergus* as one population.

The STRUCTURE analysis revealed an overall pattern of structuring of *Polyergus* individuals according to host at K=3 (Figure 5). At K=4, there was no biologically relevant fourth cluster with the exception of colony P7 (host: *F. argentea*), which was fully assigned to this cluster (Figure S7; orange). Structure Harvest output showed likelihood values for K indicating a K=7 while the delta K plot suggested a K=2 (outputs not shown).

The DAPC plot also showed a distinct pattern of clustering of *Polyergus* according to the hosts they enslave (Figure 6). Percent assignments of *Polyergus* correctly grouped with their host as calculated by the DAPC were: 92.3% (*F. accreta*), 98.9% (*F. argentea*) and 98.3% (*F. fusca*).

#### *Mitochondrial (COI) sequence data*

Cytochrome oxidase (COI) sequence data showed branching patterns that reflected clustering according to host, with the exception of colony P9 (host: *F. accreta*) for both MP and ML bootstrap consensus trees (Figure 7). Although both trees showed highly supported clades forming two distinct host groups (*Polyergus* enslaving *F. argentea* and *Polyergus* enslaving *F. fusca*), *Polyergus* enslaving *F. accreta* formed a polyphyletic group. More specifically, *Polyergus* from colony 9 (P9) that enslave *F. accreta* appear genetically distinct from the other two colonies of *Polyergus* on that same host according to COI data, though the DAPC did not support this pattern. Interestingly, COI sequence data of enslaved *Formica* representing each colony of *Polyergus* sampled also show *F. accreta* from colony 9 as not clustering with the other *F. accreta* (data not shown).

#### *Correlation analysis of pair-wise individual genetic and chemical distances*

We found a strong correlation between the genetic distance of individuals as determined by pair-wise comparisons of microsatellite genotypes and chemical distance of their chemical profiles (Mantel test:  $r= 0.3861$ ;  $p<0.0001$ ; Figure 8). Genetic distance as calculated by the p-distance between those individuals sequenced for COI (mtDNA) also correlated well with their respective chemical distances (Mantel test:  $r=0.5163$ ;  $p<0.0001$ ; data not shown).

## **DISCUSSION**

The results of our chemical and genetic analyses of *Polyergus breviceps* parasitizing three species of *Formica* strongly suggest that host specialization is occurring at our study site. The chemical profiles of *Polyergus breviceps* are distinguishable according to the three hosts they parasitize, indicating some level of chemical adaptation by the slave-maker. Both mitochondrial sequence and microsatellite loci data show genetic clustering of individuals by host, suggesting restricted gene flow among *Polyergus* that specialize on different host species. Together, these data provide evidence for host-race formation in sympatry for *Polyergus breviceps* at our study site. Interestingly, these potential host races of *Polyergus breviceps* show no obvious morphologically distinguishable

characteristics (Trager, personal communication), qualifying them as cryptic lineages as well.

The complete dependence of *Polyergus* on their *Formica* slaves likely imposes strong selection on the parasite to elude the recognition system of its hosts. Mimicry of host recognition cues, such as cuticular hydrocarbons (CHCs), is one common adaptive strategy used by such social parasites (Bagnères and Lorenzi 2010). In the case of slave-making ants, this not only maintains cohesion within a mixed colony, but may also reduce aggression by the host during slave raids (Bagnères and Lorenzi 2010). Given that CHC cues have been shown to be species-specific (Howard 1993; Akino et al. 2002; Martin et al. 2008a), we predicted that *Polyergus* that specialize on a single host species would have chemical profiles that match their host species, and that this level of matching should keep *Polyergus* populations that parasitize different species of *Formica* distinct from one another. As expected, chemical profiles of *Polyergus* workers form three groups associated with the three species of host they parasitize. Interestingly, one colony (P9) contained *Polyergus* individuals that were chemical outliers. Still, enslaved *Formica* from this colony also had chemical profiles that were somewhat distinct from conspecifics in other colonies. Moreover, the mitochondrial data indicate that colony P9 *Formica* are also be genetically distinct from conspecifics in other colonies (see below). However, despite this distinctiveness, our data indicate that *Polyergus* from colony P9 (and perhaps from other similar, unsampled colonies) specialize on these host types.

To further examine the extent to which *Polyergus* are chemically adapted to their specific host, we plotted together the cuticular profiles of both slave-makers and their slaves. We found that, although *Polyergus* parasitizing *F. accreta* and *F. fucsa* clustered closely to their respective hosts, *Polyergus* parasitizing *F. argentea* did not cluster as closely to their hosts. This suggests that *Polyergus* enslaving *F. argentea* are not as chemically well matched to their host as *Polyergus* parasitizing the other two *Formica* species. Still, we cannot be certain whether this relatively lower level of matching decreases the efficacy of *Polyergus* enslaving *F. argentea* relative to *Polyergus* enslaving other hosts. Further observations comparing *Polyergus* raiding behavior and interactions with different hosts are needed to address this question.

Previous studies have generally shown that, overall, social parasites often match their cuticular hydrocarbon profiles of their host, but the degree of matching may vary (see Bagnères and Lorenzi 2010 for review). The extent of chemical adaptation by social parasites may vary depending on the local availability of hosts and their ecological interactions with such hosts. A few studies have examined how the availability of multiple hosts in sympatry may affect the chemical adaptation of social parasites. In one study, Brandt et al. (2005b) found that the slave-making ant, *Protomognathus americanus*, had chemical profiles that clustered between sympatrically distributed host species in the genus, *Temnothorax*. However, in another location where only one species of host occurred, the slave-maker had a chemical profile that was much more closely matched to its slaves. The authors concluded that, at the site with two sympatric hosts, *Protomagnatus* acted more as a generalist than a specialist. In another study, Bauer et al. (2010) found that the

slave-maker, *Harpagoxenus sublaevis*, more closely matched one of two host species of *Leptothorax*. However, unlike *Polyergus* slave-makers, *H. sublaevis* may also enslave both species of *Leptothorax* within the same colony. In these mixed host colonies, the chemical profile of *Harpagoxenus* more closely resembled one of the two host species. Finally, Nash et al. (2008) found that the chemical profiles of the socially parasitic caterpillars of *Maculinea alcon* did not display host specificity when two species of *Myrmica* were available at the same site at equal frequencies. Instead, their profile appeared to be a mixture of cues from both hosts. In contrast, close matching of the *Myrmica* host profiles was apparent at two other sites where only one host was primarily utilized by the parasitic caterpillar.

Although we find that *Polyergus* enslaving *Formica argentea* do not seem to be as closely chemically matched to their hosts as *Polyergus* on *F. fusca* and *F. accreta*, overall *Polyergus* chemical profiles do appear to separate out by host, thus suggesting some level of host specificity. In contrast to two of the studies mentioned above (Brandt et al. 2005b; Nash et al. 2008), our chemical data suggests that *Polyergus breviceps* chemically specializes on one of three host species in sympatry. Further studies that examine the cuticular chemical profiles of free-living *Formica* from our field site and nearby populations of *Polyergus* that enslave only one host will clarify the extent and evolutionary history of this chemical adaptation.

A possible consequence of host specificity is a reduction in gene flow between parasites that use different hosts (Criscione et al. 2005; Archie and Ezenwa 2011). Because *Polyergus* newly produced slave-making queens maintain host fidelity when establishing a new slave-making colony (Goodloe and Sanwald 1985), genetic structuring by host should be evident in the maternally inherited mitochondrial DNA. Indeed, our results reveal genetic clustering of *Polyergus* according to the three species of host *Formica* in both the mitochondrial (COI) DNA sequence data, as well as at the microsatellite loci. The mitochondrial DNA data also uncovered another possible clade of *Polyergus* (P9) that formed a monophyletic group separate from other slave-makers parasitizing the three species of *Formica*. This is consistent with our general finding of genetic clustering according to host, since COI data from the enslaved *Formica* hosts also shows the same pattern of P9 as a distinct clade. However, this fourth group (P9) was not apparent from the microsatellite data: neither the STRUCTURE or DAPC analysis revealed any clustering of P9 according to bi-parentally inherited microsatellite data. Therefore, we believe *Polyergus* from P9 may be a maternal lineage that has specialized on what could be a morphologically cryptic subspecies of *Formica*. To confirm this, we need to analyze additional samples from colonies of *Polyergus* and this possible subspecies of *Formica* from our site. Data collected in a previous year suggest that other P9-type colonies exist (data not shown).

The STRUCTURE and DAPC analysis of microsatellite DNA also support the clustering of *Polyergus* into groups according to the three hosts at our field site. We also found low average fixation indices within each *Polyergus* grouped by host compared to the overall population. Together, these findings suggest limited gene flow among the lineages of *Polyergus* enslaving the three different species of *Formica*. We believe that this may be attributed to assortative mating between

slave-making ant sexuals characterized by *Polyergus* queens mating with males originating from colonies that parasitize the same host. In *Polyergus breviceps*, queens who are dispersing on foot release pheromones from their mandibular glands to attract males for mating (Greenberg et al. 2004; Greenberg et al. 2007). The specificity of this queen sex pheromone is unknown and very little is known about the mating behavior of *Polyergus*. Still, cuticular hydrocarbons (CHCs) may influence mate choice (Howard et al. 2003). A study by Beibl et al (2007) found that *Chalepoxenus mullerianus* slave-making ant sexuals obtain some of their CHCs cues from their host workers. Additionally, male slave-makers showed more interest and engaged in more mating activities with queens reared on the same host species. Beibl et al (2007) suggest that the rearing host species could influence the cuticular profile of slave-making ant sexuals such that mate choice is affected. If this is the case, males or queens of slave-makers could prefer to mate with individuals whose natal nests contain the same host species, based on host-associated CHC cues. Examining the chemical profiles of *Polyergus* sexuals, and performing choice experiments between *Polyergus* queens and males reared from different species of host would allow us to better assess whether CHCs play a role in assortative mating. Still, we cannot rule out the possibility of selection against "hybrid" slave-making ant colonies produced from the matings of sexuals originating from nests that parasitized two different hosts.

Reduced gene flow associated with possible host-race formation could be the result of differential adaptation of parasites to their host, especially if such an adaptation is result of divergent selection on that trait. Since we found chemical evidence for host specificity, we wanted to test whether chemical and genetic distances were correlated to determine the possibility that *Polyergus* chemical cues are a heritable adaptation. Recent studies on ants in the same subfamily as *Polyergus* (Formicinae), suggest that genetics plays a major role in the expression of chemical cues used in nestmate recognition (van Zweden et al. 2009; van Zweden and d'Ettorre 2010). However, in the case of social parasites, the source of chemical matching could be through direct biosynthesis of cues (indicating a genetic component) and/or through acquisition of chemical cues from social interactions such as grooming and food exchange or a combination of the two (Lenoir et al. 2001). Here, we found a strong correlation between chemical and genetic distance for *Polyergus* at our field site, suggesting an underlying genetic basis for CHC variation.

In general, providing clear geographical and population genetic evidence for of host-race formation in sympatry is difficult (Via 2001; Fitzpatrick et al. 2008). Support for host race formation in social insects parasites, in particular, has not been previously reported, and has been rarely examined (see Fanelli et al. 2005). Because our genetic data indicate biparental restricted gene flow between *Polyergus* that parasitize one of the three co-occurring hosts within a small area ( $\sim 0.10 \text{ km}^2$ ), we argue that the *Polyergus breviceps* at our study site shows evidence of host-race formation in sympatry. Additionally, we suggest that host specialization by *Polyergus* may be facilitated by a potentially heritable adaptation: production of chemical cues that closely match the specific species of enslaved *Formica*. Strong

divergent selection on a particular trait may jumpstart the speciation process and result in reduced gene flow between subpopulations (Nosil et al. 2009). Host-race formation may be viewed as an intermediate stage in the process of sympatric speciation, with complete genetic isolation and development of reproductive barriers not yet fully established. (Drès and Mallet 2002). To ascertain whether the *Polyergus* at our field site are cryptic species, cross-fostering experiments of *Polyergus* workers and mate-choice tests between slave-making queens and males originating from colonies parasitizing different hosts need to be performed. Incorporating the *Polyergus* from our study site into a broader scale phylogeographic study of several populations of *Polyergus breviceps* parasitizing different hosts will also provide a more historical background for the plausibility of sympatric speciation in this species of slave-making ant.

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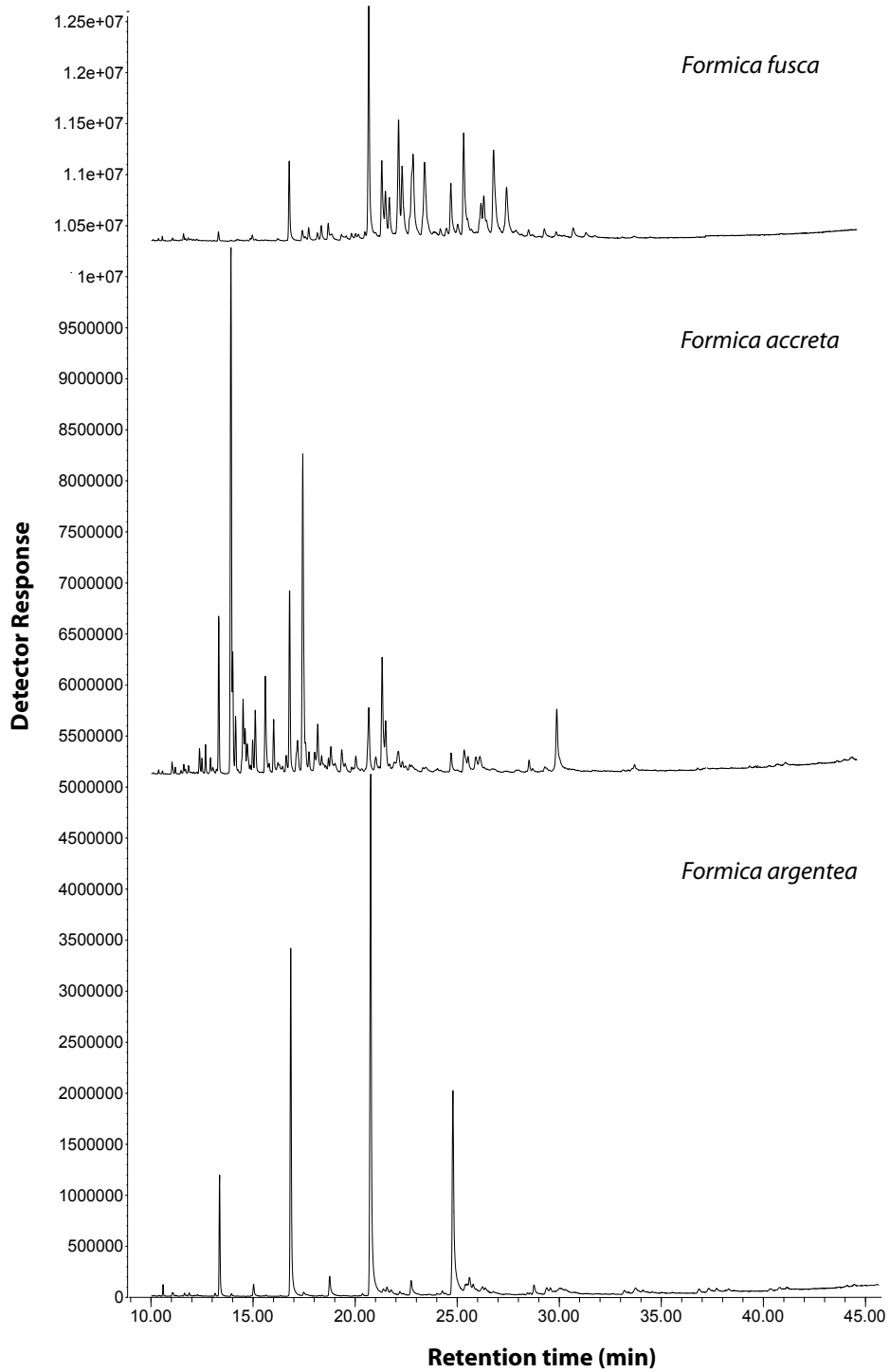
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**Table 1.** Variations in 10ul PCR conditions for 8 microsatellite loci

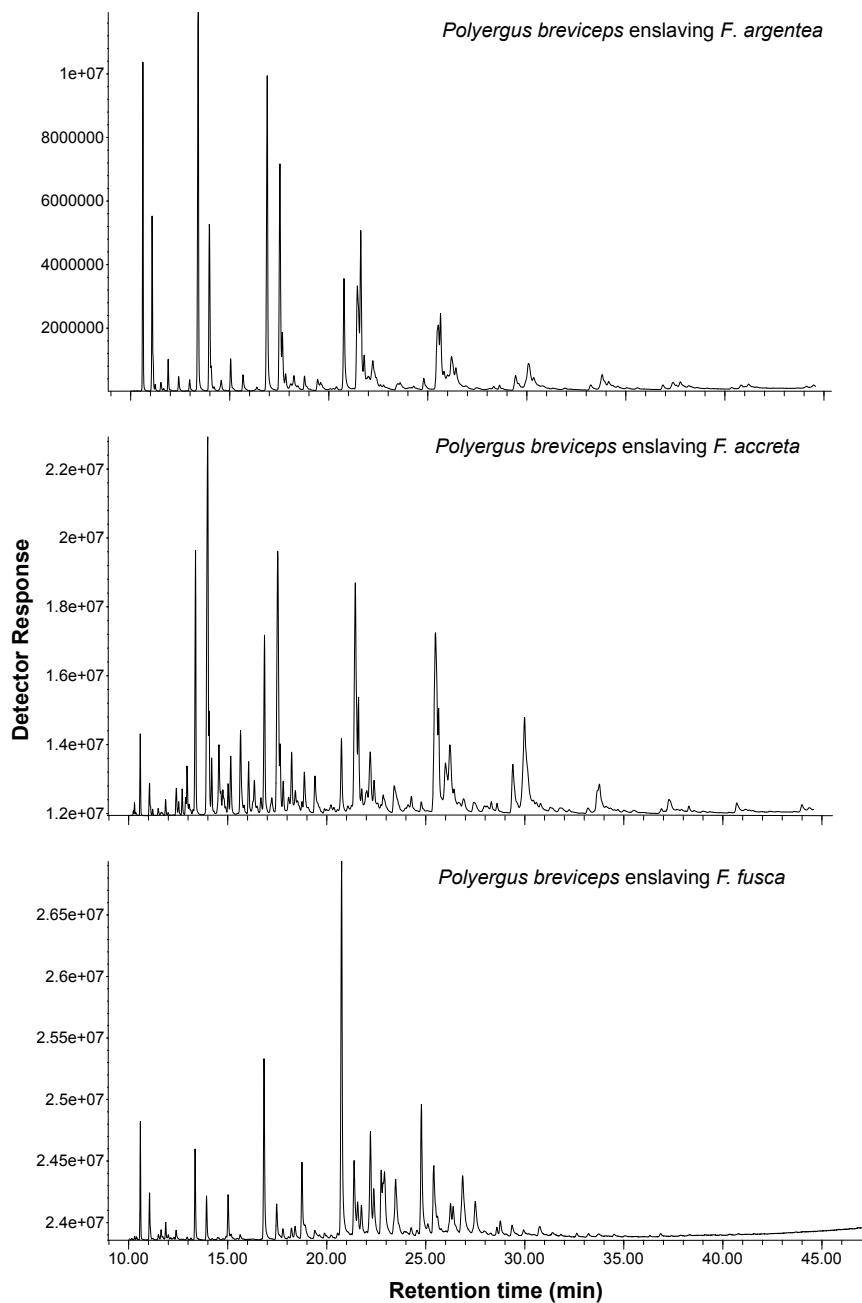
Locus	MgCl <sub>2</sub>	Taq	T <sub>a</sub> <sup>1</sup>
pol 1	1.25 mM	0.075 units	59.5°C
pol 2	2 mM	0.075 units	58°C
pol 3	2 mM	0.075 units	59.5°C
pol 4	1.25 mM	0.075 units	59.5°C
pol 5	2 mM	0.075 units	54.6°C
pol 12	1.75 mM	0.075 units	58°C
Fy 4, 13	1.25 mM	0.04 units	48°C

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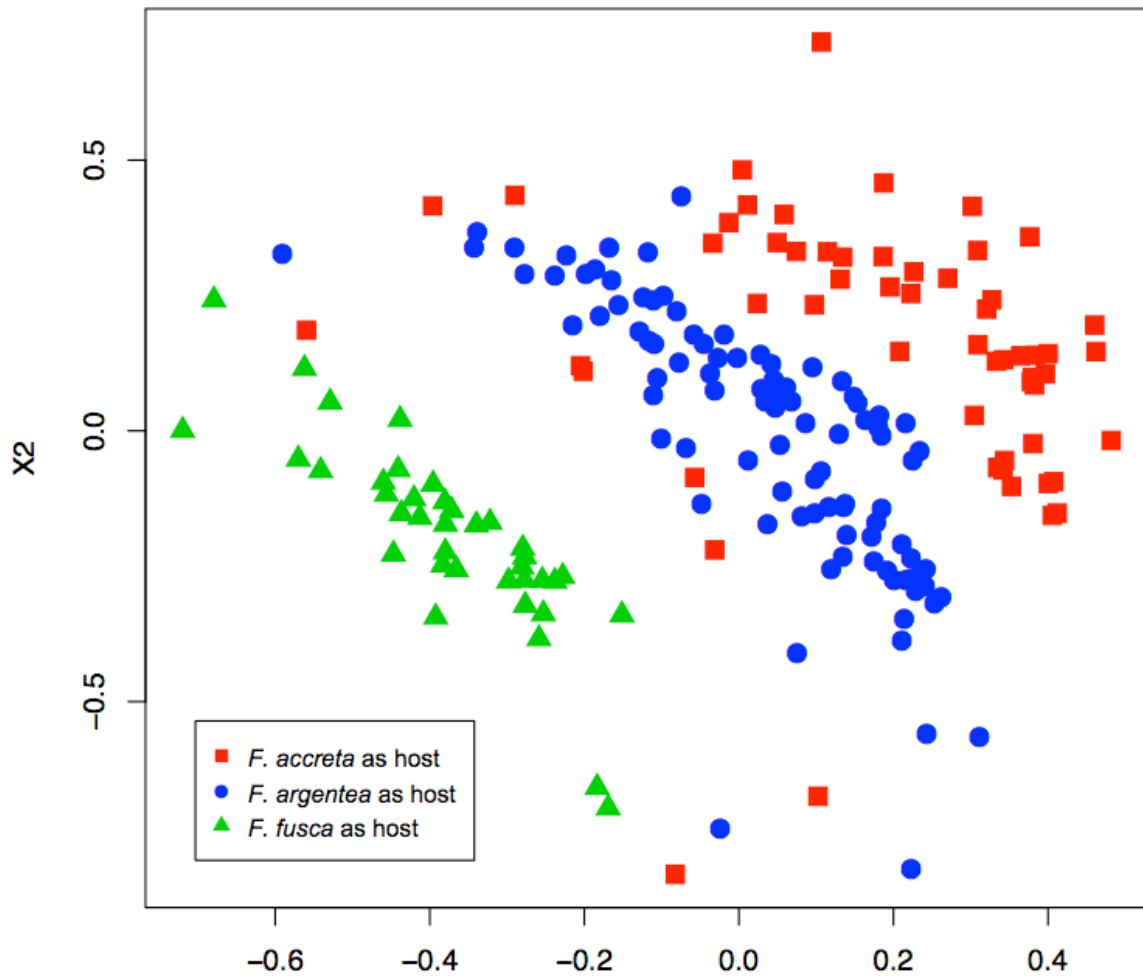
<sup>1</sup> Annealing temperature



**Figure 1.** Representative chemical profiles from one individual from each of the host species.

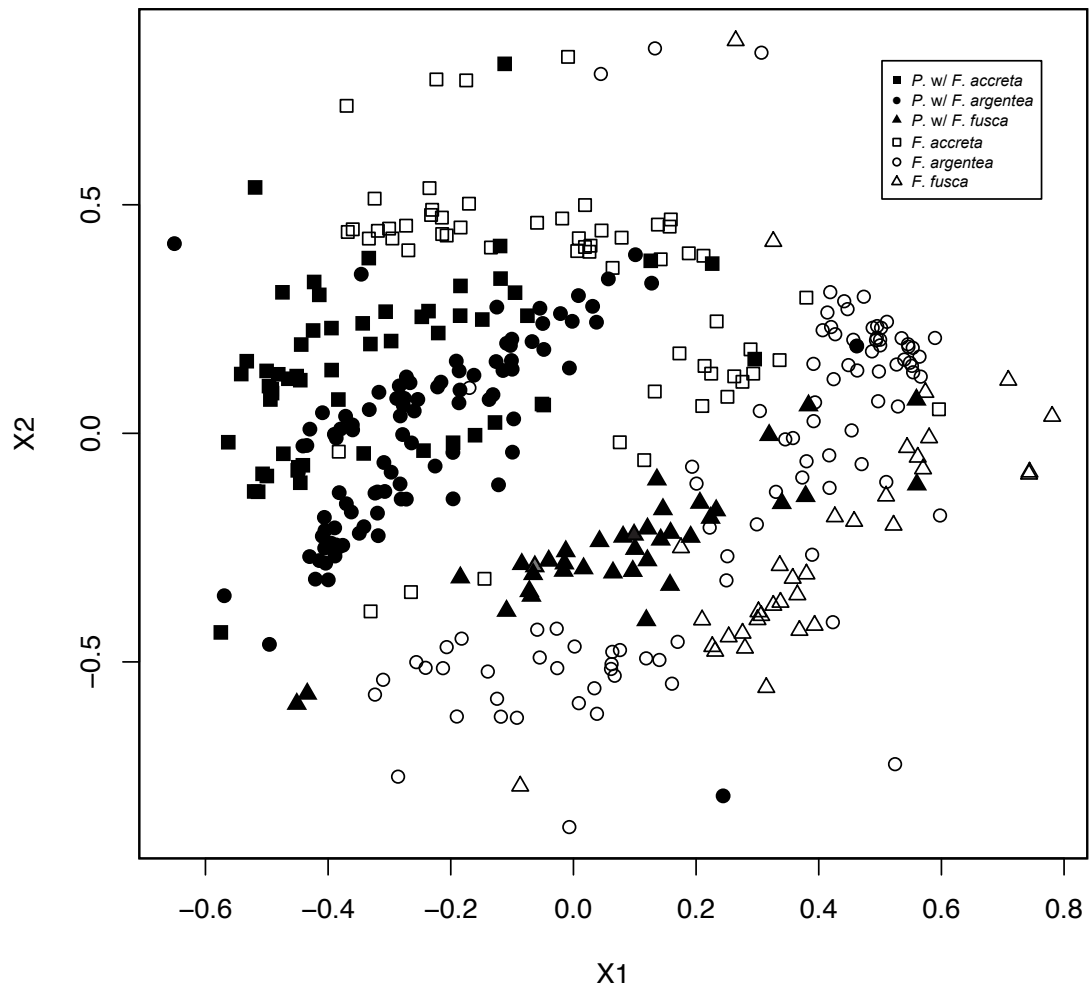


**Figure 2.** Representative chemical profiles of individual *Polyergus breviceps* workers from colonies that enslave different species of *Formica*.

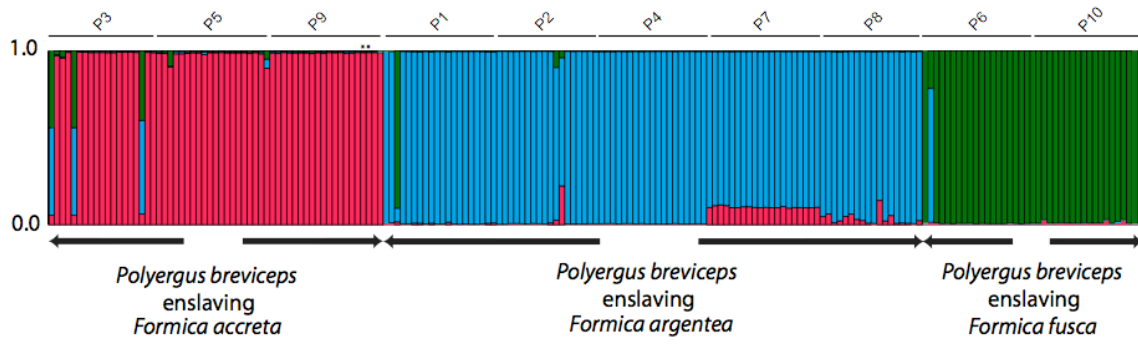


**Figure 3.** NMDS plot of individual *Polyergus breviceps* chemical profiles from colonies that enslave different species of *Formica*.

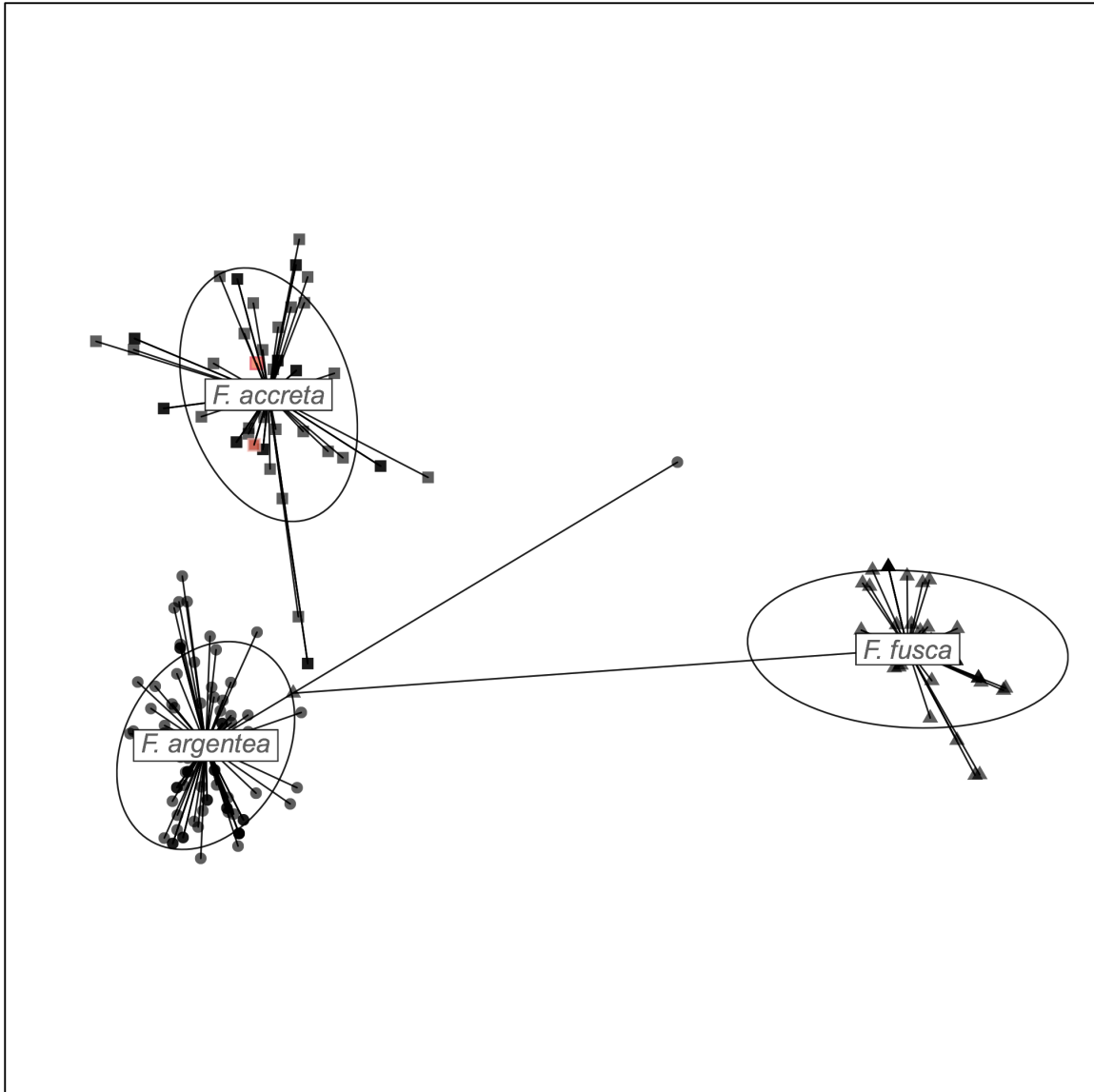




**Figure 4.** NMS plot of chemical profiles from individual *Polyergus breviceps* workers and their enslaved hosts (*Formica* sp.).

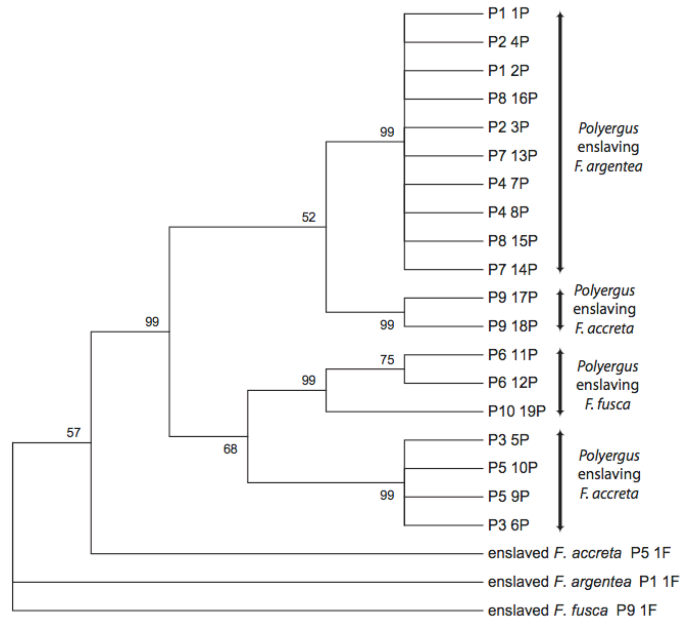


**Figure 5.** Results from the STRUCTURE analysis of 8 microsatellite loci with colors showing population cluster assignments based on  $K=3$ . Each column represents a single genotyped *Polyergus breviceps* enslaving one of three hosts: *F. accreta*, *F. argentea*, or *F. fusca*. Individuals from colony P9 used in the mtDNA analysis are indicated with small asterisks.

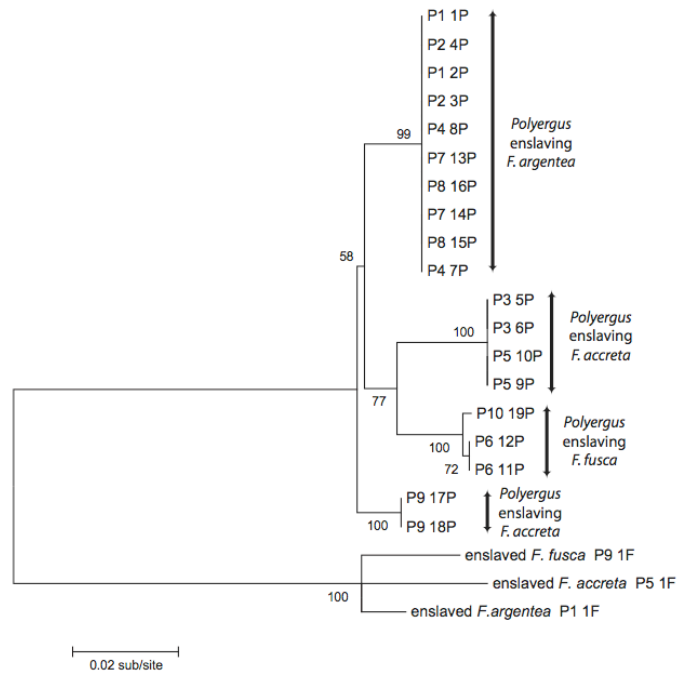


**Figure 6.** DAPC plot of *Polyergus breviceps* individuals genotypes based on microsatellite data. Individuals are plotted according to their co-inhabiting host: *F. accreta* (squares), *F. argentea* (circles) and *F. fusca* (triangles). Large circles drawn around points represent inertia ellipses that graphically summarize the cloud of points. See text for percent of *P. breviceps* individuals with genotypes that correctly assigned to clusters defined by species of host enslaved. Red colored squares indicate individuals from colony P9 used in the mtDNA analysis.

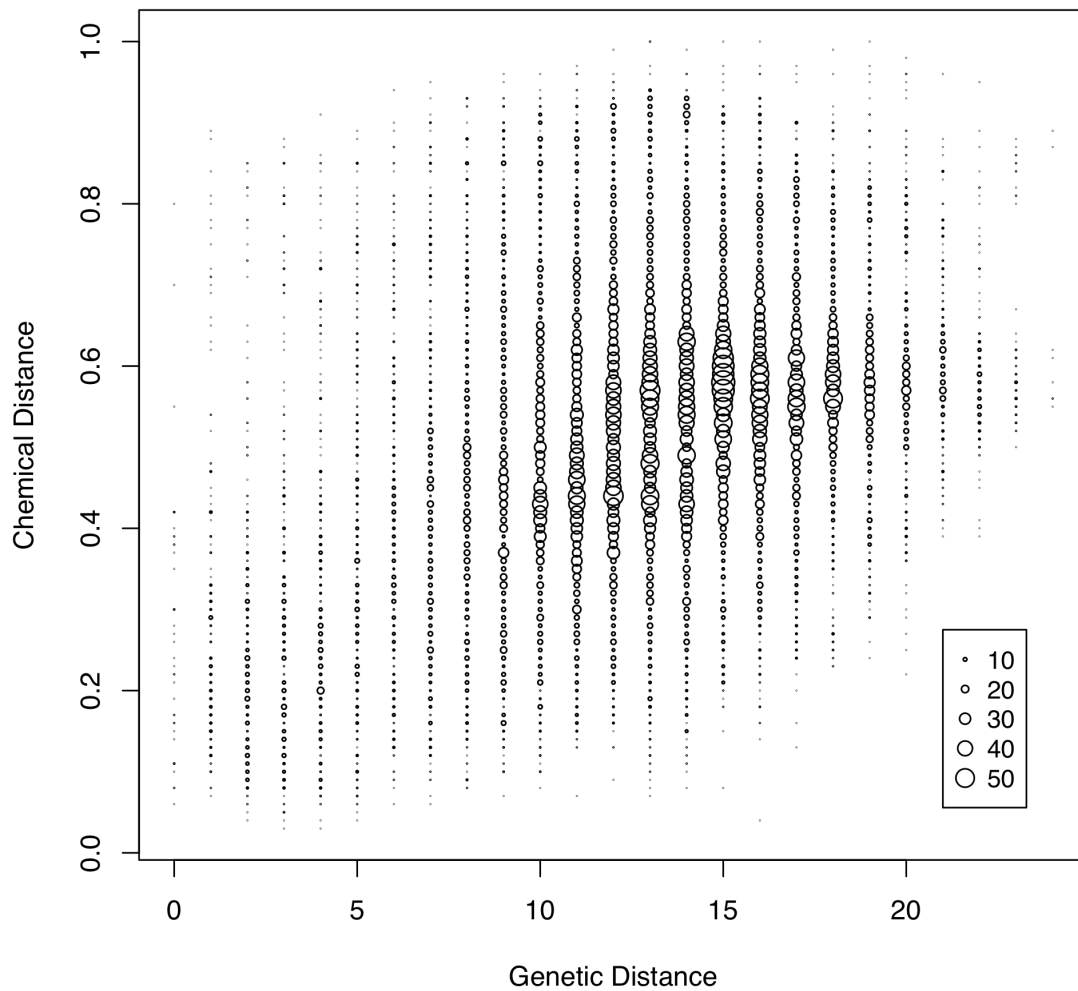
A) Maximum Parsimony tree



B) Maximum Likelihood tree



**Figure 7.** A) Maximum parsimony (MP) and B) Maximum likelihood (ML) consensus trees based on a 647bp fragment of the COI mitochondrial gene. Numbers on branches represent bootstrap values. Labels at the tips include colony name (e.g. P9) followed by individual ID number.



**Figure 8.** Scatter plot showing the correlation between pair-wise genetic distances as determined by 8 microsatellite loci and pair-wise chemical profile distances between 183 *Polyergus* workers. Different sizes of open circles represent the number of overlapping plotted individuals as indicated in the legend.

### **CHAPTER 3**

Phylogeography and population genetics of North American slave-making ants in the genus *Polyergus* parasitizing ants in the genus, *Formica*

## INTRODUCTION

Reconstructing the phylogeographic history of parasites and their hosts is critical to understanding the potential large-scale coevolutionary dynamics between them (Thompson 2005). However, the use of phylogeographic methods to study parasite and host populations has lagged behind those of other, free-living organisms (Criscione et al. 2005). Social parasites, those that exploit the vast resources of entire insect societies, have received even less attention in the parasitology literature (see D'Ettorre and Heinze 2001) and are rarely studied in a phylogeographic context (but see Beibl et al. 2007; Brandt et al. 2007). Unlike conventionally studied parasites (e.g. ectoparasites and endoparasites), social parasites may be closely related to their hosts (Emery's rule, Emery 1909) and parasitize colonies of ants, bees or wasps, rather than a single organism. This could have important and interesting impacts on the coevolutionary history between the parasite and its host. In particular, social parasites may be expected to engage in a coevolutionary arms race with their hosts (Davies et al. 1989; Brandt et al. 2005). Such is the case for so-called permanent ant social parasites whose entire life cycle depends on their phylogenetically close hosts (Buschinger 2009).

Slave-making ants in the Holarctic genus *Polyergus*, are one group of permanent social parasites that are likely to have coevolved with their hosts. Currently, there are five recognized species of *Polyergus* slave-makers that all parasitize ants belonging to a closely-related genus, *Formica* (Hölldobler and Wilson 1990). These slave-making ants are obligate social parasites that rely entirely on their hosts for brood care, nest maintenance, foraging, and colony defense. *Polyergus* colony foundation also follows a parasitic lifestyle, with *Polyergus* queens taking over existing *Formica* colonies by killing and replacing the host queen (Topoff 1999). The usurped *Formica* workers then assist the *Polyergus* queen with raising her brood: the first batch of slave-making ant workers. These highly specialized *Polyergus* workers conduct massive raids in which they steal pupae from neighboring *Formica* nests to replenish the colony's population of slaves (e.g. Topoff et al. 1985). Because of *Polyergus*' dependency on their slaves, we may expect that their hosts could affect the phylogeography and population structure of *Polyergus* species.

In this study, we focus on the phylogeography of two currently recognized North American species of slave-maker: *P. breviceps* Mayer and *P. lucidus* Mayer. As with other social parasites, the distribution of *Polyergus* populations tends to be disjunct. Although considered generally rare, *Polyergus* appear to be more abundant in areas where host density is relatively high (Fisher and Cover 2007; Buschinger 2009). *P. breviceps* is distributed mostly across the western United States but can be found from California to Michigan, and as far north as British Columbia (Creighton 1950; Wheeler and Wheeler 1986). In addition to their widespread distribution, *P. breviceps* enslaves over 10 different species of *Formica* primarily in the *fuscus* group (see MacKay and MackKay 2002; Fisher and Cover 2007). Because of the ecological diversity of their hosts within the *Formica fuscus* group, *P. breviceps* occurs in a variety of habitats, from pine forests to open alpine-level meadows. In contrast to

the wide distribution of *P. breviceps*, *P. lucidus* is primarily restricted to the eastern portion of North America (Trager et al. 2007). *P. lucidus* exclusively enslaves *Formica* from the nearctic endemic pallidefulva group that includes only five species (King and Trager 2007; Trager et al. 2007).

Most studies of *Polyergus* have focused on the behavioral and chemical ecology of particular populations. To date, no studies have examined the larger scale population genetics or phylogeography of any members of this genus. Because of *Polyergus*' obligate relationship with its host, we may expect that the genetic relationships among slave-maker ant populations to be associated with host use. However, effects of geography and restrictions on gene flow between populations of *Polyergus* may also affect the phylogeography of these slave-makers. In particular, *Polyergus*' apparently disjunct distribution, and the limited dispersal of slave-making queens, likely result in interpopulational differences (Topoff 1999; Ward 2005). Additionally, the genus *Polyergus* is currently undergoing a taxonomic revision, and morphological and ecological data suggests that both *P. breviceps* and *P. lucidus* may each be species complexes (King and Trager 2007; Trager personal communication).

Here we used molecular data from one mitochondrial gene and three more slowly evolving nuclear genes to: 1) reconstruct the phylogenetic relationships among North American *Polyergus* slave-maker populations and their slave species, 2) determine possible species boundaries for *Polyergus* using Bayesian statistical methods and 3) examine and compare the potential role of geography and host association in the genetic differentiation of populations of *P. breviceps* and *P. lucidus*. We then discuss how the genetic relationships among populations of North American *Polyergus* may have been influenced by geography and host use.

## METHODS

### Sampling

Table 1 lists the sample codes, hosts (genus: *Formica*), and locality information for the *Polyergus* colonies collected and examined in our study. We obtained samples from 41 *Polyergus breviceps* colonies and 15 *Polyergus lucidus* colonies from across North America and stored in 70-95% ethanol. Samples were acquired from museum, private, and our own personal collections. Hosts were collected with their slave-makers (*Polyergus*) whenever possible. To reconstruct more comprehensive phylogenies, we also sampled two colonies of *P. rufescens* (one enslaving *F. fusca* and one enslaving *F. gerardi*) from Europe and one sample of *P. samurai*, enslaving *F. japonica*, from Japan. *Polyergus* species were identified using current morphological standards. *Formica* species from the fusca group (host of *P. breviceps*) were identified using the key developed by Francoeur (1977) and *Formica* from the pallidefulva group were identified using a recent revision by Trager (2007).



## DNA sequencing

We extracted whole genomic DNA from the heads of ants using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA), following the manufacturer's recommended protocol. For both *Polyergus* and *Formica* samples we amplified fragments of the mitochondrial gene *cytochrome oxidase I* (COI) and nuclear genes *28S*, the F2 copy of *elongation factor-1 alpha* (EF1 $\alpha$ ), and exon II of *arginine kinase* (ArgK). We performed 10ul PCR reactions for all genes using 10X reaction buffer, 3mM of MgCl<sub>2</sub> (1.75 for ArgK), 300 $\mu$ M of each dNTP, 0.8 $\mu$ M of each primer and 0.04 units of GoTaq Flexi DNA Polymerase (Promega, San Luis Obispo, CA). We used the following temperature program for PCR amplification: an initial denaturation for 2 mins at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 49-55°C and 1 min at 72°C, and ending with a 10 min extension step at 72°C. For the COI fragment, we designed forward primers for each species of *Polyergus* sequenced (*P. breviceps*-CI13PbrevF, *P. lucidus*-COIPluc2F, *P. samauri*-COIPsam1F, and *P. rufescens*-COIPruf1F) and another forward primer for all *Formica* spp. (COIFwhlF, developed from *Formica wheeleri* sequence). We paired all these forward primers with the reverse primer CI24 (Hasegawa et al. 2002) to obtain amplification of the same region of COI. A complete list of primers used and annealing temperatures for each gene amplified is provided in Table 2. We performed a standard clean-up reaction on all PCR products using a mixture of exonuclease I and shrimp alkaline phosphatase. Sequencing reactions included approximately 33ng of DNA from the purified PCR product, 0.46 pmol of primer and 4ul of BigDye Terminator (ABI), and were run at 96°C for 1 min then 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min. Reaction cleanup was performed using Agencourt cleanSEQ (Beckman Coulter genomics, Danvers, MA) and the resulting products were sequenced on a 96 capillary 3730xl DNA analyzer.

We edited and aligned our sequences using Geneious Pro v 5.5. (Biomatters Ltd, Auckland, New Zealand). We added to our alignments sequences from *Myrmecocystus mexicanus*, obtained from GenBank (COI- DQ353330.1, 28S- DQ353639.1, EF1 $\alpha$ - EU143096.1, and ArgK-EU143007.1) to serve as an outgroup for tree reconstruction. For the final alignments used for phylogenetic analysis, we applied the Clustal alignment function in Geneious Pro under default settings and manually adjusted the alignment according to codon positions using the translation function and by comparing our sequences to similar sequences referenced from GenBank.

## Phylogenetic analysis

We constructed all phylogenies using Bayesian methods implemented in MrBayes v 3.2.1 (Ronquist et al. 2011). To determine models of evolution for each gene sequenced, we used the model selection option in MEGA5 that provides the best fit nucleotide substitution models according to Akaike information criterion (A1Cc) and Bayesian information criterion (BIC) (Tamura et al. 2011). We reconstructed separate phylogenies in MrBayes using each of the four genes sequenced. We partitioned COI by codon position and used the following models:

GTR+I+G for positions 1 and 3 and HYK for position 2. For the nuclear genes we used HKY +I for 28S and K2 for EF1 $\alpha$  and ArgK.

For species delimitation, we ran the program Bayesian Phylogenetics and Phylogeography (BPP, v.2.1; Yang and Rannala 2010) which uses a starting guide tree, coalescent-based modeling, and Bayesian methods to identify independently evolving species lineages (Fujita et al. in press). We used the topology of the COI tree produced from MrBayes as our guide tree and input sequence data from *Polyergus* and *Formica* from 28S, EF1 $\alpha$  and ArgK for the BPP analysis. Lineages for the guide tree had at least 0.6 posterior probability support in the COI tree and were bifurcating (all polytomies were collapsed into single clades). These guide tree lineages were also used to label the COI tree and for subsequent analyses (see below). For accuracy, BPP requires assignment of at least two samples per lineage, so we excluded any single samples that did not fall into the lineages of the guide tree (i.e. JT019\_1P and the outgroup). We ran BPP using a gamma prior distribution of G(2,400) for ancestral population size ( $\theta$ ), and G(2,330) for the age of the root of the species tree ( $\tau$ ). The Dirichlet prior was applied for all other divergence time parameters. Since we found no significant difference in the posterior probability values produced by the different rjMCMC algorithms, we present here the results from running algorithm 1 under default settings with a burn in of 5000, sampling every 5<sup>th</sup> individual for a total of 100,000 samples. We considered any nodes with less than 0.99 posterior probability values obtained from the BPP analysis to have lineages that are unsupported as separate species and therefore collapsed them into a single species lineage for the subsequent species tree inference using \*BEAST (see below).

We applied two methods for reconstructing phylogenies using multi-locus data: a phylogenetic reconstruction of concatenated sequences of all four genes built in MrBayes using the aforementioned evolutionary models (partitioned by codon for COI and by gene for the nuclear sequences) and a species tree reconstruction applying the \*BEAST method in the program BEAST v1.7.2. \*BEAST uses Bayesian MCMC methods and the multi-species coalescent to infer a species tree within which several gene trees are co-estimated (Heled and Drummond 2010). Since \*BEAST assumes no gene flow between species lineages, we assigned our samples to lineages (candidate species) based on those determined by the BPP analysis (individual JT019\_1P was lumped with lineages A and B since both lineages plus this sample were found to form a single clade and JTL525 was assigned its own lineage, N). Additionally, we removed any *Polyergus* samples originating from the same localities that also parasitized the same species of *Formica*. We included all four genes in the \*BEAST analysis and applied the following substitution models for each gene: GTR+I for COI, HKY+I for 28S and HKY for EF1 $\alpha$  and ArgK as determined by the model selection option in MEGA. We applied the Yule model for the species tree prior and used the uncorrelated lognormal relaxed clock model under default settings. The substitution models, clock rate, and tree topologies were unlinked for the four genes. We ran \*BEAST using a random start tree for 10,000,000 generations and sampled parameters every 1000 generations. The resulting species tree from the \*BEAST analysis was viewed using TreeAnnotator v 1.7.2.

### **Population genetic analysis of *P. breviceps* and *P. lucidus***

We used MEGA5 to calculate mean sequence diversity among groups of well-supported lineages produced from the COI tree (Figure 1) and between the two species of North American *Polyergus* using the Kimura 2-parameter (KP2) model of sequence evolution. To determine how populations of *P. breviceps* and *P. lucidus* are structured, we performed an analysis of molecular variance (AMOVA), a Mantel test, and a partial Mantel test for each species (as they are currently recognized by morphological standards) implemented in the program Arlequin v 3.1 (Excoffier et al. 2007). For the AMOVAs, we grouped samples into levels according to statistically supported lineages from the reconstructed COI phylogeny (see Figure 1) to examine the partitioning of genetic variance within and among these lineages. We ran the AMOVA under the Kimura 2-parameter model with 1000 permutations and determined significance of resulting  $\Phi_{ST}$  values using 110 permutations. We also calculated nucleotide diversity within lineages of *P. breviceps* and *P. lucidus* using Arlequin.

For the Mantel test, we compared pair-wise genetic distances between samples of *Polyergus* and the *Formica* they enslaved to test for statistical correlation between them. To determine the possible role of geography (isolation by distance) and/or host in the genetic differentiation between populations of *P. breviceps* and *P. lucidus*, we performed a partial Mantel test (i.e. partial correlation in Arlequin). Partial Mantel tests allowed us to examine whether there was a correlation between genetic distances of *Polyergus* and geography while controlling for host, and vice versa. We tested for correlations between a matrix of pair-wise genetic distances between samples of *Polyergus* ( $Y_1$ ) with a matrix of their geographic distances ( $X_1$ ) calculated using Geographic Distance Matrix Generator v 1.2.3 (Ersts 2012) and a host matrix ( $X_2$ ) indicating whether *Polyergus* enslaved the same (0) or different (1) species of *Formica*. Pair-wise genetic distances of *Polyergus* and *Formica* samples used for Mantel and partial Mantel matrices were calculated in the program MEGA5 (Tamura et al. 2011) under the Kimura 2-parameter (KP2) model for sequence evolution. We ran partial and full Mantel tests using 10,000 permutations. For these population genetic analyses, we removed any duplicate samples that were from the same location and parasitized the same host to avoid biases in the correlations.

## **RESULTS**

### **Phylogenetic analysis**

For COI, we sequenced a total of 123 individuals (48 *P. breviceps*, 15 *P. lucidus*, 3 *P. rufescens*, 1 *P. samurai*, 39 *Formica* from the fusca group, 13 *Formica* from the pallidefulva group, 2 *Formica fusca* from Europe, 1 *Formica japonica*, and 1 *Formica gerardi*). Final alignments for individual genes were 741 bp for COI, 530 bp for 28S, 540 bp for EF1 $\alpha$  and 351 bp for ArgK.

For phylogenies inferred from all four genes used in our study (COI, 28S, EF1 $\alpha$ , and ArgK) we included 91 individuals (29 *P. breviceps*, 12 *P. lucidus*, 3 *P.*

*rufescens*, 1 *P. samurai*, 28 *Formica* from the fusca group, 13 *Formica* from the pallidefulva group, 2 *Formica fusca* from Europe, 1 *Formica japonica*, 1 *Formica gerardi* and the *Myrmecocystus mexicanus* as the outgroup). The final concatenated four gene alignment consisted of 2160 bp including gaps from indels.

The Bayesian tree inferred from COI mitochondrial DNA recovered both *Polyergus* and *Formica* genera, and *P. breviceps* and *P. lucidus* as monophyletic groups (Figure 1). This pattern was also true for the concatenated tree incorporating all four genes analyzed (Figure 2). For both the COI and concatenated trees, *P. samurai* clustered with *P. rufescens* from Bulgaria (lineage D) while *P. rufescens* from France and Austria formed a separate clade (lineage C). However, the placement of the Eurasian *Polyergus* differed between the COI and concatenated gene trees. While the COI data grouped the two Eurasian lineages to with *P. breviceps*, the concatenated tree placed these two Eurasian clades as sister to all North American *Polyergus*. Both the COI data and the concatenated data from all four genes recovered the *Formica pallidefulva* species group as monophyletic. In contrast, the *Formica fusca* group was polyphyletic, with *F. moki* and *F. gnava* falling outside the rest of the fusca group clade for the COI and concatenated gene trees (L and M in Figures 1 and 2). Neither the COI data nor the concatenated four gene data produced a phylogeny that clearly defined the relationships among the different species of *Formica* and several *Formica* species did not come out as monophyletic (Figures 1 and 2).

All individual nuclear DNA gene trees supported the genus *Polyergus* as a monophyletic group. For *Formica*, only taxa belonging to the *Formica pallidefulva* group were consistently monophyletic across all three genes. As with the concatenated tree, *F. moki* and *F. gnava* formed clades distinct from the rest of the *Formica* in the fusca group. 28S sequence data produced the most unresolved tree with regard to the *Polyergus* clade (S1), but did place all *Polyergus* from outside North America as a clade separate from the North American *Polyergus*. Both EF1 $\alpha$  and ArgK sequence data produced limited resolution of possible groups within *P. breviceps*, although some individuals originating from California and Washington grouped together (S2, S3). However, none of the nuclear genes could resolve the relationship between the currently recognized (by morphology) *P. breviceps* and *P. lucidus*.

Mitochondrial COI sequence data produced the most well-resolved phylogeny for *Polyergus*, dividing the morphologically identified *P. breviceps* and *P. lucidus* samples into distinct lineages, nearly all with  $\geq 0.99$  posterior probability support (Figure 1). *P. breviceps* samples were grouped into three bifurcating lineages (E, F and G) and *P. lucidus* into two lineages (A and B), with one sample falling outside lineages A and B (JT019\_P) but still within the monophyletic *P. lucidus* clade. The concatenated four gene tree also agreed with this splitting of *P. breviceps* lineages, but the lineages A and B within *P. lucidus* were not monophyletic for the concatenated gene tree (Figure 2).

The *Polyergus* lineages generally followed large-scale phylogeographic patterns across North America, with lineage E distributed along the Pacific coast, lineages F and G extending from the southern tip of the Rocky Mountain Range

eastward, and lineages A and B restricted mostly to eastern portion of North America (Figure 3). Lineage E consists of populations of *P. breviceps* from California, Washington and British Columbia. Interestingly, samples from a single site in California (Sagehen Creek) and samples from the state of Washington did not form separate monophyletic clades based on their sampling locations (Figure 1). Lineage F includes *P. breviceps* from Illinois, an individual from Alberta, Canada and, oddly, one individual from Arizona parasitizing *Formica fusca marcidia*. Lineage G includes the majority of the samples of *P. breviceps* from the southwestern United States (Arizona, Colorado and Utah) but also those from Missouri and Arkansas. Although the COI tree showed some *Formica* samples from the same or geographically close locations as monophyletic, the overall geographic patterns seen in *Polyergus breviceps* was not uncovered for those *Formica* that they enslave (lineages J, L and M, Fig 1).

For *Polyergus lucidus*, lineage A consisted of samples from Florida, New York, Massachusetts, Mississippi, and Wisconsin while lineage B contained *P. lucidus* from Ontario, Canada and Iowa (Figure 1). Only one sample of *P. lucidus*, JT019\_P, parasitizing *F. incerta* in Missouri, fell outside lineages A and B but within the *P. lucidus* clade. It should be noted that this sample was collected from the same county as *P. breviceps* parasitizing *F. subsericia*. The pallidefulva group, those *Formica* enslaved by *P. lucidus*, also broke up into lineages in the COI phylogeny (lineages H and I, Figure 1) but these lineages did not follow the same geographic patterns as that of their slave-makers.

### Species delimitation and species tree reconstruction

Posterior probability node support values from the BPP analysis suggests North American *Polyergus* can be divided into three species groups (Figure 4). The splitting of lineages A and B had very weak node support and thus *P. lucidus* can be inferred as a single species (lineage AB). In contrast, *Polyergus breviceps* separated into two species groups with the combined lineages E and F as one species and lineage G as the other. *Formica* lineages remained distinct, as they were in the original guide tree with the exception of lineages H and I, which could be collapsed into a single lineage consisting of the *Formica* from the pallidefulva group (Figure 4).

For the species tree based on the \*BEAST analysis, we examined the relationships between *Polyergus* and *Formica* lineages identified as species by the BPP analysis (noted above). As with the concatenated data, the species tree produced from \*BEAST strongly supports *Polyergus* and *Formica* as monophyletic groups and places North American *Polyergus* as a sister clade to those from Eurasia (Figure 5). Within *Polyergus*, however, the \*BEAST analysis inferred the currently recognized *P. breviceps* as a paraphyletic group with *P. lucidus* (lineage AB) nested within the clade of North American *Polyergus*. Although *P. lucidus* grouped more closely with lineage G of *P. breviceps*, support for the clade that combines the two is relatively low. The \*BEAST tree inferred the *Formica* pallidefulva group as a sister clade to the rest of the *Formica* which included all *Formica fusca* and Eurasian

*Formica*. There is no resolution, however, between the paraphyletic *Formica fusca* group (lineages J, L and M) and the Eurasian *Formica*.

### **Population genetic analysis of *P. breviceps* and *P. lucidus***

Between *P. breviceps* and *P. lucidus* we found an average percent sequence divergence of 12.05% for COI. Within the *P. breviceps* lineages, the average percent sequence divergences were 5.85% between lineage F and G, 7.24% between E and G, and 6.92% between E and F. Nucleotide diversity within each *P. breviceps* lineage was as follows: 2.45% for lineage E, 1.0% for lineage F, and .007% for lineage G. For *P. lucidus*, the average percent sequence divergence between lineages A and B was 1.39% and the nucleotide diversity within lineage A and B was 1.0% and 0.07%, respectively.

The AMOVA for *P. breviceps* had high statistical support for genetic structuring according to E, F and G lineages as designated by the COI phylogeny, with 76.57% of the variance explained among these lineages (Table 3). *P. lucidus* also showed significant genetic structuring according to COI lineages A and B, though not as high as *P. breviceps* and only 27.96% of the variance could be attributed to differences among lineages for *P. lucidus*.

Results from the Mantel test comparing genetic distances between pairs of *P. breviceps* populations (samples belonging to lineages E, F and G of the COI tree) and those of their enslaved *Formica* species (lineages J, L, N, M) showed only a weak correlation between them (Table 3). In contrast, *P. lucidus* populations (lineages A and B) pair-wise genetic distances were strongly and significantly correlated with those of their hosts from the pallidefulva group.

Partial Mantel analysis on *P. breviceps* revealed that both host identity (correcting for geographic distance) and geographic distance (correcting for host ID) were significantly correlated with pair-wise genetic distances between samples (Table 3). In contrast, *P. lucidus* genetic distances were only significantly correlated with host association, and not with geographic distance, when controlling for geography or host, respectively. A closer examination of the genetic distance and geographic distance matrices suggests that this lack of correlation may be due to several individuals being genetically close but geographically distant from one another relative to others and vice versa. For example, although two *Polyergus* samples (BRS010 and CJ033) are only about 29 km apart, there was a genetic distance of 0.01 (KP2 value) between them. Conversely, two other samples that were 1663.23 km apart (JMG018 and BRS010) had a genetic distance of 0.006. Compared to *P. breviceps*, *P. lucidus* genetic distances were more strongly correlated with host association when controlling for geography.

## **DISCUSSION**

In this study we used sequence data from one mitochondrial gene (COI) and three nuclear genes (28S, EF1 $\alpha$ , and ArgK) to examine the phylogeography and population structure of two currently recognized North American species of slave-

making ant (*Polyergus breviceps* and *Polyergus lucidus*) and their slaves (*Formica* spp). For the COI phylogeny, the concatenated phylogeny and the \*BEAST species tree, we found *Polyergus* and *Formica* grouped monophyletically by genus in agreement with previous studies involving these genera (Hasegawa et al. 2002; Munoz-Lopez et al. 2011). Mitochondrial DNA sequence from COI provided the most information with regard to resolving phylogeographic patterns for *Polyergus*. Nuclear DNA sequence data, on the other hand, was not as phylogeographically informative. However, it did provide additional information that allowed us to ascertain that North American *Polyergus* could be divided into three distinct species instead of the two currently recognized by morphology. Additionally, COI data allowed us to determine the possible role of geographic distance and host association in population structuring of *P. breviceps* and *P. lucidus*. We found that the relative role of geography and host differs between the two species. This likely reflects contrasting population genetic structures, evolutionary histories, and host adaptation abilities.

### **Phylogeography and phylogenetics of *Polyergus* and *Formica***

In general, we found that *P. breviceps* populations fall into three lineages that follow west-east phylogeographic patterns as expected from their widespread distribution across North America. The COI phylogeny and the concatenated four gene tree (included COI, 28S, EF1 $\alpha$ , and ArgK) supported splitting *P. breviceps* into lineages E, F and G. Lineage E is restricted to localities along the Pacific coast of North America and represents the most geographically isolated lineage. Lineages F and G have samples that extend from the Rocky Mountains eastward into the midwestern United States. However, two phylogenetic observations of note do not seem to conform strictly to phylogeographic expectations. First, within lineage E, California and Washington samples are polyphyletic with Washington and British Columbia samples (Pacific Northwest) embedded within a clade of California samples. This contrasts with other studies of organisms distributed across the Pacific coast, which have shown a clear phylogenetic division between the Pacific Northwest and California populations of the same species (e.g. Bradley Shaffer et al. 2004; Recuero et al. 2006; Rich et al. 2008; Turmelle et al. 2011). Additional and faster evolving genetic markers may resolve the relationships among populations within lineage E. The second pattern we noted was that two populations of *P. breviceps*, one from lineage F and one from G, had nearly overlapping geographic locations in mid-eastern Arizona (~26km apart). This is despite the fact that lineage F is more closely related to lineage E than G according to COI data and that lineage G may actually represent a species distinct from lineages E and F (see next section below). As such we believe this Arizona location may represent a contact zone between the lineages F and G and a similar geographic pattern has also been shown in a survey of amphibians distributed across North America (Rissler and Smith 2010). To test this hypothesis and gain a better understanding of the distribution of these lineages, increased sampling should be conducted to further explore *Polyergus* population demographics (i.e. presence or absence of migration, population

expansion, and genetic structuring) in the context of possible glacial refugia (e.g. Beibl et al. 2007; Brandt et al. 2007).

In contrast, *P. lucidus* populations did not follow any obvious phylogeographic patterns. For the COI tree, lineages A and B had similar distributions in the localities surrounding the Great Lakes and in the mid Western US. Within lineage A, we found no clear resolution between possible phylogeographic breaks that have been found in other studies within eastern North America (see Soltis et al. 2006 for review). One sample JT019, is placed as a root to both A and B lineages but still groups with these lineages forming a clade including all *P. lucidus*. Interestingly, this sample was collected from the same locality as *P. breviceps*. The most parsimonious explanation for this overlap is that these samples may represent ancestors from a geographic location where North American *Polyergus* could have originated. This assumes *P. breviceps* expanded westward while *P. lucidus* expanded eastward. Again, this speculation of origin is best addressed with more extensive population sampling of *P. breviceps* and *P. lucidus* populations to look for patterns of population expansion and to establish which populations are the likely ancestors with additional molecular markers that provide better resolution at the population and species level.

Host association at the phylogenetic level could not be analyzed in a clear or rigorous manner given the results we received from our phylogenetic reconstruction and BPP species delimitation analysis (i.e. we could not apply phylogenetic analyses to test for matching branching patterns between *Polyergus* and *Formica* lineages). In particular, sequence data from the four genes used in this study could not clearly resolve the relationships among different species of hosts (*Formica*) and often didn't recover some species of *Formica* as monophyletic. A phylogenetic analysis using both nuclear and mitochondrial loci to examine the relationships among *Formica* in the subgenera *Serviformica* and Eurasian slave-makers *P. rufescens* and *P. samurai*, also showed a lack of clear relationship among the different species of *Formica* (Munoz-Lopez et al. 2011). Since relationships among the *Formica* could not be determined and formation of distinct host clades could not be established from phylogenetic reconstruction, assessing the congruency between *Polyergus* and *Formica* branch topology could not be conducted with confidence. Additionally, the number of distinct clades uncovered for *Polyergus* were not high enough to perform traditional tests for cospeciation.

One exception to the overall lack of apparent host-association at the phylogenetic level was the *Formica pallidefulva* group (lineages H and I) that includes all hosts of *P. lucidus* (lineages A and B) and consistently formed a monophyletic group separate from the other *Formica* in our phylogenetic analyses. In contrast, hosts from the currently recognized *Formica fusca* group were polyphyletic (lineages J, N, L, and M) and did not reflect the branching patterns of their parasites, *P. breviceps*. Of particular note are *Formica subpolita* (lineage N), *Formica moki* (lineage L), and *Formica gnava* (lineage M) that form clades separate from the remainder of the *Formica fusca* group. Interestingly, lineages N, L and M contain species which each originate from a distinct, currently recognized species complexes and are the only bicolored *Formica* from the fusca group represented in



this study (Francoeur 1973). The *P. breviceps* that parasitizes these "outgroup" fusca group lineages, do not follow this phylogenetic pattern. Overall, no recognizable phylogeographic patterns within the *Formica pallidefulva* group or *Formica fusca* group could be detected from phylogenetic reconstruction. As such, we believe examining each currently recognized slave-making ant species using population genetic methods, rather than the phylogenetic analyses, was the most appropriate way of examining the roles of host and/or geography on population structure of North American *Polyergus*. Host switching may be one mechanism by which the patterns of relationship among two interacting antagonists may become discordant when comparing their respective phylogenies (Page and Charleston 1998). This could explain the lack of congruence between the phylogenetic patterns found in *Polyergus* and their hosts *Formica*.

### **Species delimitation for North American *Polyergus***

Overall, our results from the BPP species delimitation analysis suggest that North American *Polyergus* can be divided into three distinct species: *P. lucidus* as it is currently recognized and two species of what has been morphologically identified as *P. breviceps*. These two new species of the currently recognized *P. breviceps* include the combined lineage "EF" (from the Pacific Coast, Illinois, Canada, and one sample from Arizona parasitizing *F. fusca marcida*) and lineage "G" (from the southwestern US, Missouri and Arkansas). The relationships among these species however could not be clearly resolved based on nuclear data alone and we found a disagreement between the inferred concatenated gene tree and the Bayesian species tree analysis using \*BEAST. In particular, the \*BEAST analysis suggests a weakly supported clade (0.57 posterior probability value) that groups *P. lucidus* with lineage G from *P. breviceps* and renders *P. breviceps* as a paraphyletic grouping. We believe that our sequence data and limited sampling were unable to uncover the true relationships between these North American *Polyergus*. One limitation of \*BEAST is that it does not account for recombination within loci (Heled and Drummond 2010) and this could potentially have an affect on our estimate of the species tree since we incorporated three nuclear genes into the analysis.

Morphological analysis and ecological observations have lead to the belief that *P. breviceps* and *P. lucidus* may each be species complexes (King and Trager 2007; Trager et al. 2007). To address these suspicions, James C. Trager is currently conducting a worldwide revision of *Polyergus* based on extensive sampling and morphological data. Our genetic data do not currently support dividing *P. lucidus* into a species complex, though additional genetic data from more rapidly evolving genes and a more extensive sampling of *P. lucidus* individuals may shed light on whether subspecies exists. Currently, no data has been published implying the separation of *P. breviceps* into more than one species but recent morphological analysis suggests that *P. breviceps* should not be treated as a single species based on morphology (Trager, personal communication). Comparison of the results the forthcoming revision of *Polyergus* and this study will provide a more complete picture of the evolutionary history of this genus of slave-maker.

## **Population genetic analysis of *P. breviceps* and *P. lucidus* and the role of geography and host in population structuring**

COI sequence data provided the best resolution for examining population level genetic structure based on geographic distance and host association for *P. breviceps* and *P. lucidus*. Considering all population genetic results obtained from the nucleotide diversity analysis, AMOVA, the Mantel tests, and the partial Mantel tests, we can make two general conclusions. First, *P. breviceps* is more genetically structured than *P. lucidus*. Second, *P. lucidus* populations appeared more structured by host while *P. breviceps* populations seemed more strongly structured by geography. *P. breviceps* had over double the percentage of genetic variance explained among lineages compared to *P. lucidus* which had the majority of the genetic variance explained within lineages. This is not surprising since our analysis of *P. breviceps* included lineages that were shown to be two putative species according to the BPP analysis and *P. lucidus* could be considered a single species. Still, nucleotide diversity was also higher among lineages within *P. breviceps* than *P. lucidus*. This may be due to the fact that *P. lucidus* populations could be smaller and parasitize a more limited number of *Formica* species compared to *P. breviceps* populations, and thus genetic diversity might be lower. We also cannot rule out the fact that we had more limited sampling for *P. lucidus* compared to *P. breviceps*.

*P. lucidus* populations showed a stronger genetic correlation with their hosts compared to *P. breviceps* for both the Mantel (tested for the correlation with host genetic distance) and partial Mantel tests (tested whether host was the same species or not). In contrast, *P. breviceps* population differentiation showed a strong correlation with geographic distance while *P. lucidus* showed none at all (see partial Mantel test). These results seem to generally agree with the overall patterns found in the phylogenetic analysis with *P. breviceps* being more variable and structured according to geography and *P. lucidus* having a more clear association with their hosts and showing a lack of phylogeographic structure.

Though slave-making ants generally appear specialized on their hosts, these social parasites may alternate or shift to different hosts depending on host availability (Brandt et al. 2005). If this is the case, our data indicate that *P. breviceps* is perhaps more likely to have undergone host switching than *P. lucidus*. Additionally, *P. lucidus* is only known to parasitize five species belonging to the *Formica pallidefulva* group while *P. breviceps* enslaves several different species from the phylogenetically diverse *Formica fusca* group. As such, we suspect that *P. lucidus* may be under more phylogenetic constraints with regard to host switching and perhaps more specialized on their hosts than *P. breviceps* through evolutionary time. This could explain why we found higher host association with *P. lucidus* than *P. breviceps*, especially with regard to the results of the Mantel test. However, the weak correlation of genetic distances among *P. breviceps* populations with their hosts still suggests some level host association. We suspect that that perhaps *P. breviceps* are more likely to be locally adapted to their hosts but may still retain the ability to switch hosts over evolutionary time. The role of local adaptation in shaping larger phylogeographic patterns will need to be explored to better understand possible population dynamics between these parasites and their hosts (see Thompson 2005).

## Conclusions

Little is known about the ecology and natural history of North American *Polyergus* beyond the knowledge gained from behavioral studies performed on a small representation of *Polyergus* populations and hosts. The results from this study provides the first population-level and phylogenetic analysis of North American slave-making ants in the genus *Polyergus* in the context of their geographic distribution and the hosts they enslave in the genus *Formica*. Here we examine several populations of *Polyergus* from across their North American distribution and present data on how both geography and host association may have shaped the relationships among slave-making ant populations. We also uncovered a putative third species of North American *Polyergus* that should inform the future revision of this genus and future research on this genus. This study lays the foundation for future work on the dynamics of interactions between different populations of *Polyergus* and their hosts and is important for understanding whether these slave-makers and slaves are coevolving.

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**Table 1.** List of *Polyergus* and host (*Formica*) samples with their locality information. Sample codes reference the labels on the tips of phylogenetic trees included in this study. See footnotes below for the key to symbols associated with sample sources.

Sample code (host if different)	<i>Polyergus</i> species	<i>Formica</i> (host) species	Locality	Coordinates	
BRS010*	<i>P. lucidus</i>	<i>F. incerta</i>	Long Island, Medford, NY, USA	40.817778	-72.986111
CAJcol 1†	<i>P. lucidus</i>	unknown	Suffolk Co., NY, USA	40.897534	-72.937187
CD030* (CD025*)	<i>P. breviceps</i>	<i>F. podzolica</i>	Larimer Co., CO, USA	40.65	-105.46
CJ033*	<i>P. lucidus</i>	<i>F. dolosa</i>	Suffolk Co., NY, USA	40.94	-72.68
CJ04-161‡	<i>P. breviceps</i>	unknown	White River, AZ, USA	34.006825	-109.859268
CLL011*	<i>P. rufescens</i>	<i>F. gerardi</i>	Banyuls, FRANCE	n/a	
CWT068‡	<i>P. breviceps</i>	<i>F. gnava</i>	Portal, AZ, USA	31.909633	-109.148483
CWT096‡	<i>P. breviceps</i>	<i>F. occulta</i>	Barfoot Creek, AZ USA	31.91607	-109.27763
CWT105‡	<i>P. breviceps</i>	<i>F. accreta</i>	Sagehen Creek Field Station, CA, USA	39.438333	-120.252222
GU034*	<i>P. lucidus</i>	<i>F. pallidefulva</i>	Pinery Pr. Pk., ON, CANADA	43.259958	-81.827821
JG009*	<i>P. lucidus</i>	<i>F. pallidefulva</i>	Richland Co., WI, USA	43.38	-90.43
JG015*	<i>P. breviceps</i>	<i>F. podzolica</i>	Sturgeon Co., AB, CANADA	53.802222	-113.649722
JG040*	<i>P. lucidus</i>	<i>F. incerta</i>	Sauk Co., WI, USA	43.43	-89.94
JMG007*	<i>P. breviceps</i>	<i>F. subsericea</i>	Conway Co., AR, USA	35.264444	-92.698889
JMG016*	<i>P. lucidus</i>	<i>F. dolosa</i>	Oktibbeha Co., MS, USA	33.43	-88.88
JMG018*	<i>P. lucidus</i>	<i>F. dolosa</i>	Winston Co., MS, USA	33.09	-89.04

\* Sample obtained from James C. Trager of the Shaw Nature Reserve in Missouri who is currently working on a global revision of *Polyergus* and includes some from museum collections and his own personal collection.

† Sample obtained from private or museum collections housed outside the University of California, Berkeley.

‡ Sample from the authors' private collection at the University of California, Berkeley

Sample code (host if different)	<i>Polyergus</i> species	<i>Formica</i> (host) species	Locality	Coordinates	
JRK1000‡ (CWT170)	<i>P. lucidus</i>	<i>F. archboldi</i>	Leon Co., FL, USA	30.279895	-84.340082
JRK1001‡ (CWT169)	<i>P. lucidus</i>	<i>F. pallidefulva</i>	Leon Co., FL, USA	30.279895	-84.340082
JRK1002‡ (CWT171)	<i>P. lucidus</i>	<i>F. dolosa</i>	Leon Co., FL, USA	30.279895	-84.340082
JT001*	<i>P. breviceps</i>	<i>F. subsericea</i>	Franklin Co., MO, USA	38.473386	-91.048678
JT004*	<i>P. breviceps</i>	<i>F. subsericea</i>	Franklin Co., MO, USA	38.473386	-91.048678
JT005*	<i>P. breviceps</i>	<i>F. subsericea</i>	Franklin Co., MO, USA	38.473386	-91.048678
JT006*	<i>P. breviceps</i>	<i>F. subsericea</i>	Franklin Co., MO, USA	38.473386	-91.048678
JT019*	<i>P. lucidus</i>	<i>F. incerta</i>	Franklin Co., MO, USA	38.473386	-91.048678
JTL3916†	<i>P. breviceps</i>	<i>F. accreta</i>	Snoqualmie National Forest, WA, USA	46.9	-121.41667
JTLC1252†	<i>P. breviceps</i>	<i>F. argentea</i>	Colville, WA, USA	48.73333	-117.88333
JTLC1288†	<i>P. breviceps</i>	unknown	Cortes Island, BC CANADA	50.1	-125.03333
JTLC1562†	<i>P. breviceps</i>	<i>F. accreta</i>	Chinook Pass, WA, USA	46.87958	-121.28178
JTLC525†	<i>P. breviceps</i>	<i>F. subpolita</i>	Cleman Mt. Lookout, WA, USA	46.81667	-120.85
KI013*	<i>P. rufescens</i>	<i>F. fusca</i>	BULGARIA	n/a	
LG024*	<i>P. breviceps</i>	<i>F. moki</i>	San Bernardino Co., CA, USA	34.83	-116.19
LR008*	<i>P. breviceps</i>	<i>F. montana</i>	Cook Co., IL, USA	42.14	-88.204722
LR027*	<i>P. breviceps</i>	<i>F. montana</i>	Cook Co., IL, USA	42.14	-88.204722
LRD014*	<i>P. lucidus</i>	<i>F. archboldi</i>	Clay Co., FL, USA	29.98	-81.86
LRD022*	<i>P. lucidus</i>	<i>F. pallidefulva</i>	Decatur Co., IA, USA	40.741389	-93.780833
MY1601†	<i>P. samurai</i>	<i>F. japonica</i>	Osato, Saitama, JAPAN	36.1	139.216667
NDT360‡	<i>P. breviceps</i>	<i>F. sp. c.f. argentea</i>	Rist Canyon, CO, USA	40.593706	-105.088939
NDT362‡	<i>P. breviceps</i>	<i>F. occulta</i>	Rist Canyon, CO, USA	40.593706	-105.088939
NDT407‡	<i>P. breviceps</i>	<i>F. altipetens</i>	Williams Valley, AZ, USA	33.8575	-109.221983

Sample code (host if different)	<i>Polyergus</i> species	<i>Formica</i> (host) species	Locality	Coordinates	
P1‡	<i>P. breviceps</i>	<i>F. argentea</i>	Sagehen Creek, CA, USA	39.436111	-120.261111
P10‡	<i>P. breviceps</i>	<i>F. fusca</i>	Sagehen Creek, CA, USA	39.43625	-120.260611
P2‡	<i>P. breviceps</i>	<i>F. argentea</i>	Sagehen Creek, CA, USA	39.435778	-120.26075
P3‡	<i>P. breviceps</i>	<i>F. accreta</i>	Sagehen Creek, CA, USA	39.441778	-120.245778
P4‡	<i>P. breviceps</i>	<i>F. argentea</i>	Sagehen Creek, CA, USA	39.436444	-120.261
P5‡	<i>P. breviceps</i>	<i>F. accreta</i>	Sagehen Creek, CA, USA	39.437472	-120.256667
P6‡	<i>P. breviceps</i>	<i>F. fusca</i>	Sagehen Creek, CA, USA	39.441583	-120.245639
P7‡	<i>P. breviceps</i>	<i>F. argentea</i>	Sagehen Creek, CA, USA	39.441333	-120.244194
P8‡	<i>P. breviceps</i>	<i>F. argentea</i>	Sagehen Creek, CA, USA	39.441444	-120.246333
P9‡	<i>P. breviceps</i>	<i>F. accreta</i>	Sagehen Creek, CA, USA	39.435833	-120.2615
PSW15251†	<i>P. breviceps</i>	<i>F. moki</i>	Mount Laguna, CA, USA	32.867465	-116.418793
PSW15985†	<i>P. breviceps</i>	<i>F. sp. cf. argentea</i>	Blue Canyon Lake, CA, USA	38.298633	-119.66525
SPC6620†	<i>P. breviceps</i>	<i>F. fusca marcida</i>	Greenlee Co, AZ, USA	33.642667	-109.326333
SPC6672†	<i>P. breviceps</i>	<i>F. occulta</i>	El Paso, Co., CO, USA	38.969167	-104.8015
SPC6949†	<i>P. breviceps</i>	<i>F. altipetens</i>	Williams Valley, AZ, USA	33.862667	-109.219667
SPC7221†	<i>P. breviceps</i>	<i>F. podzolica</i>	Garfield Co., CO, USA	39.5905	-108.816833
SPC7610†	<i>P. breviceps</i>	<i>F. gnava</i>	Santa Cruz Co., AZ, USA	31.674167	-110.558
SPC7686†	<i>P. lucidus</i>	<i>F. dolosa</i>	Plymouth Co., MA, USA	41.873833	-70.651833
SPC7863†	<i>P. breviceps</i>	<i>F. podzolica</i>	San Juan Co., UT, USA	38.368	-109.196333
TL003 *	<i>P. breviceps</i>	<i>F. occulta</i>	Pine Co., AZ, USA	32.366667	-110.816667
VB021 *	<i>P. rufescens</i>	<i>F. fusca</i>	Klagenfurt, AUSTRIA	n/a	

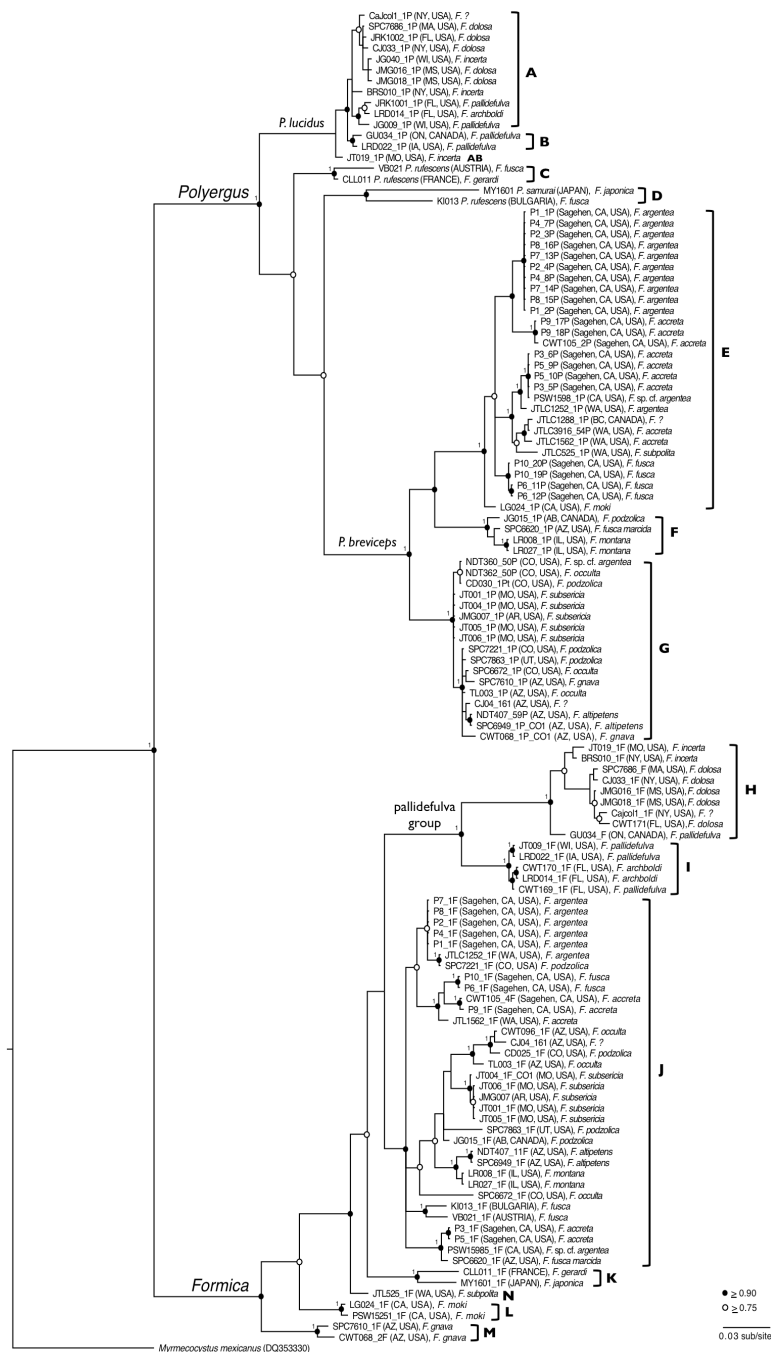


**Table 2.** Primer sets used to sequence cytochrome oxidase I (COI), ribosomal subunit 28S, the F2 copy of elongation factor 1 alpha (EF1 $\alpha$ ) and exon II of arginine kinase (ArgK).

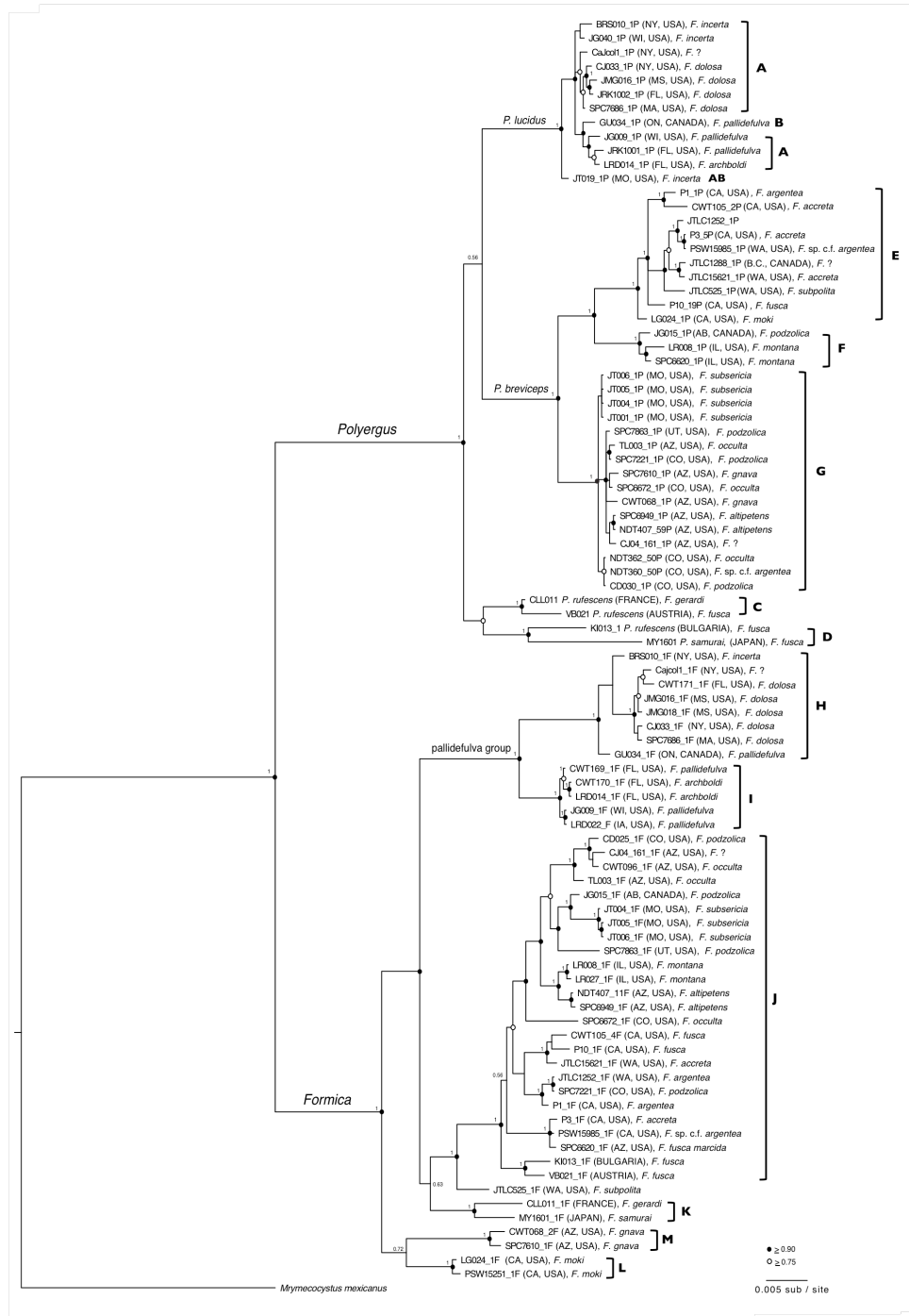
Gene	Primer	Sequence (5'-3')	Annealing temperature	Reference
COI	CI13PbrevF	CACTGCAATTTTACTTCTTT	49°C	this publication
	COIPluc2F	TCCTACTTCTATTATCCCTGCCTG	49°C	this publication
	COIPsam1F	TCTTTACCTGTCCTAGCTGGAGCA	49°C	this publication
	COIPruf1F	TCCCCTTATTAGTATGATCAAGTTT	49°C	this publication
	COIFwhlF	TTCCTTTGCTTGTATGATCAATTT	49°C	this publication
	CI24	TCCTAAAAAATGTTGAGGAAA	49°C	Hasegawa, 2002
28S	28S-3665F	AGAGAGAGTTCAAGAGTACGTG	49°C	Belshaw and Quicke, 1997
	28S-4068R	TTGGTCCGTGTTTCAAGACGGG	49°C	Belshaw and Quicke, 1997
EF1 $\alpha$	F2-557F	GAACGTGAACGTGGTATYACSAT	51°C	Brady et al., 2006
	F2-1118R	TTACCTGAAGGGGAAGACGRAG	51°C	Brady et al., 2006
ArgK	AK346EF	AGGGTGARTACATCGTRTCHACT	55°C	Jansen and Savolainen, 2010
	AK720ER	ACCTGYCCRAGRTCACCRCCCAT	55°C	Jansen and Savolainen, 2010

**Table 3.** Summary of population genetic statistics for *Polyergus breviceps* and *Polyergus lucidus*. AMOVA  $\phi_{ST}$  values are based on genetic structuring according to COI *Polyergus* lineages (Figure 1). Mantel tests results were from analyses of the correlation between pair-wise genetic distances of *Polyergus* and those of their hosts (*Formica*). Partial Mantel analyses tested for the correlation between genetic distance and geography (controlling for the effect of host) and genetic distance and hosts (controlling for the effect of geography).

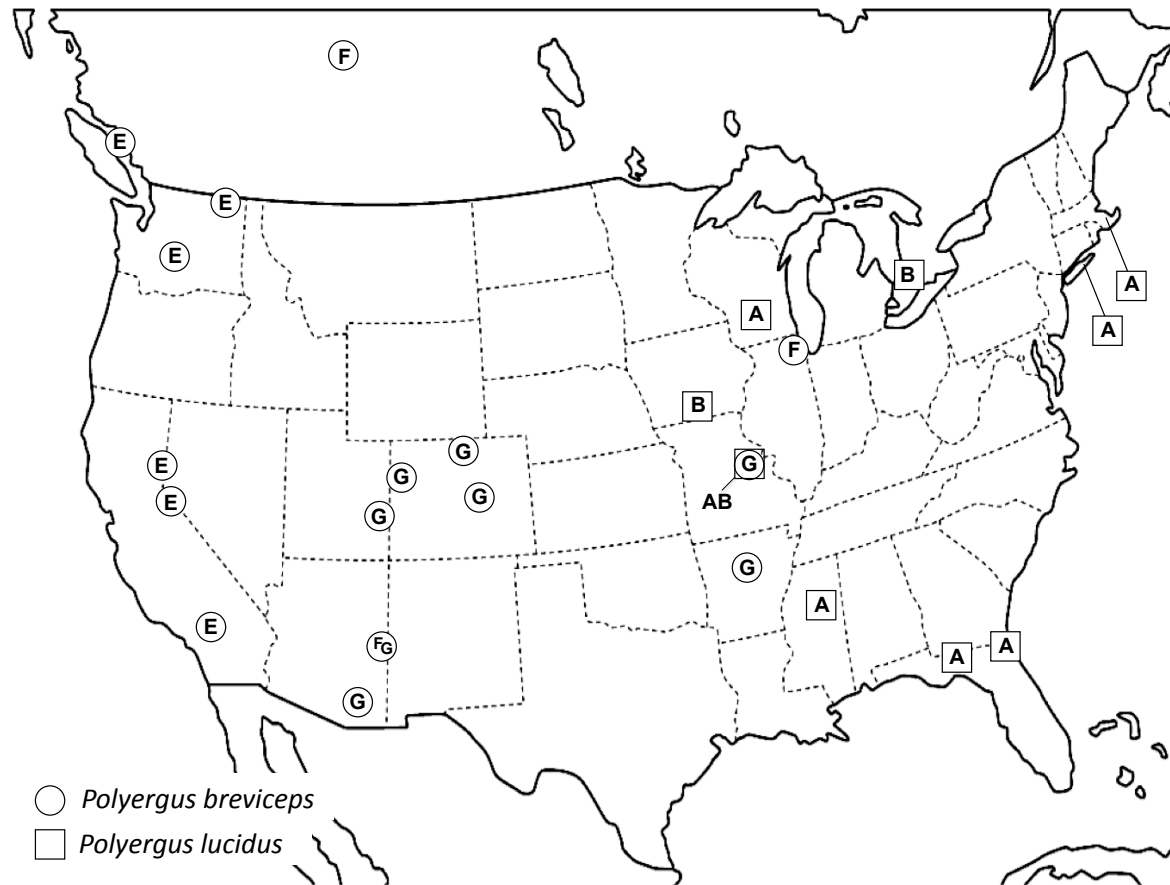
<i>Polyergus</i> species	AMOVA			Mantel		Partial Mantel			
	$\phi_{ST}$	p value	% var explained	r value	p value	Geography r value	p value	Host r value	p value
<i>P. breviceps</i>	0.76	<0.0001	76.57 %	0.107	0.065	0.408	<0.0001	0.097	0.044
<i>P. lucidus</i>	0.28	0.026	27.96 %	0.563	0.0061	-0.094	0.6	0.4653	0.003



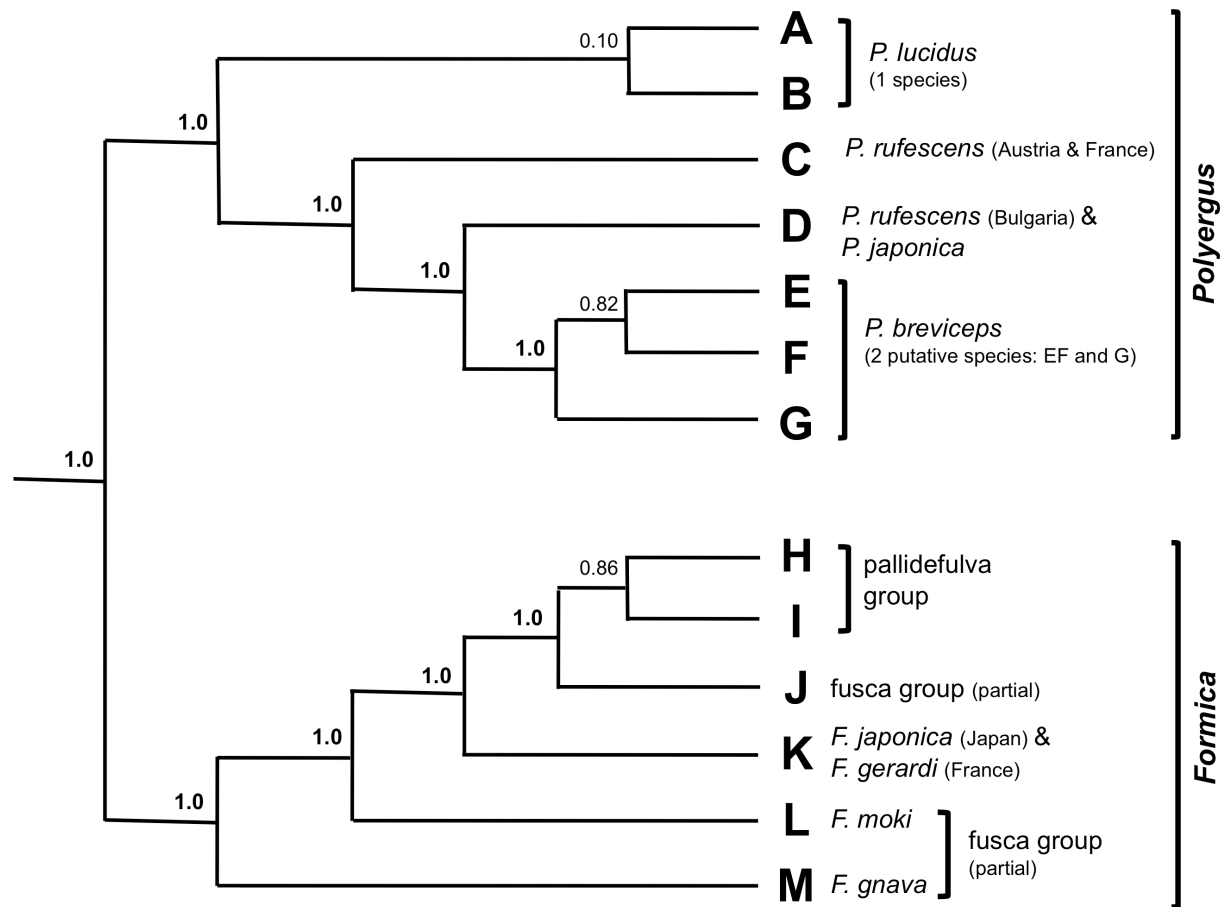
**Figure 1.** Bayesian tree inferred from mitochondrial COI sequence data from *Polyergus* and *Formica*, rooted with *Myrmecocystus mexicanus*. Letters represent lineages referred to in the text. Closed and open dots, and numbers at nodes represent posterior probability values. Unlabeled nodes have >0.5 posterior probability support. Tips are labeled with sample code\_individual ID, (locality), and host species (see Table 1).



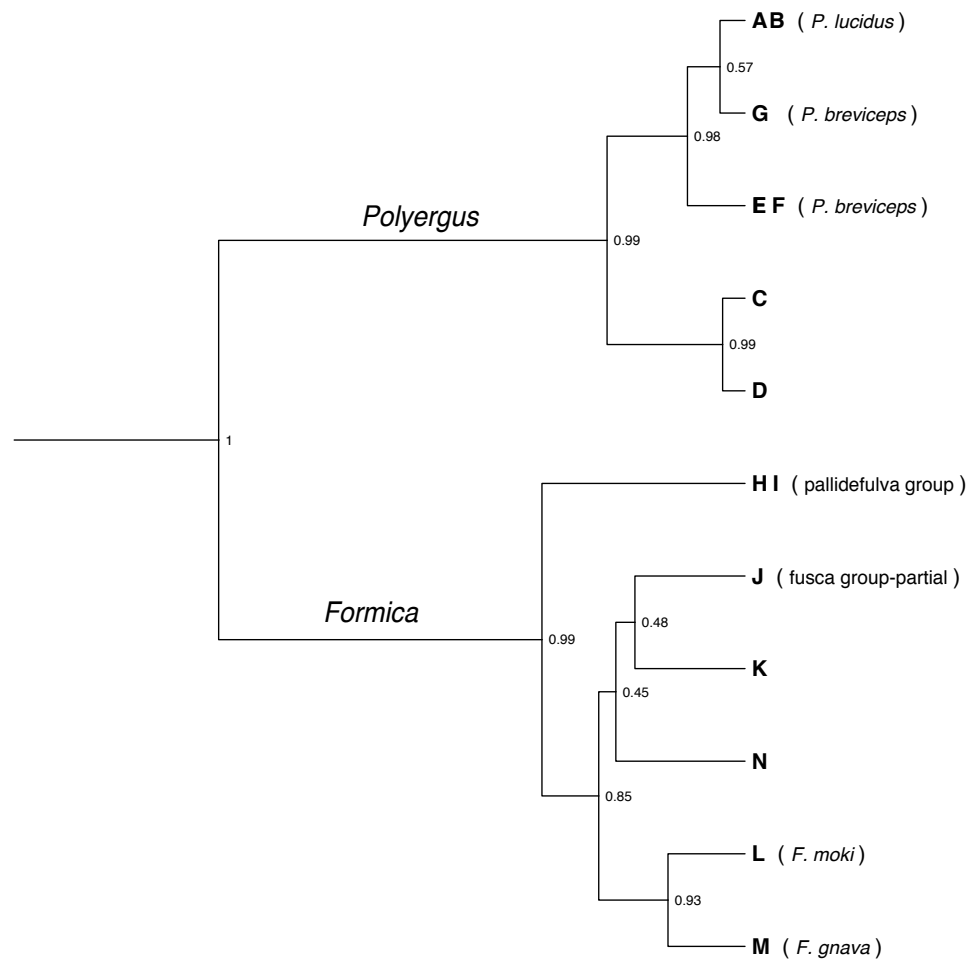
**Figure 2.** Bayesian tree inferred from concatenation of COI, 28S, EF1 $\alpha$ , and ArgK sequence data from *Polyergus* and *Formica*, rooted with *Myrmecocystus mexicanus*. Closed and open dots, and numbers at nodes represent posterior probability values. Any unlabeled nodes have  $>0.5$  posterior probabilities. Tips are labeled with sample codes\_individual IDs, (locality), and host species (see Table 1).



**Figure 3.** Map representing the North American distribution of supported lineages of *P. breviceps* and *P. lucidus* as determined by the Bayesian tree inferred from mitochondrial COI sequence data (see Figure 1). Some plots represent geographically close but different locations belonging to more than one sample.



**Figure 4.** Phylogram representing the output from the Bayesian Phylogenetics and Phylogeography (BPP) analysis using a guide tree based on lineages from the COI tree (Figure 1). Numbers on nodes are posterior probability values with bolded values representing clades ( $\geq 0.99$ ) supported for species delimitation.



**Figure 5.** \*BEAST species tree output of inferred relationships between lineages of *Polyergus* and *Formica* (see Figure 1 and 4) with outgroup excluded. Numbers on nodes are posterior probability support values.

## CONCLUSION

Previous research on other species of obligate slave-making ants strongly suggest such social parasites undergo a co-evolutionary arms race with their hosts (Brandt et al. 2005). However, to gain complete knowledge of possible coevolutionary processes occurring between a parasite and its host, one must first understand the dynamics of their ecological interactions at the local scale and the traits likely involved in this interaction. It is also useful to examine how several genetically distinct populations of the same parasite and host species interact with one another across a larger geographic landscape through ecological and evolutionary time. Combining studies of ecology and population genetics at and above the population level can lay the foundation for studying how parasites and hosts may coevolve with one another across broad geographic landscapes (Thompson 2005).

Despite their widespread distribution and the diversity of host species they enslave, there have been relatively few studies on North American *Polyergus* slave-makers that address their interaction with their host beyond the ecology of their raiding behavior and raiding biology. Of particular note is *P. breviceps*, in which only two populations have been studied (see: Topoff 1999; Johnson et al. 2005; Bono et al. 2006; Bono et al. 2007). In this dissertation, I attempt to remedy some of the gaps present in the *Polyergus* literature, and provide a more comprehensive overview of the coevolution between this slave-maker with its host.

In the first chapter of my dissertation, I focused on how social parasitism by *Polyergus* affects the nestmate recognition system of its host. Proper functioning of this recognition system in social insects is vital to maintaining the integrity of their societies so that only those closely related colony members benefit (Hölldobler and Wilson 1990). The results of my research indicate that enslavement by *P. breviceps* dramatically alters the chemical and genetic context that their hosts experience. In particular, enslaved *Formica* are exposed to a wider variety of chemical cues, as enslaved *Formica* are captured from several, often genetically distinct, colonies. Since chemical cues learned by ants early in adulthood are later used to distinguish nestmates from non-nestmates (Crozier and Pamilo 1996), differences in cue exposure could have important consequences for recognition behavior. Indeed, this may be the case for enslaved *Formica* since they were comparatively less aggressive towards non-nestmates than free-living *Formica*.

The second chapter of my dissertation presents both chemical and genetic evidence for host-race formation in a population of *Polyergus breviceps* that parasitizes three species of *Formica* in sympatry. To avoid rejection by their hosts and achieve colony integration, social parasites often use chemical mimicry or camouflage (Bagnères and Lorenzi 2010). My results show that *P. breviceps* worker ants are chemically distinguishable from one another based on the species of *Formica* they enslave, thus showing some level of host-specific adaptation. Both maternally inherited mitochondrial and biparentally inherited nuclear DNA markers provide evidence for genetic structuring according to host. Together, these data suggest that *P. breviceps* queens and males mate assortatively by host and this could



possibly be mediated by host-specific chemical cues present on the reproductive partners. However, we cannot rule out the possibility that selection against "hybrids" produced from matings between reproductives parasitizing two different hosts could also result in this pattern. Currently, only a few well-studied examples exist for host race formation in sympatry including some species of brood parasites in birds (Marchetti et al. 1998; Sorenson et al. 2003) and phytophagous insects (Feder et al. 1988; McPherson et al. 1988; Drès and Mallet 2002). To my knowledge, the results from this dissertation chapter provide the first example of possible host-race formation in any social insect parasite.

In the final chapter of my dissertation, I use mitochondrial and nuclear DNA sequence data to study the phylogeography and population genetics of *Polyergus* and their hosts across their North American distribution. I also applied a Bayesian method of species delimitation (Yang and Rannala 2010) to determine whether the currently recognized *P. breviceps* and *P. lucidus* slave-makers (Bolton 1995) may actually be comprised of more than just two species. Our data suggest that *P. lucidus* should remain a single species while *P. breviceps* can be divided into two putative species. Phylogenetic reconstruction and population genetic analysis revealed that *P. breviceps* populations are more structured according to geography than are populations of *P. lucidus*. A general west-east phylogeographic pattern could be determined among three distinct lineages of *P. breviceps* while no clear phylogeographic structuring could be found for *P. lucidus*. While both species showed evidence of population structuring according to host, differentiation in *P. lucidus* populations appeared to be more driven by host association than in *P. breviceps*. Together, the findings from this chapter represent the first population-level study of *Polyergus* slave-makers explored in the context of geographic distribution and host association.

Examining the ecological and evolutionary interactions between slave-making ants in the genus *Polyergus* and their *Formica* slaves offers a unique opportunity to study parasite-host coevolutionary dynamics. This is because, unlike organisms that are conventionally studied in parasitology, *Polyergus* social parasites are closely related to their hosts, and their exploitation of entire *Formica* colonies provides insight into the ecology and evolution of social insect societies. One phenotype that is likely to play a vital role in this host-parasite interaction is the chemical profile that is present on the exoskeleton of ants and is used in nestmate recognition. In this dissertation I show that social parasitism by *Polyergus* slave-makers dramatically affects the nestmate recognition system of their slaves, that the presence of multiple species of hosts in sympatry may have resulted in possible host race formation in slave-making ants and that North American *Polyergus* populations are shaped by both geography and host use. Overall, these results address several gaps in the knowledge we have about these species of slave-maker and lay the foundation for future research on the ecological and evolutionary interactions between *Polyergus* slave-makers and their *Formica* slaves. My dissertation also illustrates the ecological and evolutionary diversity of these slave-makers and emphasizes the importance of studying several different populations slave-making ants to gain a better understanding of how such a parasitic relationship have

evolved through time with a diverse group of hosts. Future studies should focus on obtaining better sampling of different populations of *Polyergus* and *Formica* and comparing the same ecological (host use and raiding behavior), chemical (cues used in nestmate recognition) and genetic components among them. Additional genetic markers that are more informative at the population level than the ones presented here would provide the most valuable information in this regard.

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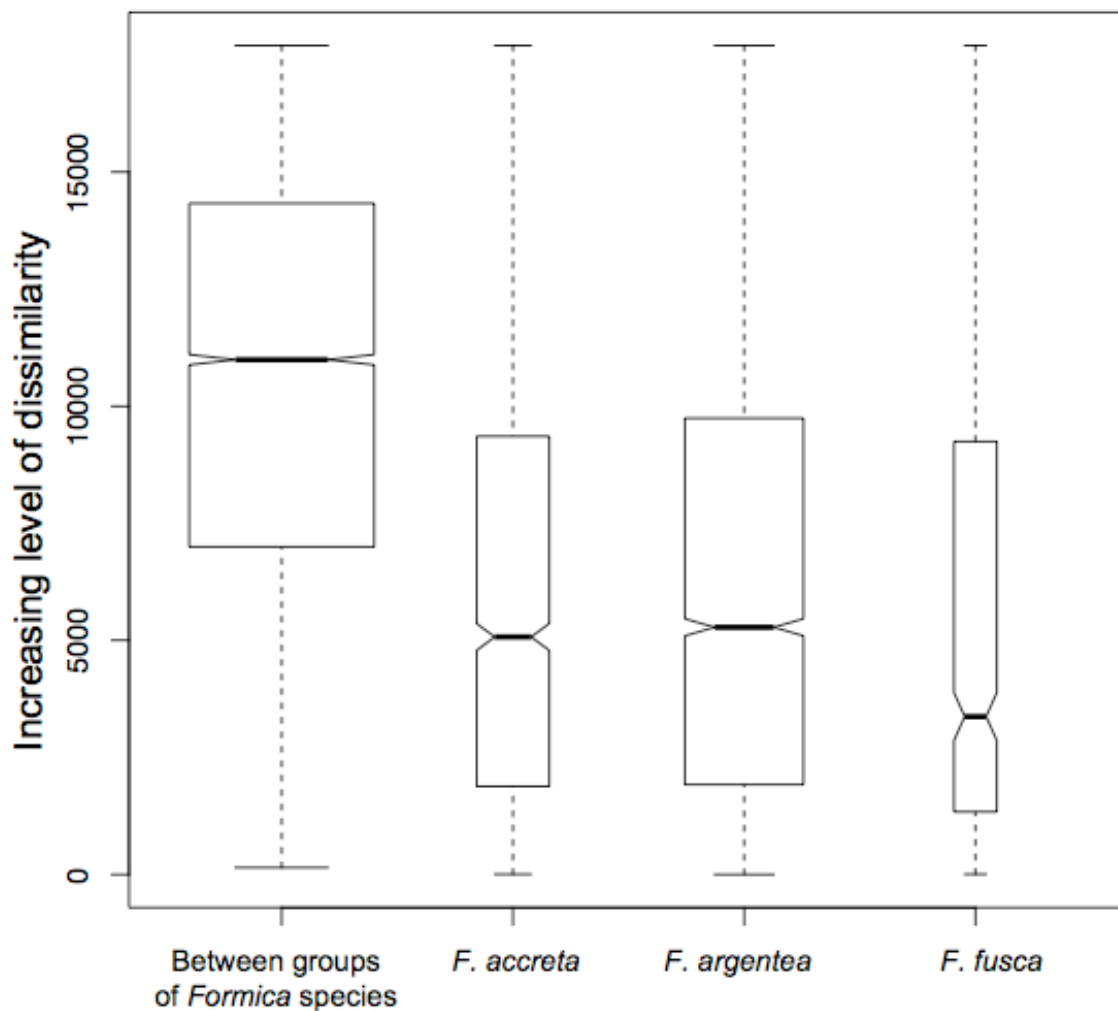
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Topoff H (1999) Slave-making queens. *Scientific American* 281:84-90

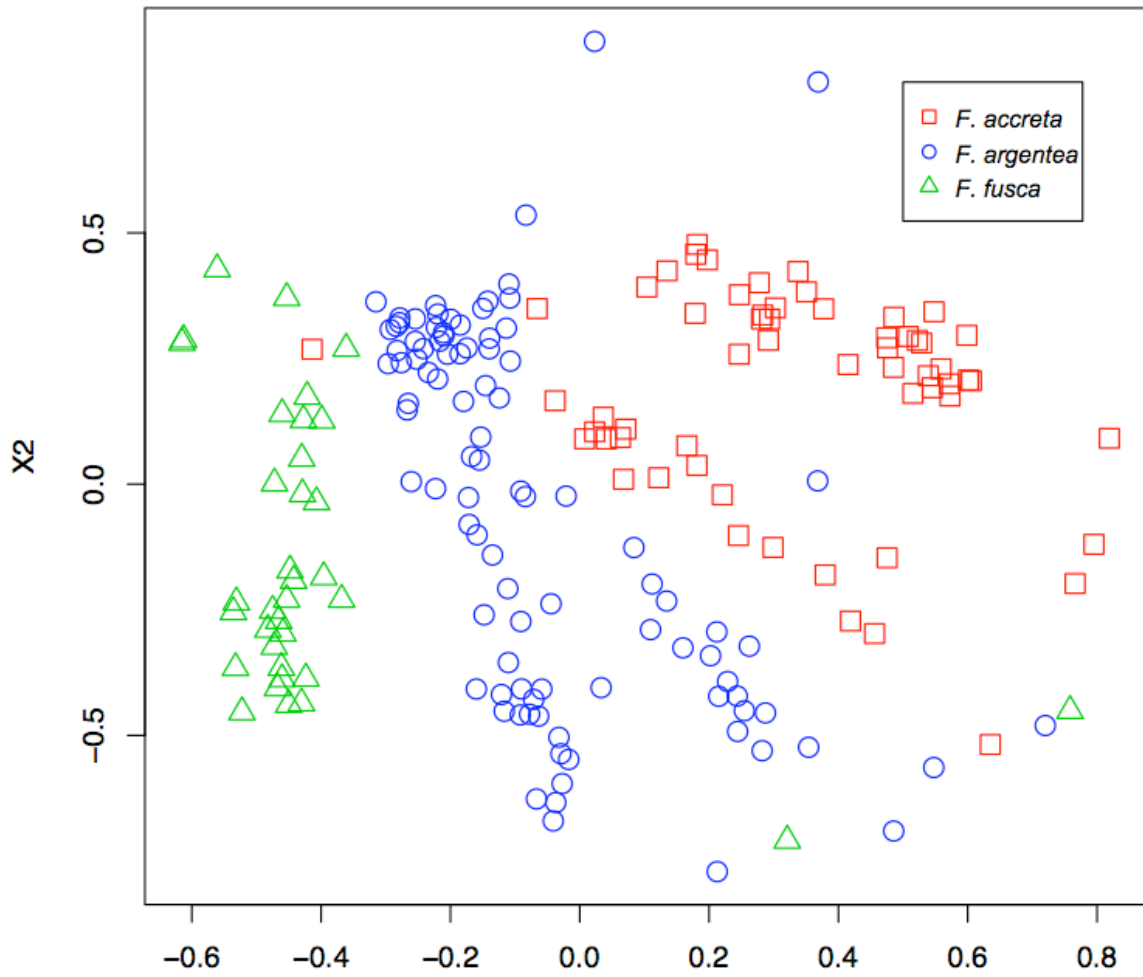
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**APPENDIX 1:**

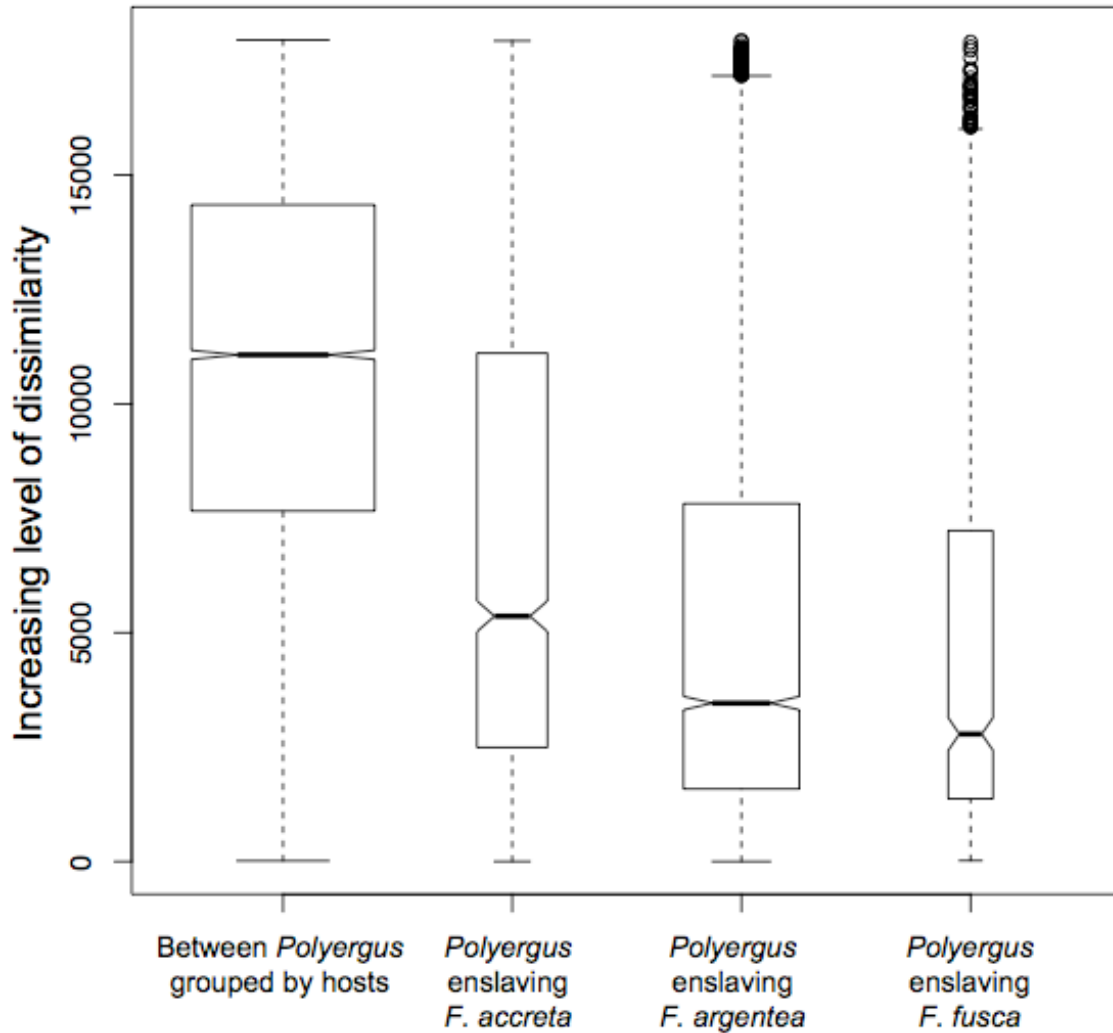
Chapter 2 supplementary figures



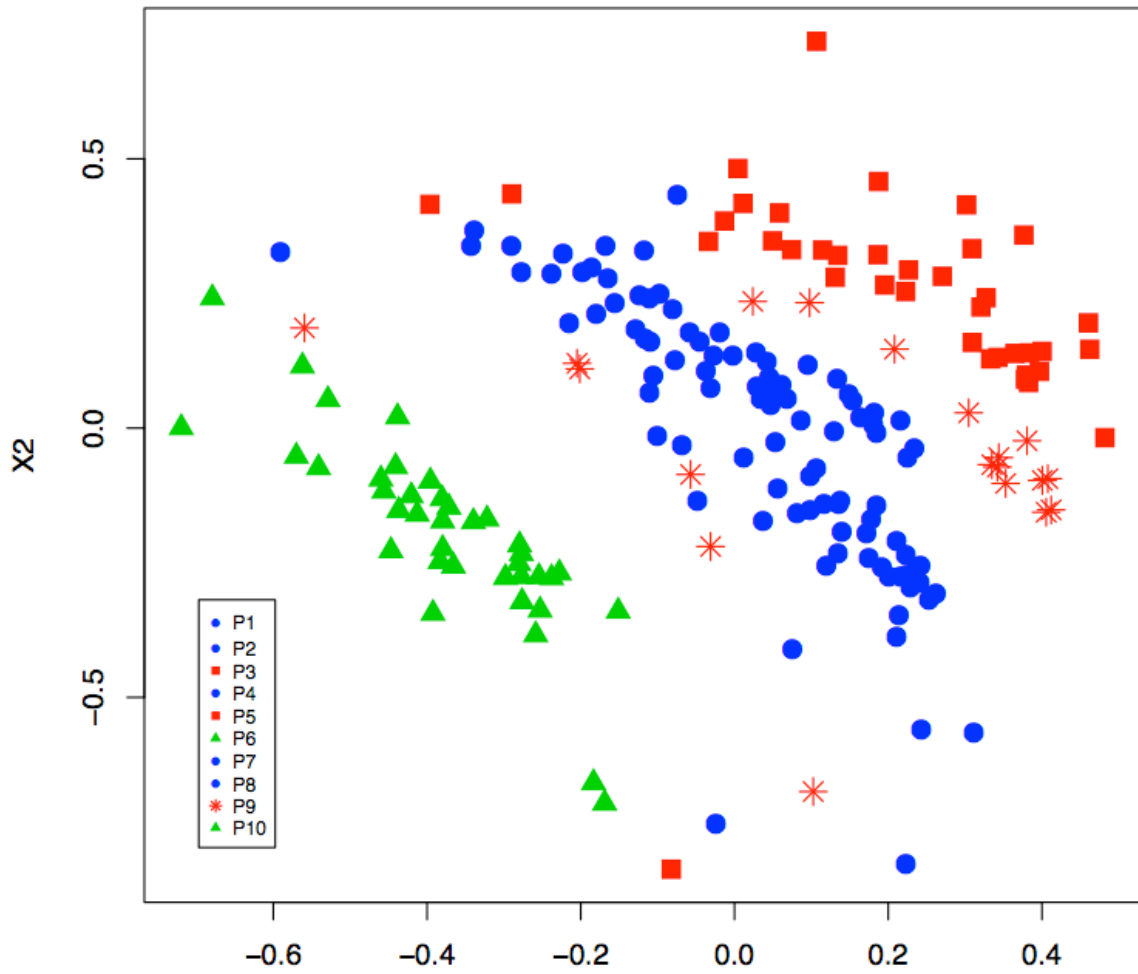
**S1.** ANOSIM results shown as box plots of individual *Formica* chemical profiles testing for grouping by species. The three box plots to the right show the spread of rank dissimilarities between pairs of individual *Formica* within groups categorized by the species. The box plot to the far left shows the spread of the rank dissimilarities between pairs of individual *Formica* across groups categorized by species.



S2. NMDS of individual chemical profiles of enslaved *Formica* plotted by species.

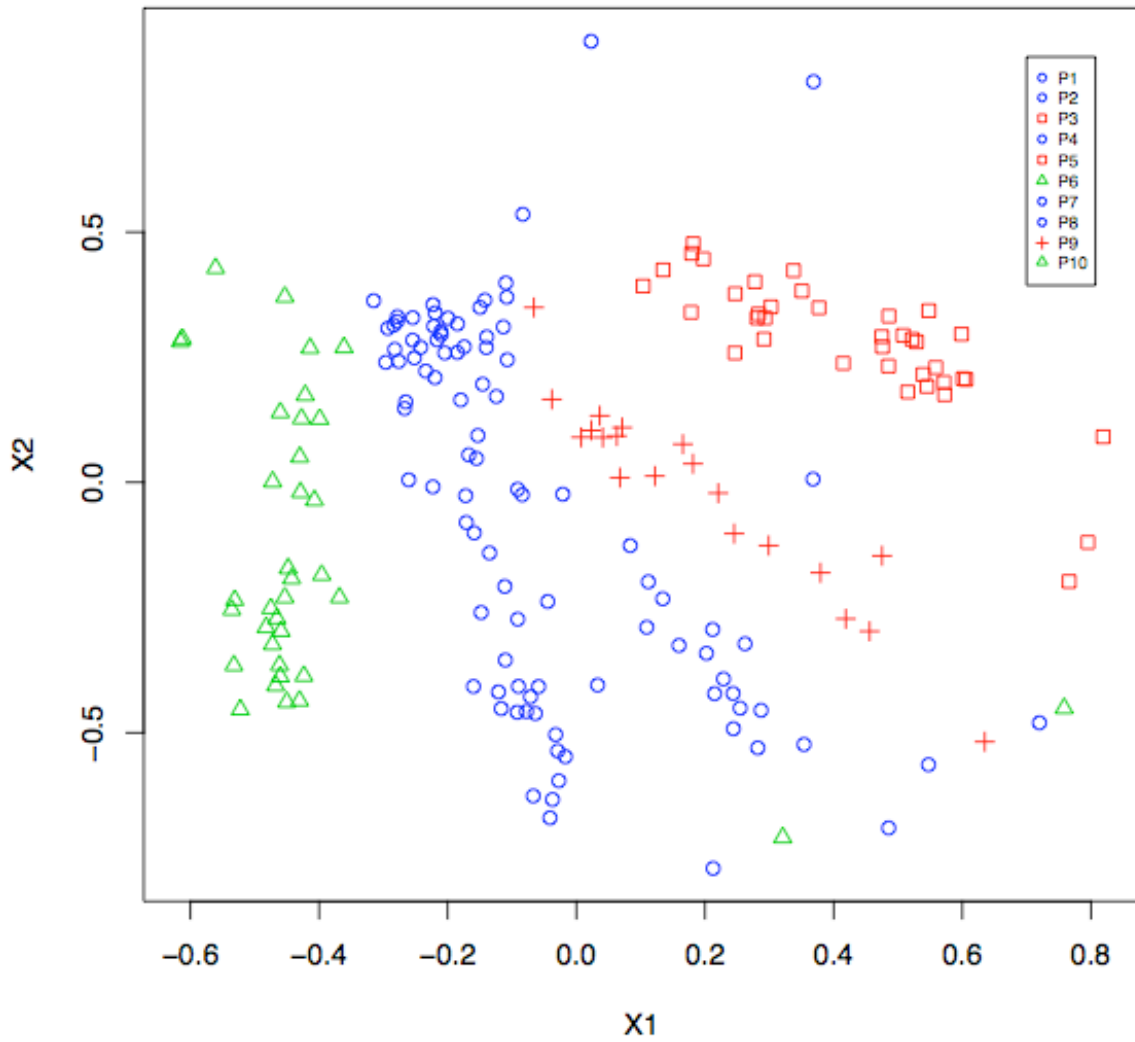


**S3.** ANOSIM results shown as box plots of individual *Polyergus* chemical profiles testing for grouping by host. The three box plots to the right show the spread of rank dissimilarities between pairs of individual *Polyergus* within groups categorized by the three species of host. The box plot to the far left shows the spread of the rank dissimilarities between pairs of individual *Polyergus* across groups categorized by host.

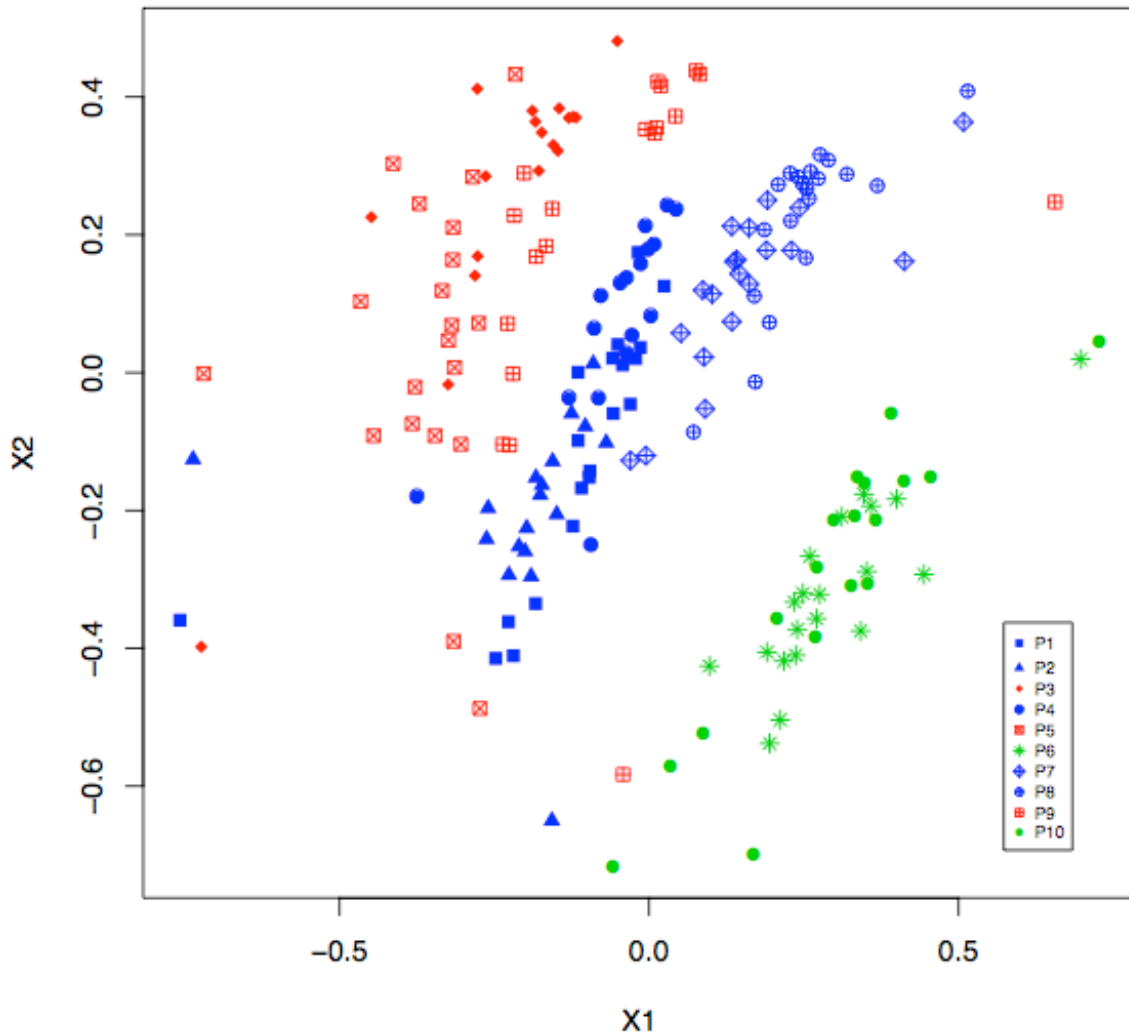


**S4.** NMDS of individual chemical profiles of *Polyergus* plotted by the host they enslave with colony “P9” indicated as red asterisks.



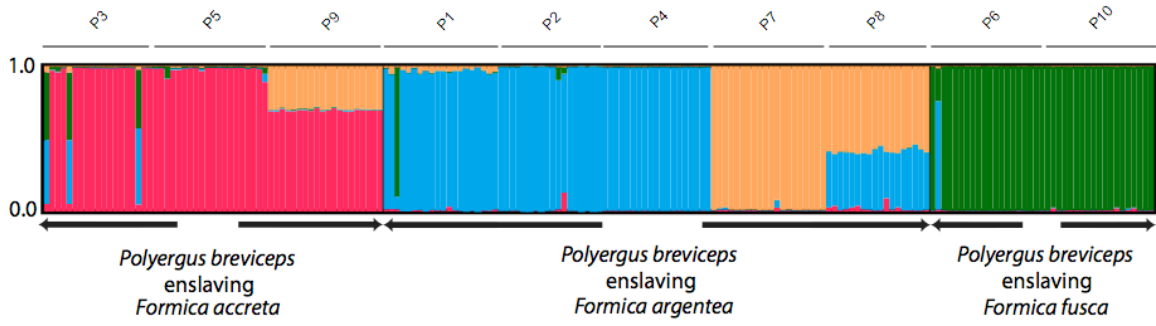


**S5.** NMDS of individual chemical profiles of enslaved *Formica* plotted by species with those belonging to colony “P9” indicated by red crosses.



**S6.** NMDS<sup>1</sup> of individual chemical profiles of *Polyergus* plotted by colony. Red symbols= *Polyergus* enslaving *F. accreta*, blue symbols= *Polyergus* enslaving *F. argentea*, and green symbols= *Polyergus* enslaving *F. fusca*.

<sup>1</sup> Differences in plotting from Fig 3 and S4 reflects multiple solutions produced from NMDS analysis



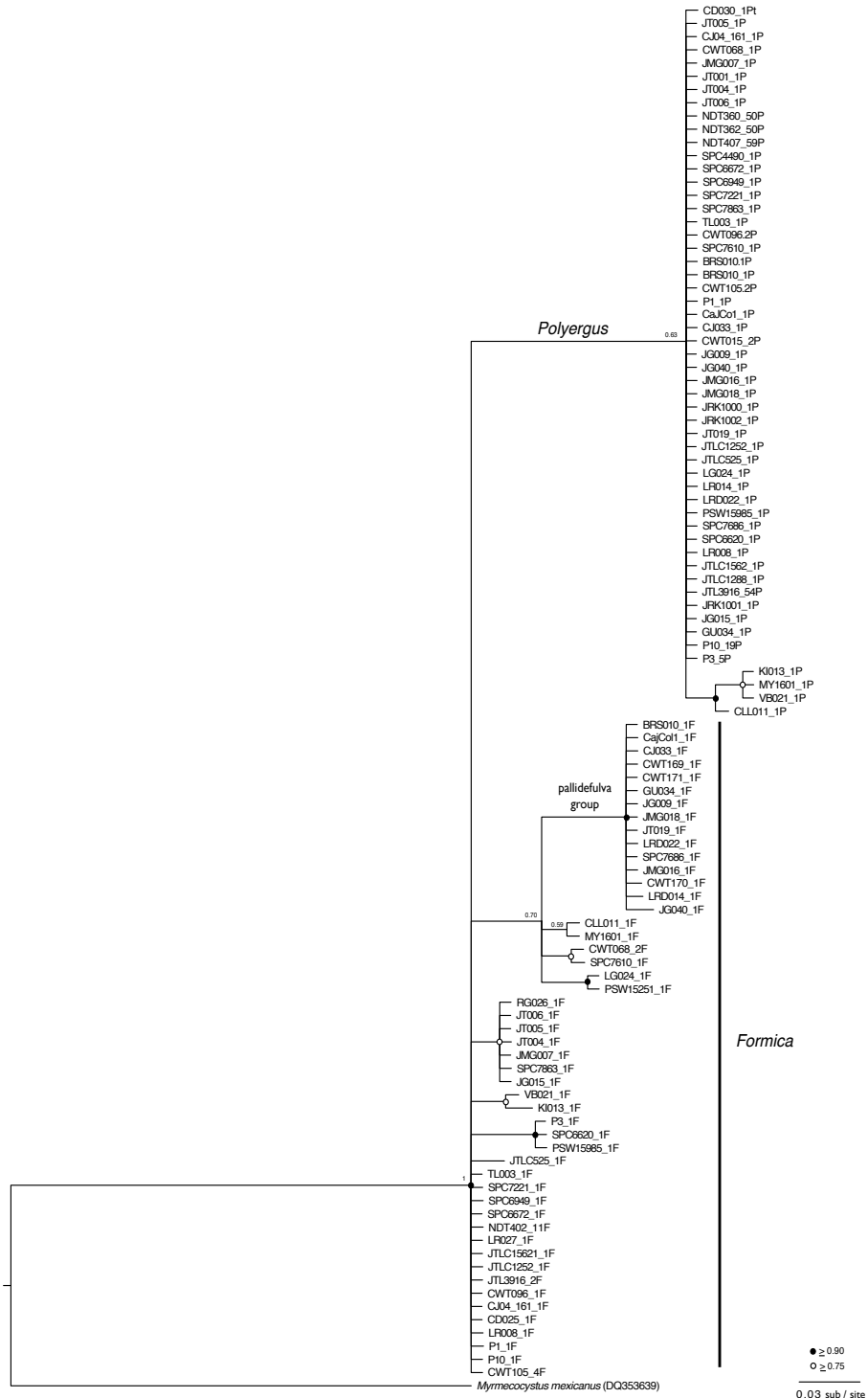
**S7.** Results from the STRUCTURE analysis of 8 microsatellite loci with colors showing population cluster assignments based on K=4. Each column represents a single genotyped *Polyergus breviceps* enslaving one of three hosts: *F. accreta*, *F. argentea*, or *F. fusca*.

**S8.** Chemical peaks that account for >50% of the differences between *Polyergus* individuals grouped by the host they enslave.

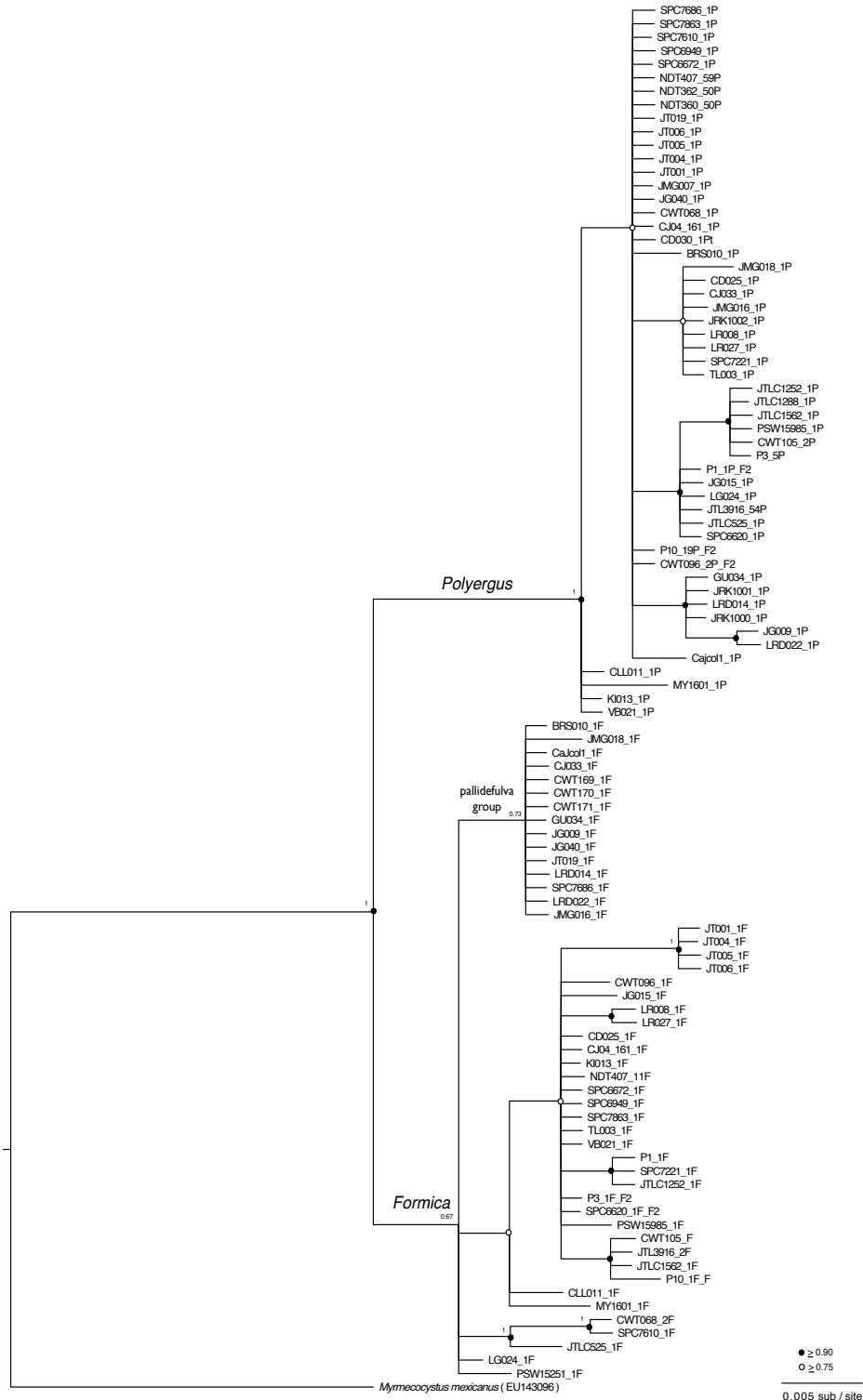
Retention time	<i>Polyergus</i> enslaving <i>F. accreta</i> vs. <i>F. argentea</i>	<i>Polyergus</i> enslaving <i>F. fusca</i> vs. <i>F. accreta</i>	<i>Polyergus</i> enslaving <i>F. fusca</i> vs. <i>F. argentea</i>
10.62	X		
11.088	X		
13.414	X	X	X
13.98	X	X	
16.897	X	X	X
17.539	X	X	X
20.777	X	X	X
21.451	X	X	
21.566	X		
22.233		X	X
22.394			X
22.857		X	X
23.499		X	X
24.794			X
25.403	X	X	X
25.491	X	X	
25.911	X		
26.866		X	X
29.86	X		
30.091	X		
33.809	X		

**APPENDIX 2:**

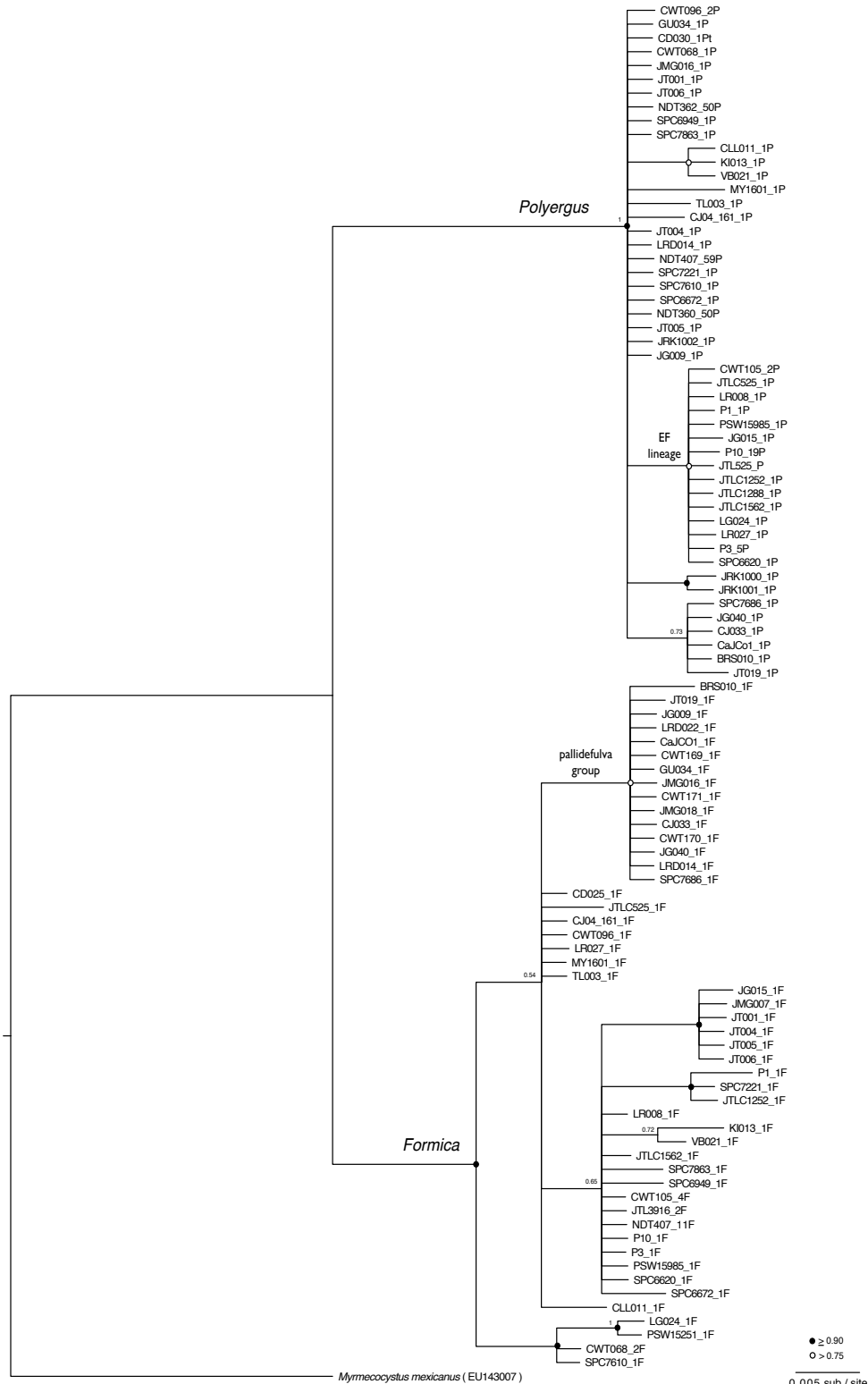
Chapter 3 supplementary figures



**S1.** Bayesian tree inferred from 28S nuclear sequence data from *Polyergus* and *Formica* with *Myrmecocystus mexicanus* as the outgroup. Numbers and dots on nodes are posterior probability support values.



**S2.** Bayesian tree inferred from EF1α nuclear sequence data from *Polyergus* and *Formica* with *Myrmecocystus mexicanus* as the outgroup. Numbers and dots on nodes are posterior probability support values.



**S3.** Bayesian tree inferred from ArgK nuclear sequence data from *Polyergus* and *Formica* with *Myrmecocystus mexicanus* as the outgroup. Numbers and dots on nodes are posterior probability support values.