Acacia-inhabiting *Pseudomyrmex* ants — integrating physiological, behavioral, chemical and genetic data to understand the maintenance of ant-plant mutualisms

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Abstract

Mutualisms are interspecific interactions that benefit all partners involved. These interactions often include reciprocal adaptations of the partners. Acacia ant-plants secrete sucrose-free extrafloral nectar and I demonstrated that the defending *Pseudomyrmex* ant mutualists correspondingly almost completely lacked invertase activity (15 to 19 ng glucose released μg^{-1} ant fresh weight min^{-1}). In contrast, generalist and exploiting ant species possessed invertase activity (89 to 107 ng glucose μg^{-1} min^{-1}). In experiments, sucrose uptake induced invertase activity in generalist (300%) and exploiting workers (250%) as well as in larvae of all species (170-310%) investigated, but not in mutualist workers. Thus, the mutualists loose invertase during their ontogeny. This reduced metabolic capacity ties the mutualists to their host plants, but it does not prevent the mutualism from exploitation. A molecular phylogeny demonstrated that the exploiter species did not evolve from former mutualists but from generalists. Thus, being physiologically specialized and dependent on their host plants prevents mutualists from evolving into exploiters, while other mechanisms are required to stabilize a mutualism against the exploitation by species that evolved from generalists.

The colonization patterns of a mutualistic and a parasitic acacia-inhabitant were compared using three complementary approaches: observations of aggression behavior, chemical analyses of cuticular hydrocarbon profiles and genetic microsatellite analyses. Genetic data indicated that one colony of the mutualist *Pseudomyrmex ferrugineus* inhabited up to two host plants (ø 1.56), while several parasitic *P. gracilis* colonies (ø 2.23) shared the same individual host. In both species, ant individuals inhabiting the same acacia possessed characteristic chemical profiles. In behavioral experiments under field conditions, inhabitants sharing the same acacia showed no aggression, which is in line with cuticular hydrocarbon profiles. Genetic, chemical and behavioral patterns were concordant for *P. ferrugineus*, while genetic heterogeneity and low relatedness (R_{min}=0.00±0.18) of *P. gracilis* was in contradiction to chemical and behavioral data. The non-aggressive coexistence of different colonies of *P. gracilis* seems to prevent the inhabitation of hosts by other species. Genetic identity of ant colonies, the ants' chemical profiles and their behavior towards conspecifics shape the colonization pattern of acacias and can determine the outcome of plant-ant interactions.

Abstract

Large and fast growing colonies are advantageous in competitive environments to allow for efficient nest defense and foraging. How the obligate acacia-ant *Pseudomyrmex peperi* forms colonies was investigated in the present study. The species establishes distinct, but highly polygynous colonies that can inhabit large clusters of host trees. Analyzing workers, males, queens and virgin queens (264 individuals) from two supercolonies with eight polymorphic microsatellite markers indicated that colonies are founded by one singly mated queen and supercolonies are established by intranidal mating among colony-derived males with daughter queens. This allows colonies to constantly grow by budding without having to found new colonies. Ancestral states reconstruction revealed that polygyny represents the derived state among acacia-ants and has evolved at least twice independently. The extreme polygyny of *Pseudomyrmex peperi* achieved by intranidal mating may play an important role for species coexistence in a dynamic and competitive habitat.

I was able to identify physiological, genetic and behavioral adaptations of mutualistic acacia-ants to their specific life style. Large colonies with high numbers of individuals, which allow for efficient host defense, seem to be a key adaptation of mutualistic acacia-ants. Constant colony growth is possible especially in the polygynous acacia-ant *Pseudomyrmex peperi* and seems to be a higher adaptation towards the life style as acacia-inhabitant. High relatedness among individuals colonizing the same host reduces conflicts and increases inclusive fitness of the individuals. The genes and consequently the behavior of the mutualist workers are then passed on to the next generation and allow the mutualism to persist in evolutionary terms. In contrast, ant species that parasitize the mutualism of acacias were not adapted to their hosts. They do not depend on the host plant and decrease plant fitness. These parasites can only persist in evolutionary terms because they coexist with mutualistic ant species that assure the maintenance of myrmecophytic traits in host plant populations.

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CONTENTS

List of Figures

List of Tables

Abbreviations

A	observed number of alleles in a given sample
abd-A	abdominal-A
AMOVA	analysis of molecular variance
ANOVA	analysis of variance
B/MCMC	Bayesian analysis using Markov chain Monte Carlo methods
BW	bind and wash (buffer)
BS	bootstrap support
BSA	bovine serum albumin
bp	base pairs
COI	cytochrome c oxidase subunit I
DA	discriminant analysis
ddH_2O	double distilled water
df	degrees of freedom
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTP	dideoxynucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
EFN	extrafloral nectar
EL	eye length
et al.	et alii
F	measurement of distance between individual distributions; as F goes up, P (probability) goes down (i.e., more confidence in there being a difference between two means)
F_{CT}	measures inbreeding among individuals relative to the supercolony of which they are part
F_{IS}	measures inbreeding among supercolonies
F_{IT}	measures inbreeding among all individuals included in the analysis
F_{SC}	measures inbreeding in sub-samples relative to the supercolony to which they belong
F_{ST}	measures inbreeding in sub-samples relative to all individuals included in the analysis
fwt	fresh weight
GC-MS	gas chromatography – mass spectrometry
GLM	general linear model
GPS	global positioning system
H_E	expected heterozygosity
H_O	observed heterozygosity
HK	hexokinase
HL	head length
HW	head width
kb	kilo bases

LSD	Fisher's protected least significant differences test (i.e., post hoc test)
LW *Rh*	long-wavelength rhodopsin
M	molar / mol l^{-1}
MDS	multi dimensional scaling
ML	Maximum Likelihood
mt	mitochondrial
mtDNA	mitochondrial DNA
N	sample size
nu	nuclear
P	probability
p.A.	pro analysis
PBS	phosphate buffered saline
PCA	principal components analysis
PCR	polymerase chain reaction
pers. comm.	personal communication
pers. obs.	personal observation
pp	posterior probability
p.q.	per queen
R	measurement of genetic relatedness
R_{min}	lowest value of relatedness observed in a given study
r	Pearson product moment correlation (i.e., measure of correlation; varies from 1 to 0)
REL	relative eye length [calculated as eye length (EL) per head length (HL)]
RT	room temperature
rDNA	ribosomal DNA
SE	standard error of the mean
SD	standard deviation
SS	sum of squares
T_a	annealing temperature
TAE	Tris-acetate EDTA
Taq	*Thermus aquaticus* (polymerase)
TBE	Tris-borate EDTA
TE	Tris EDTA
spec., sp.	species
unpubl. data	unpublished data
wg	wingless
w/v	weight per volume
28S	large ribosomal subunit
*	indicates a significant result ($P<0.05$)
**	indicates a significant result ($P<0.01$)
***	indicates a significant result ($P<0.001$)
ø	mean (bzw. Durchschnitt)

1 Introduction

No organism is an autonomous entity. In nature, organisms interact in multiple ways with each other and their environment. The relationship between two species in an ecosystem can be categorized as mutualistic, antagonistic or neutral. Mutualisms are interspecific interactions, in which one partner provides a 'service' to be 'rewarded' by the other and which maximize the net fitness of all partners involved (Bronstein 1994). Mutualisms can be exploited, however, by species that take advantage of the resources that are provided by one partner without reciprocating (Bronstein 2001; Sachs & Simms 2006; Yu 2001). Such exploiters do not pay the cost of providing services and, thus, should be fitter than mutualists (Doebeli & Hauert 2005; Doebeli *et al.* 2004; Doebeli & Knowlton 1998; Hoeksema & Bruna 2000; Sachs *et al.* 2004; Sachs & Simms 2006). Since the presence of exploiters that are competitively superior to the mutualists should destabilize a mutualism, explaining the maintenance of mutualisms remains 'one of the greatest problems for evolutionary biology' (Kiers *et al.* 2003).

Several mechanisms have been proposed for the stabilization of mutualisms against exploitation. For example, 'host sanctions' mean that hosts cease the provisioning of rewards when they do not receive the respective service, while 'partner choice' mechanisms allow the preferential selection of suitable partners based on certain traits that are used as 'keys' for partner identification (Bull & Rice 1991; Sachs *et al.* 2004; Sachs & Simms 2006). Different mechanisms are required to stabilize a mutualism against different types of exploiters and therefore the term 'cheater' is explicitly used for exploiters that have evolved from former mutualists which ceased the service (Segraves *et al.* 2005), while exploiters that invaded the system without having an evolutionary history as a mutualist are termed 'parasites' (Bronstein 2001). If this differentiation based on the phylogenetic history is made, it becomes obvious that — in contrast to theoretical expectations — little empirical evidence exists for the occurrence of cheaters in nature (Sachs & Simms 2006), although cheaters indeed can evolve under laboratory conditions (Rainey & Rainey 2003). Moreover, cheaters can retain the traits on which partner choice mechanisms are based from their evolutionary past and cheating is, thus, more likely prevented by host sanctions, which control the quantity or quality of rewards provided.

Ant-plant protection mutualisms have a model character for a great range of mutualisms (Bronstein 1998) and serve as model systems to investigate various

general questions in ecology and evolutionary biology (Heil & McKey 2003). In the tropics, these symbioses involve species of over 100 genera of angiosperms and 40 genera of ants (Davidson & McKey 1993b). For instance, in the Neotropics the plant genera *Acacia* (Fabaceae), *Ateleia* (Fabaceae), *Cordia* (Boraginaceae), *Tachigali* (Fabaceae) and *Triplaris* (Polygonaceae) are known to comprise myrmecophytic species associated with *Pseudomyrmex* (Janzen 1966; Ward 1991). Other ant-plant mutualisms from the South American tropics are formed by *Piper* plants (Piperaceae) with *Pheidole* ants (Letourneau 1983) and *Cecropia* plants (Cecropiaceae) with *Azteca* ants (Longino 1989, 1991). In Southeast Asia and Africa, many members of the ant-plant genus *Macaranga* (Euphorbiaceae) live in a mutualistic association with ants of the genera *Crematogaster* or *Camponotus* (Davidson & McKey 1993a) and in Cameroon, the plant species *Leonardoxa africana* is inhabited by *Petalomyrmex phylax* (Dalecky *et al.* 2005; Dalecky *et al.* 2007; McKey 1984). Ants are the insect worlds major mutualists (Moreau *et al.* 2006).

As in any mutualism, ant-plant mutualisms are characterized by the reciprocal exchange of resources. The plants nourish the ants with extrafloral nectar (EFN; i.e., nectar secreted on vegetative organs that is not functionally involved in pollination) and/or food bodies and, in some cases, provide housing for them. In return, the ants defend 'their' plant against herbivores, pathogens and encroaching vegetation (Buckley 1982; Heil & McKey 2003; Hölldobler & Wilson 1990). Protective ant-plant interactions, however, differ in their degrees of specificity (Fiala 1996; Heil & McKey 2003); such derivations can even be found within the same plant genus. The genus *Macaranga*, for instance, displays various degrees of ant specificity from facultative to obligate — including specific coccids (sap-sucking insects producing honeydew) as a third symbiotic partner (Fiala 1996; Heckroth *et al.* 1999).

In Central America, some acacia species live in an obligate association with specific species of the ant genus *Pseudomyrmex*. These acacia species are termed myrmecophytes: They always produce EFN and protein-rich food bodies (Janzen 1974). Additionally, they provide hollow swollen-thorns (domatia) that are permanently inhabited by nesting ants (Belt 1874). The ants exclusively feed on plant-derived cellular protein-rich food bodies and on EFN being constitutively produced in high rates by the plant (Janzen 1966, 1974). Both partners seem highly adapted to this mutualism (Raine *et al.* 2002). The ant-plants possess swollen thorns, food bodies, enlarged foliar nectaries and year-round leaf production, while the plant-

ants are extremely aggressive towards the plants' enemies, make use of the offered food rewards and reside on the plant. Thus, the ants patrol the plants' surfaces permanently and serve as indirect defense for the plant (Janzen 1966).

Other acacia species interact with ants on a facultative basis and are termed myrmecophilic plants. They offer food rewards to foraging, non-specialized ants (generalists) in form of EFN, but only in response to herbivore-caused damage (Heil *et al.* 2004b). Thus, the extrafloral nectar serves as an indirect defense mechanism against herbivores by the attraction of ants. These interactions seem, however, of minor importance for both plant and ant partners (Janzen 1966). In general, obligate interactions are more effective than facultative ones concerning the protective benefits for the plant (Heil *et al.* 2001). Whereas the EFN secretion forms a constitutive trait in myrmecophytic acacias, it is inducible in other species of the same genus (Heil *et al.* 2004b), thus, serving different functions in the two forms of ant-plant interaction. Specialized plant-ants recruit actively to parts of their plant that are currently under herbivore attack. Non-specialized ants are only attracted to myrmecophilic plants by EFN on a facultative basis (Agrawal & Rutter 1998; Bentley 1977; Koptur 1992), but protective effects for these plants have nevertheless been demonstrated repeatedly (Costa *et al.* 1992; del Claro *et al.* 1996; Koptur 1992; Oliveira *et al.* 1999; Sobrinho *et al.* 2002).

1.1 Physiological adaptations of acacia-inhabiting *Pseudomyrmex* ants

In addition to quantitative differences of nectar flow, myrmecophytes show qualitative differences to non-myrmecophytes in the composition of EFN. Heil and co-workers (2005b) demonstrated a post-secretory regulation of the carbohydrate nectar composition by showing high invertase activity in EFN of obligate acacia ant-plants — a phenomenon that could not be detected in myrmecophilic acacia species. Invertase is a digestive enzyme that hydrolytically cleaves the disaccharide sucrose into its two monomers, glucose and fructose. Consequently, the myrmecophytes' EFN contains only little or no sucrose. Sucrose, which is common in other EFNs as well as in phloem sap, usually attracts non-symbiotic ants (Blüthgen & Fiedler 2004; Boevé & Wäckers 2003; Galetto & Bernardello 1992; Koptur 1994; Stapel *et al.* 1997). Adding sucrose to sucrose-free nectars, thus, increases the attractiveness to generalist ants (Heil *et al.* 2005b). As EFN is a valuable source of carbohydrates and amino acids to

Box 1 List of terms used in the present study

Altruism	behavior by an individual that increases the fitness of another individual and decreases the actor's fitness
Colony	several individuals of the same species that cooperate
Kinship	relationship of workers that share a genealogical origin through descent
Hamilton's rule	C < R x B; where C it the cost in fitness to the actor, R the genetic relatedness between the actor and the recipient and B is the fitness benefit to the recipient. Fitness costs and benefits are measured in fecundity.
Monogyny	colonies contain one queen
Polygyny	colonies contain several queens
Monoandry	single mating by the queen
Polyandry	multiple mating by the queen
Polydomy	several nests are inhabited by one colony and individuals interact socially
Coevolution	an evolutionary process, in which different species undergo reciprocal adaptations
Symbiosis	generally describes the interaction between different species, regardless of the outcome of this interaction
Mutualism	mutually beneficial interaction between different species
Parasitism	interaction between different species, in which one organism benefits and harms the other
Exploiter	generally describes species that take advantage at the expense of the other in an interspecific interaction
Parasite	an exploiter without evolutionary past as mutualist
Cheater	an exploiter that evolved from a mutualist and is, thus, likely to carry traits of the mutualist (e.g., physiological or morphological adaptations)

foraging ants (Baker & Baker 1973; Lanza 1991; Smith *et al.* 1990), the sucrose-free EFN of the myrmecophytic acacias is, therefore, unattractive to generalists. Consistently, the *Pseudomyrmex* workers that are specialized to live on myrmecophytic acacia plants display almost no invertase activity in their digestive tracts and prefer sucrose-free EFN, whereas the non-symbiotic ant species do have active invertase (Heil *et al.* 2005b). Consequently, myrmecophytes appear to preferentially reward mutualists in comparison to non-mutualists by secreting a chemically specialized EFN. However, the existence of exploiters of this mutualism (Clement *et al.* 2008; Janzen 1975; Raine *et al.* 2004) demonstrates that the described filter does not confer absolute protection against non-reciprocating ants.

1.2 The social structure of a mutualist and a parasite

Ants are eusocial. The key trait of eusocial (= truly social) species is that most of the colony members give up their own chances of reproduction and help raise the offspring of nestmates, which are highly fecund. According to Wilson (1977) eusociality is defined by (i) the partition of reproduction among the colony members, with sterile or subfertile workers and highly fecund sexuals or reproductives, (ii) overlapping adult generations and (iii) cooperative brood care. All ants and termites, some bees and wasps, ambrosia beetles (Kent & Simpson 1992), aphids (Aoki 1977; Ito 1989), thrips (Crespi 1992), shrimp (Duffy 1996), possibly spiders (Vollrath 1986) and several naked mole rats (Burda & Kawalika 1993; Jarvis 1981; Sherman *et al.* 1991) are eusocial.

The recognition cues used by social insects to distinguish nestmates from foreign individuals are non-volatile chemicals (usually hydrocarbons) that are expressed on the cuticle (Vander Meer & Morel 1998). Members of a colony share a common chemical signature created by the admixture of individual profiles through allogrooming, trophallaxis (i.e., mouth to mouth feeding) and physical contact (Crozier & Dix 1979). When workers emerge from pupation, they learn their colony's recognition cues, thus, forming an internal template that is then compared to the profile of subsequently encountered conspecifics (Breed & Bennett 1987). Individuals whose chemical signature deviates from the template are recognized as foreign and are often attacked. Aggression between colonies is generally negatively correlated with overall hydrocarbon similarity (e.g., Suarez *et al.* 2002; Vasquez & Silverman 2008). As cues for individual recognition cuticular hydrocarbons also play role in mate choice, parental care, conflicts and cooperation (Sherman *et al.* 1997).

In addition to the role of cuticular hydrocarbons as cues in insect communication, these molecules are also involved in other functions, such as protection against desiccation and pathogens (Howard & Blomquist 2005). The cuticular hydrocarbon profile is shaped by endogenous, genetic factors and exogenous, environmental factors (e.g., Provost 1991; Vander Meer & Morel 1998). Because of their multiple biological functions, these molecules are subjected to complex selective pressures. However, the relative importance of neutral and selective processes in shaping the diversity of these biochemicals is poorly known (Howard & Blomquist 2005).

So far, the social organization and mechanisms for nestmate recognition has not been investigated in pseudomyrmecine plant-ants. Social organization and environmental factors should have a great impact on cuticular hydrocarbon profile of colony members and, thus, affect nestmate recognition and aggression towards conspecifics. The life-style of a species — such as acacia-inhabitant — should shape the evolution of recognition cues.

1.3 Polygyny in the acacia-mutualist *Pseudomyrmex peperi*

The evolution of eusociality contradicted Darwin's theory of evolution through natural selection (Darwin 1859). About a century later, Hamilton's famous theory of kin selection provided an elegant explanation for the widespread occurrence of altruism among social insects (Hamilton 1964a,b; see Box 1). Kin selection theory predicts that individuals within a social group should be related to a higher degree than two random individuals within the population in order to express altruistic behavior (Hamilton 1964a,b). Otherwise, individuals that help others would not increase their inclusive fitness via this behavior and should be counter-selected (Helanterä *et al.* 2009; Seppä *et al.* 2008). Inclusive fitness comprises both a direct and an indirect fitness component. 'Direct fitness' describes the impact on the individual's fitness via its own reproduction, while 'indirect fitness' comprises any impact on the fitness of its kin that carries the same genes (Ricklefs & Miller 2001).

The male-haploid genetic system of Hymenoptera has been interpreted as preadaptation for the evolution of eusociality. In general, workers share three-quarters of their genes by descent with their sisters and this high sister relatedness in social insect societies favors the evolution of altruism through kin selection in females (e.g., West-Eberhard 1975). Given that the costs and benefits are equal, ant colonies headed by one singly mated queen and consequently showing high relatedness among nestmates, should maximize colony survival and production of sexuals (Crozier & Pamilo 1996; Hamilton 1964a,b, 1972) better than polygynous colonies (i.e., colonies with more than one queen) or polyandrous colonies (i.e., colonies with queens that mate with multiple males). Kin selection theory predicts that altruistic behavior that does not increase one's genes in the next generation is maladaptive and evolutionary unstable (Helanterä *et al.* 2009; Queller & Strassmann 1998; Strassmann & Queller 2007).

Nevertheless, some selective pressures may drive the evolution of polygynous and/or polyandrous colonies. Possible examples include limited nest site availability and habitat saturation as well as cooperative brood raising of different founding colonies, which may lead to such transitions in mating system and colony structure (Herbers 1993; Pedersen & Boomsma 1999; Rissing *et al.* 1989; Seppä 1995). Striking examples of polygyny are found among invasive ant species. These 'tramp ants' often show increased queen numbers and may completely lose colony boundaries in the introduced range, often resulting in unicoloniality, i.e., populations consisting of a network of non-aggressive nests among which free exchange of individuals takes place (Crozier & Pamilo 1996; Heinze *et al.* 2006). This 'unicolonial' population structure differs from the social organization of native ant species, which typically show high aggression against neighboring nests (Chapuisat *et al.* 1997). Unicoloniality significantly contributes to the ecological dominance of invasive ants as it allows establishing huge colonies and very high nest densities, thus, greatly increasing foraging efficiency (Bourke & Franks 1995). In contrast, 'supercolony' generally describes large aggregations of nests that are non-aggressive to each other in species where aggression between nests can occur as for example in *Formica paralugubris* and *F. yessensis* (Chapuisat *et al.* 1997). Invasive ant colonies are independent of unrelated mating partners in the introduced range as they have generally lost the typical mating flights that characterize most ants, but rather mate inside the nest and found new colonies by nest-budding – a breakaway of queen(s) and workers to form a new colony (Passera 1994; Tsutsui & Suarez 2003).

Polygyny and polydomy have been described as an adaptation of invasive ant species to their competitive environment (e.g., Ugelvig *et al.* 2008), but are also found in other ant species (Debout *et al.* 2007). For example, some mutualistic plant-ant systems show these traits. In the system of the myrmecophyte *Leonardoxa africana*, which is inhabited by the ant *Petalomyrmex phylax*, ants are facultatively polygynous (Dalecky *et al.* 2005) and polygyny has also been reported for the *Macaranga triloba* - *Crematogaster* association (Feldhaar *et al.* 2000; Feldhaar *et al.* 2005). The most extreme form of polygyny has been discovered in the acacia-ant *Pseudomyrmex veneficus* — colonies, which may comprise hundreds of thousands of queens and millions of workers and which can colonize clusters of several hundreds of acacias (Janzen 1973). These colonies are among the largest of all

social insects (Ward 1993). Workers leave individual trees and freely enter other individual plants suggesting unicoloniality and extensive polydomy (Janzen 1973).

In most ant-plant mutualisms, several closely related species participate in the mutualism on the side of both plants and ants (Davidson & McKey 1993b). Different mechanisms have been proposed to enable species coexistence in ant-plant mutualisms and competition-colonization trade-offs have been postulated (Stanton *et al.* 2002). Better competitors are more successful in founding of colonies on young and small hosts, while colonizers succeed in inhabiting mature hosts (Stanton *et al.* 2002). As individual plants are rarely occupied by more than one ant colony (Davidson *et al.* 1989; Yu & Davidson 1997), co-occurring plant-ant species compete intensively for host plants (Clement *et al.* 2008; Davidson & McKey 1993b; Janzen 1975) resulting in limited nest site availability and habitat saturation. Moreover, myrmecophytes present a highly dynamic environment to their ant-inhabitants, since many myrmecophytes are pioneer trees (e.g., Dejean *et al.* 2008; Fiala *et al.* 1989; McClure *et al.* 2008). In such competitive and dynamic environments, fast growing and large colonies might be advantageous and could lead to transitions in mating system and colony structure (Herbers 1993; Pedersen 1996; Rissing *et al.* 1989; Seppä 1995).

1.4 DNA sequences as molecular markers

During the last decades, the importance of molecular methods in evolutionary research has greatly increased (e.g., Pagel 1999a). In comparison to phylogenetic reconstructions based merely on morphological traits, using DNA sequences is often preferable due to some properties. A very large amount of information can be gathered from a single gene fragment while numbers of morphological characters employed for phylogenetic studies are usually much lower, each single character can be treated as being independent from the others and data is comparable over a wide range of organisms (Ridley 2004). These features allow to compare even organisms of different domains employing conserved molecular markers (Woese & Fox 1977).

Previous systematic studies have demonstrated that some genes are better suitable than others for the reconstruction of evolutionary relationships among taxa at particular levels of divergence (Simon *et al.* 1994). In distantly related taxa, only slowly evolving genes are comparable and allow construction of a robust tree,

whereas in recently diverged taxa, only the most rapidly evolving nucleotide sites will have accumulated substitutions and are useful for comparisons (Kocher *et al.* 1989; Simon *et al.* 1994). In animals, mitochondrial DNA (mtDNA) generally evolves much faster than nuclear DNA (nuDNA) (Avise 2000; Halliburton 2004; Hey & Machado 2003) with mutation rates in mtDNA in insects being estimated to be 2 to 9 times higher than in nuDNA (Ballard & Whitlock 2004; deSalle *et al.* 1987; Monteiro & Pierce 2001; Moriyama & Powell 1997). Mitochondrial molecular markers, thus, are often employed in studies comparing more closely related invertebrate taxa. In general, some features explain the popularity of mtDNA in phylogenetic studies of animals. They are inherited maternally and clonally (Attardi & Schatz 1988; Brown 1985; Wolstenholme 1992) and mitochondria only possess one chromosome. As to these reasons and as a result of clonal inheritance, mitochondrial genes are generally free of recombination (Avise 2000; Birky 2001).

There are also a number of disadvantages involved in using mitochondrial sequence data (Ballard & Whitlock 2004; Lin & Danforth 2004). They involve very high evolutionary rates for single positions, which can lead to homoplasy (i.e., when characters are similar, but not derived from a common ancestor) due to multiple mutations at the same site. Looking solely at mtDNA may more often show evolutionary bias compared to the rest of the genome based on its small total length (Ballard & Whitlock 2004). Further, mitochondria are maternally inherited and, thus, lack power to detect hybridization events. Despite these problems, studies involving very closely related taxa generally depend on the higher overall rates of nucleotide substitution found in mitochondrial genes. Mitochondrial loci, thus, usually present a suitable tool for resolving close relationships (Avise 2000; Lin & Danforth 2004).

Sequences from the mitochondrial genome are frequently used in phylogenetic studies for various taxa. Universal primers have been published to amplify virtually any fragment of this circular genome (Kocher *et al.* 1989; Simon *et al.* 1994). The cytochrome c oxidase subunit I (COI) gene has been used extensively in phylogenetic analyses of Hymenoptera (repeatedly in bees), at both lower and higher taxonomic levels (Crozier *et al.* 1989; Leys *et al.* 2000; Pedersen 1996, 2002). COI is part of the coding region for the cytochrome c oxidase protein, which is involved in oxidizing ubiquinol and reducing oxygen to water in the mitochondrial electron transport chain on the way to ATP formation (Ballard & Whitlock 2004).

In this study, part of the gene coding for mtCOI was employed as a sequence marker. Additionally, parts of the gene regions coding for the 28S (nuclear large subunit) as well as the protein-encoding genes *abd*-A (abdominal-A), LW *Rh* (long-wavelength rhodopsin) and *wg* (wingless) were sequenced in order to embed specialized ant species of the *ferrugineus* group into a large dataset comprising several *Pseudomyrmex* species of different species groups published by Ward and Downie (2005).

1.5 Microsatellites as molecular markers

While DNA sequences often serve to investigate phylogenies among different taxa, more variable markers are required to examine small-scale phenomena within a single species. Microsatellites are DNA regions with tandemly repeated motifs of one to six nucleotides. They are found in all prokaryotic and eukaryotic genomes (Zane *et al.* 2002). They are also known as Short Tandem Repeats (STR) or Simple Sequence Repeats (SSR). These repeats are usually characterized by a high degree of length polymorphism (Zane *et al.* 2002). Microsatellites are mostly found in non-coding regions of the genome such as introns and rarely occur in coding regions (exons) (Hancock 1995). Microsatellites have been detected in higher frequencies than expected in the genome of almost every organism studied so far (Hancock 1999). They seem to be distributed evenly throughout the genome. Edwards and co-workers (1996) investigated microsatellite loci in the human genome and observed at least one microsatellite repeat per 300 to 500 kb. $(GT)_n$ seems to be the most common repeat motif in the human genome (Lagercrantz *et al.* 1993), while $(GA)_n$ seems to be the most common repetitive motif in ants (A. Trindl, pers. comm.).

Microsatellite loci are highly polymorphic due to high mutation rates. Their mutation rate is higher as compared to rates of point mutation, which have an order of 10^{-9} to 10^{-10} (Hancock 1999). In general, the mutation rate of microsatellite sequences is estimated to be 10^{-3} to 10^{-5} per locus and generation (Bowcock *et al.* 1994; Edwards *et al.* 1996; Forbes *et al.* 1995; Schlotterer & Tautz 1992).

The high mutation rate of microsatellite markers is caused by DNA polymerase slip-strand mispairing and/or unequal crossing over (Eisen 1999; Fresco & Alberts 1960; Li *et al.* 2002). According to the slip-strand mispairing model, slippage of DNA polymerase occurs during DNA replication and causes the template strand and the

newly replicated strand to be temporarily unaligned. In order to continue DNA replication, the strands must realign and a mutation occurs in the case of errors in this realignment. The presences of repeats in template DNA increase DNA polymerase slippage, since repeats can easily be looped out of the DNA double helix (Streisinger *et al.* 1966). In contrast, unequal crossing over occurs during the recombination of two homologous chromosomes, which are imperfectly aligned during crossing over (Smith 1973). The presence of repeat motifs generally increases the likelihood of misalignment between homologous chromosomes (Eisen 1999).

Understanding the mutational process of the evolution of microsatellites is necessary to optimize the information obtained from these markers (Estoup & Cornuet 1999). Several theoretical mutation models have been developed. The infinite allele model (Kimura & Crow 1964) predicts that any number of tandem repeats can evolve and always results in a new allele status that did previously not exist in the population. Under the assumption of the stepwise mutation model (Kimura & Ohta 1978), each mutation creates a novel allele either by adding or deleting a single repeat with the same probability in both directions. Consequently, alleles of similar sizes would be evolutionarily closer than alleles of higher size differences. Under the K-allele model (Crow & Kimura 1970), there are K possible allelic states and any allele has a constant probability of mutating towards any of the K-1 allelic states existing before.

Due to their high mutability, microsatellites play a significant role as molecular markers for evolutionary and population genetic studies. Microsatellites offer several advantages compared to other molecular markers: they are highly reproducible, highly polymorphic, PCR-based and readily portable within a species (Edwards *et al.* 1996). All these positive attributes coupled with their multi-allelic nature, co-dominant transmission, relative abundance, extensive genome coverage and requirement of only a small amount of template DNA have contributed to the extraordinary increase of development of microsatellite markers for many organisms (Zane *et al.* 2002).

1.6 Aims of the present study

The overall aim of the present thesis was to understand mechanisms that promote the maintenance of the specific acacia-*Pseudomyrmex* interaction with special emphasis on coevolutionary aspects. I used a comparative approach by investigating *Pseudomyrmex* ants, which represent all the following types of interactions: (i) generalists, i.e., ants with no known association with acacia plants, (ii) obligate mutualists and (iii) exploiters of acacia myrmecophytes.

I quantified invertase activity in workers and larvae of mutualists, exploiters and generalists to test whether the ants' feeding preferences have a physiological basis. A putative substrate-induction was considered, since workers of obligate acacia-inhabitants naturally feed on sucrose-free EFN only. Larvae of the specialized *Pseudomyrmex* species feed on sucrose-containing food bodies (Heil *et al.* 2004a) and, thus, were also included. Additionally, I established a molecular phylogeny of *Pseudomyrmex* ants to understand the evolutionary history of species that inhabit acacia myrmecophytes as mutualists or as exploiters. Thereby, I wanted to understand at which level (behavior, physiology or phylogeny) the putative filter against exploitation of the acacia-*Pseudomyrmex* mutualism functions and why certain ant species can still exploit the resources provided by the acacias.

Further, I aimed at analyzing whether ant-plant interactions affect nestmate recognition, the ants' cuticular hydrocarbon profiles and the genetic structure of the resident ants. I therefore applied a comparative approach integrating observations of aggression behavior, chemical analyses of cuticular hydrocarbon profiles and genetic microsatellite analyses. My aim was to evaluate the efficiency of these three different approaches to detect colony boundaries and to test whether they give consistent information. I tried to obtain an estimate of how many colonies inhabit one host tree and on how many host trees can be colonized by the offspring of one queen. In my experiments, I compared the observed patterns of the mutualist *Pseudomyrmex ferrugineus* to the parasitic species *P. gracilis* in order to identify how these different interspecific interactions can shape the social structures of participating ant-inhabitants.

A further aspect of this thesis was investigating the genetic colony structure of the polygynous ant mutualist *Pseudomyrmex peperi*. As described for other members of the *P. ferrugineus* group, *P. peperi* exclusively nests in the hollow thorns of myrmecophytic acacia plants and feeds only on the directly plant-derived food sources extrafloral nectar and food bodies (Clement *et al.* 2008; Heil *et al.* 2004a; Janzen 1966). Congeneric ant species of the *P. ferrugineus* group, which occur sympatrically, target at inhabiting the same host plant species. Polygynous colonies have been described for *P. peperi* as well as for three other acacia-ants belonging to the same species group, i.e., *Pseudomyrmex janzeni*, *P. satanicus* and *P. veneficus*, while monogyny has been described for five species (*P. ferrugineus*, *P. flavicornis*, *P. mixtecus*, *P. nigrocinctus* and *P. spinicola*) (Ward 1993). Thus, I asked how *P. peperi* establishes polygynous colonies and how these colonies are genetically structured and whether polygyny in *P. peperi* is a consequence of interspecific competition. To address these questions, I applied genetic microsatellite data and investigated the colony structure. Finally, I employed a phylogeny based on DNA sequence data of obligate acacia-ants to address the evolution of polygyny in acacia-ants and to test the hypothesis that polygyny evolved within this clade as a consequence of interspecific competition among acacia-ants. Taken together, this study provides a framework to better understand if and how polygyny represents an adaptation of certain obligate plant-ants to high competition in such a successful mutualistic interaction.

2 Materials and Methods

2.1 Study sites

Field studies and collection of samples in Mexico were conducted during three research trips each from August to the end of the rainy season in November (2005, 2006 and 2007). In the present study, three main field sites were included. Site 1 was located in the vicinity of Puerto Escondido in the state of Oaxaca at the Pacific coast of Mexico. Site 2 was located in the inland of Oaxaca State with Matias Romero the largest town nearby. Additional samples were collected in the Los Tuxtlas area (site 3), which is located close to the city San Andres Tuxtla in the state of Veracruz at the Gulf coast of Mexico. All three sites are located in the south of Mexico and are climatically considered to be part of the 'tierra caliente' (hot land) belonging to the outer tropics. Site 1 and site 3 are separated by the Isthmus of Tehuantepec and are some 400 km apart, while site 2 is situated in the Isthmus of Tehuantepec. The Isthmus is the region in Mexico where both coasts are closest to each other. It is considered the geographic border between North and South America separating the Sierra Madre del Sur from the mountain range of Chiapas by forming a plateau with an elevation of approximately 300 m. Samples were collected at and in the vicinity of all three sites as well as along roadsides between those sites (Fig. 2.1.1). Behavioral studies and collection of cuticular compounds were conducted at sites 1 and 2.

Fig. 2.1.1 Geographic location of the main study sites in Mexico.
Three main study sites were included in the present study: Site 1 (Puerto Escondido – blue marker) is located at the Pacific coast, site 2 (Matias Romero – green marker) in the Isthmus of Tehuantepec and site 3 (Los Tuxtlas – red marker) at the Gulf coast.

2.2 Species investigated

The New World ant genus *Pseudomyrmex* comprises ca. 200 species. Most of these are generalists that nest in dead twigs, but about forty species are specialized inhabitants of myrmecophytes (Ward & Downie 2005). Prominent examples among the plant-ants are those inhabiting swollen-thorn acacias. These include ten species of obligate mutualists in the *Pseudomyrmex ferrugineus* group and four distantly related acacia-ant species that appear to be specialized exploiters of acacia (Ward 1993).

Invertase activity was quantified in seven *Pseudomyrmex* species: *P. ferrugineus* F. SMITH 1877, *P. mixtecus* WARD 1993 and *P. peperi* FOREL 1913 belong to the *ferrugineus* group (Ward 1989; Ward 1993) and are obligate acacia-mutualists. *Pseudomyrmex gracilis* FABRICIUS 1804 and *P. nigropilosus* EMERY 1890 are exploiters (Clement *et al.* 2008; Janzen 1975), although their interaction with acacia myrmecophytes appears to be obligate in the case of *P. nigropilosus*, yet facultative for *P. gracilis*. Generalist ant species included were *P. salvini* FOREL 1913 and the undescribed species *P.* spec. PSW-06. *Pseudomyrmex salvini* belongs to the *elongatulus* group; *P*. spec. PSW-06 is placed in the *pallidus* group (P.S. Ward, unpubl. data). Both species have no known affinity towards acacia plants. Species were determined according to (1993; in prep.). Some terms that are relevant for the identification of *Pseudomyrmex* ants are explained in Box 2.

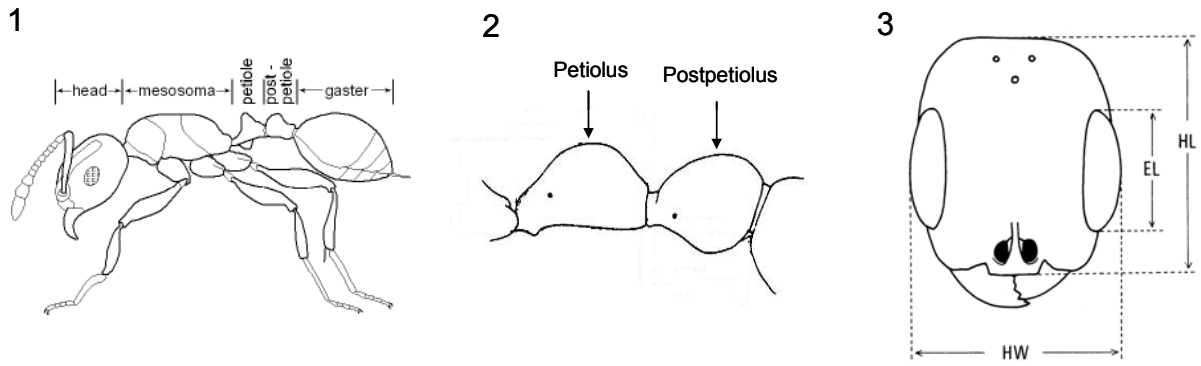
Additional species included in the phylogenetic analysis were kindly provided and determined by P.S. Ward totaling 30 species (*P. flavicornis* F. SMITH 1877, *P. godmani* FOREL 1913, *P. haytianus* FOREL 1913, *P. laevifons* WARD 1989, *P. major* FOREL 1913, *P. nigrocinctus* EMERY 1890, *P. perboscii* WARD 1993, *P. spinicola* EMERY 1890, *P. satanicus* WHEELER 1942, *P. veneficus* WHEELER 1942, *P.* spec. PSW-01, *P.* spec. PSW-02, *P.* spec. PSW-37 and *P.* spec. PSW-54). *Tetraponera rufonigra* (the sister taxon to *Pseudomyrmex*) and *Apis mellifera* (honey bee) were used as outgroup species.

In the experiment, in which I compared behavioral, chemical and genetic data, I selected *P. ferrugineus* as an obligate acacia-mutualist and *P. gracilis* as an exploiter. Additionally, I investigated the social structure within colonies of the polygynous acacia-ant *P. peperi*.

Box 2 Terms on morphology of ants.
In the order Hymenoptera, the posterior extremity of the first abdominal segment is pinched off, separating the **mesosoma** from the **gaster** and fuses the first abdominal segment to the thorax. Thus, hymenopteran bodies are not divided into **caput** (head), thorax (middle body with appendages including legs and wings) and abdomen like other insects, but into caput, mesosoma (thorax including frontal part of the abdomen) and gaster (abdomen excluding first abdominal segment). Ants (family Formicidae) have one segment called the **petiole** between the thorax and the abdomen. It is derived from the second abdominal segment that is constricted. In the subfamily Pseudomyrmecinae, the third abdominal segment becomes also constricted and builds the so-called **postpetiole** (Baroni Urbani 1989).

Some measurements of the head are relevant to distinguish *Pseudomyrmex* species from one another. The **head width** (HW) refers to the maximum width of the head, including the eyes and the **head length** (HL) to the midline length of the head excluding the mandibles. The **eye length** (EL) describes the length of the compound eye (main eye, besides three small eyes – ocelli - on 'forehead'). The **relative eye length** (REL) is the ratio of EL/HL. All parameters are measured with the head in full-face view (Ward 1985).

The term **pilosity** indicates groups of thick, long hair while short, fine hair is called **pubescence**. Both can cover any part of the ants body and are often used as morphological characters in keys for determination of species from the genus *Pseudomyrmex* (Ward 1985, 1990, 1993, 1999).



Figures. Schematic views of an ant. 1. Generalized ant body with head, mesosoma, petiole, postpetiole and gaster (from Shattuck 2000). 2. and 3. Generalized *Pseudomyrmex* worker, illustrating some terms. 2. Lateral view of petiole and postpetiole (from Ward 1985). 3. Frontal view of head. EL, eye length; HL, head length; HW, head width (from Ward 1985).

2.2.1 *Pseudomyrmex gracilis* FABRICIUS 1804

Fig. 2.2.1 *Pseudomyrmex gracilis*.
Lateral, dorsal and frontal view (www.antweb.org; kindly permitted by P.S. Ward).

Species of the *P. gracilis* group have relatively large and elongate eyes with eye length more than one-half of the head length (worker and queen REL 0.52-0.65). The outer surfaces of the tibiae are usually covered with standing pilosity, which may be very short. Ants of this species group are the largest within the genus (worker

HW>1.2). The color of *P. gracilis* is extremely variable and ranges from unicolored black with lighter appendages to unicolored orange-brown. Many intermediate and bicolored combinations exist. Central American species usually have a black gaster and head with a lighter coloration of the mesosoma (Ward 1993).

Pseudomyrmex gracilis is widely distributed and ranges from the United States (California to Florida; Hawaii) to Argentina and Brazil, including the Caribbean islands. These generalist ants inhabit a variety of habitats, from fields and roadsides to forests (Ward 1993). Nests are often located in dead twigs or hollow branches of all kinds of plants. This opportunistic species also has been found to nest in cracks within buildings and other stoneworks (Buren & Whitcomb 1977; Cassani 1986; Klotz *et al.* 1995; Ward 1993). Several colonies have been recorded to nest in swollen thorns of Central American acacias ranging from Mexico to Panama (Janzen 1975; Ward 1999; Wheeler 1942). The species expresses behavior different from the specialized plant-ants and does not show any protective traits. It appears, in contrast, to be a parasite to myrmecophytic acacias (Clement *et al.* 2008). Occasionally, *P. gracilis* has been found to nest on the same plant with specialized species of the *ferrugineus* group and occasionally they share host plants with other non-symbiotic species, e.g., *P. nigropilosus* and *Crematogaster* spec. (pers. obs.).

2.2.2 *Pseudomyrmex nigropilosus* EMERY 1890

Fig. 2.2.2 *Pseudomyrmex nigropilosus*.
Lateral, dorsal and frontal view (www.antweb.org; kindly permitted by P.S. Ward).

Pseudomyrmex nigropilosus is also a member of the *gracilis* group and has the morphological traits of this group as described for *P. gracilis* (see above). The standing pilosity of this species is long and conspicuously black. The petiole is short and high, in contrast to the petiole of *P. gracilis*. The color of *P. nigropilosus* is variable, usually pale or bicolored orange and black. The species is distributed in Central America, ranging from Mexico to Costa Rica (Ward 1993). Ants of this

species have exclusively been found nesting in swollen-thorn acacias and the species is considered an obligate acacia-ant (Janzen 1966). As described by Janzen (1975), *P. nigropilosus* is a secondary parasite of the acacia-*Pseudomyrmex* mutualism. It nests in the domatia provided by the host and feeds on the EFN and food bodies without providing any kind of protection to the plant.

2.2.3 *Pseudomyrmex salvini* FOREL 1913

Pseudomyrmex salvini is a member of the *elongatulus* group. It has not been reported to live in association with acacias and usually nests in dead twigs. The species is of medium size within the genus. The head is black with large eyes. The mesosoma and gaster are usually orange. Variants in color occur, in which the gaster is black.

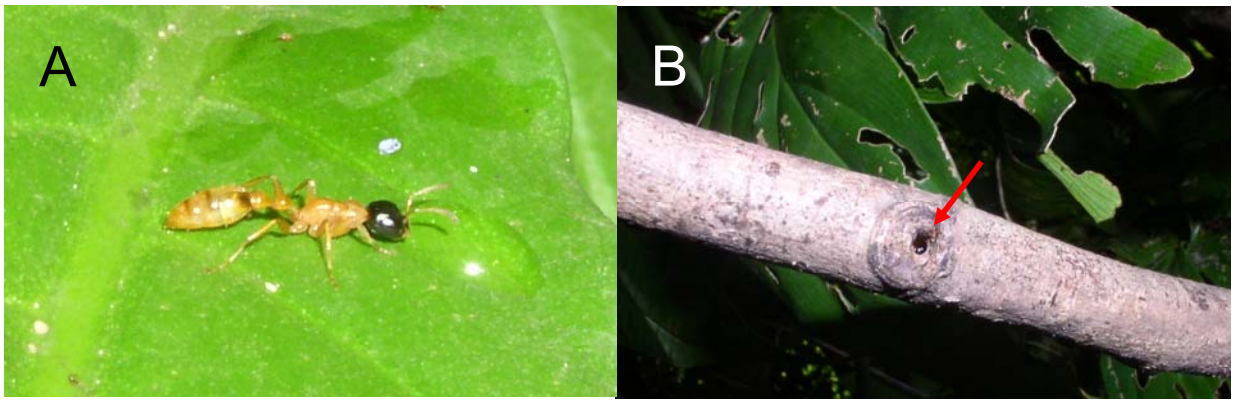


Fig. 2.2.3 *Pseudomyrmex salvini*.
A. Worker feeding on experimentally offered sugar solution. B. Dead twig inhabited by *P. salvini* with entrance hole (indicated by a red arrow). The black head of a colony member is visible in the entrance hole. Photos by S. Kautz.

2.2.4 *Pseudomyrmex* spec. PSW-06

The species *P.* spec. PSW-06 is not yet described. Ward places it in the *pallidus* group (pers. comm.). The species has been found in the vicinity of Puerto Escondido, Mexico, nesting in dead twigs as well as in a blade of grass. Workers of this species are relatively small. They are unicolored light brown to orange.

Fig. 2.2.4 *Pseudomyrmex* spec. PSW-06.
Twig inhabited by ants including three workers, several larvae and a queen whose abdomen is distended with eggs (http://www.myrmecos.net/ants/PseNrPal1.html, kindly permitted by A. Wild).

2.2.5 *Pseudomyrmex ferrugineus* F. SMITH 1877

Fig. 2.2.5 *Pseudomyrmex ferrugineus*.
Lateral, dorsal and frontal view (www.antweb.org; kindly permitted by P.S. Ward).

Members of the *ferrugineus* are medium-sized (worker HW<1.28) and have smaller eyes than other *Pseudomyrmex* ants with a length usually less than one half of the head length (worker and queen REL 0.38-0.50). All *ferrugineus* group members are obligate plant-ants living in association with swollen-thorn acacias. They are extremely aggressive (Ward 1993).

Pseudomyrmex ferrugineus is of medium size (worker HW>0.91) with a color varying from light reddish- or yellowish-brown to very dark brown. The gaster and head are darker than the mesosoma. The distribution ranges from southern Mexico to El Salvador and Honduras. The species is very common and has been recorded for all swollen-thorn acacia species growing within its distribution, i.e., *Acacia chiapensis*, *A. collinsii*, *A. cookie*, *A. cornigera*, *A. gentlei*, *A. globulifera*, *A. hindsii*, *A. janzenii*, *A. mayana* and *A. sphaerocephala* (Ward 1993).

2.2.6 *Pseudomyrmex mixtecus* WARD 1993

Fig. 2.2.6 *Pseudomyrmex mixtecus*.
Lateral, dorsal and frontal view (www.antweb.org; kindly permitted by P.S. Ward).

Pseudomyrmex mixtecus has the common traits of the *ferrugineus* group. It is smaller than *P. ferrugineus* (worker HW<1.04). The color is very dark brown to black with lighter appendages (Ward 1993). The species has only been found on *Acacia hindsii*, *A. collinsii* and *A. cornigera* in the Mexican states Guerrero and Oaxaca.

2.2.7 *Pseudomyrmex peperi* FOREL 1913

Fig. 2.2.7 *Pseudomyrmex peperi*.
Lateral, dorsal and frontal view (www.antweb.org; kindly permitted by P.S. Ward).

Pseudomyrmex peperi is a small species (worker HW<0.92) of the *ferrugineus* group. The head is moderately elongate. The color is light to medium brown, hardly ever dark brown. Sometimes the gaster is darker than the rest of the body, the appendages are usually lighter. The species is widely distributed and has been found from eastern Mexico to Nicaragua. Collections were made from *Acacia chiapensis*, *A. collinsii*, *A. cornigera*, *A. gentlei*, *A. globulifera* and *A. hindsii*. The species is usually polygynous (having more than one queen per colony) and occurs in large colonies that can inhabit several plants (Ward 1993). The species shows protective behavior, but does not seem as aggressive as *P. ferrugineus* and *P. mixtecus* (pers. obs.).

2.3 Physiological adaptations

2.3.1 Sampling of ants

Mutualists and exploiters were collected by cutting off swollen acacia thorns, whereas generalists were baited by pipetting aqueous sucrose solution (10% w/v) onto branches of the background vegetation: the ants took up the offered liquid (Fig. 2.2.3), returned to their nest site and, thus, nests could be localized. Ants of each mutualist or exploiter colony were divided into three sub-samples, one of which was not fed (‘starved’), while the two others were fed for five days with a solution (10% w/v) of glucose or sucrose *ad libitum* prior to quantification of invertase activity. The generalists could not be included in the study of starved ants, because sugar solution had been taken up by an unknown proportion of the workers during collection. Thorns were kept intact to protect larvae, which were fed by the respective workers with the offered sugar solution. Starved ants were dissected on the day after collection. Ten different colonies of each species from different sites were included in the study with exception of *P.* spec. PSW-06, of which four colonies were used.

2.3.2 Preparation of ants

Ants were dissected in ‘Insect Ringer’ solution (10.4 g NaCl, 0.32 g KCl, 0.48 g $CaCl_2$ and 0.32 g $NaHCO_3$ in 1 l water). The sting and poison glands were removed from the gaster and discarded. Head and gaster were opened with tweezers and all inner parts were collected, the exoskeleton and mesosoma were discarded as well. All glands that might contain digestive enzymes and the entire digestive tracts were included in the analyses. The content of one to eight ants from one sample were pooled, transferred into 300 µl 50 mM sodium phosphate buffer (pH 6.0) with 25 µl of proteinase inhibitor (one Complete Mini Tablet, Roche Diagnostics, Branchburg, NJ, USA in 1.5 ml water) and homogenized with sand. One complete larva was used for each extraction, transferred to sodium phosphate buffer with proteinase inhibitor and homogenized with sand. The extracts were filtered with micro membranes (‘Rotilabo Spritzenfilter’, 13 mm, 0.2 µl Nylon, Carl Roth, Karlsruhe, Germany), before incubation at 4° C for 1 h. Until otherwise noted, all chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

2.3.3 Quantification of invertase activity

Invertases (β-fructofuranosidase, EC 3.2.1.26, also termed β-fructosidase, saccharase, or sucrase) are glycoside hydrolases (EC 3.2.1.-) that catalyze the cleavage of sucrose (α-D-glucopyranosyl-β-D-fructofuranoside) into the two monosaccharides, glucose and fructose (Henrissat & Bairoch 1993; Naumoff 2001; Sturm & Tang 1999). Invertase activity was quantified spectrophotometrically based on the kinetics of glucose release from sucrose. The 'Glucose (HK) kit' (Sigma-Aldrich) was used as described in Heil (2005a). Ten microliter of extract and 100 µl of 'Glucose (HK) reagent' (prepared according to the manufacturer's protocol; HK, hexokinase) were introduced into cuvettes (70 µl micro disposable cuvettes, Plastibrand®, Brand, Wertheim, Germany) and immediately spectrophotometrically measured at 340 nm (Genesys 20 Spectrophotometer, ThermoSpectronic, Cambridge, UK). Ten microliters of sodium phosphate buffer and 100 µl of 'HK' reaction solution were used as negative controls. Absorption at 340 nm was recorded every ten minutes until a steady state was reached (30-60 min). In cases of very high initial absorptions (>0.3) or high increases in absorption, the samples were further diluted with 'HK' reaction solution and sodium phosphate buffer. After the steady state was reached, 20 µl of sucrose with water as solvent (25% w/v) were added to each sample and absorption was quantified for 1.5-2 h in order to calculate invertase activity as the amount of newly produced glucose (Heil *et al.* 2005a). If samples had to be diluted, a proportional amount of sucrose was added. The 'Glucose (HK) kit' is designed to determine glucose quantitatively and with a high degree of specificity. It combines hexokinase and glucose-6-phosphate dehydrogenase. Glucose present in the sample solution (in the present case appearing *de novo* as product of invertase-catalysed hydrolysis of sucrose) is phosphorylated by hexokinase. The resulting glucose-6-phosphate is then oxidized to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. In this reaction, NAD is reduced to NADH, which absorbs UV-light at 340 nm. The increase in absorbance at 340 nm is directly proportional to glucose concentration (www.sigmaaldrich.com; Fig. 2.3.1). Different ant species of the genus *Pseudomyrmex* were used to quantify invertase activity. Extinction at 340 nm was measured and plotted against reaction time. Ant extracts first showed an increase in absorption due to the presence of the substrate sucrose as well as the product glucose of the enzymatic reaction in the extract. Depending on the species and number of ants used, 20 to 60 minutes were required to reach the equilibrium,

after which sucrose could be added. Sucrose was added at excess (Heil *et al.* 2005a). Resulting from dilution with the sucrose solution that is not absorbent at 340 nm, absorption was slightly reduced after sucrose solution was added. After 10-15 min, the resulting curves showed almost linear slopes (Fig. 2.3.2) and invertase activity could be calculated from these slopes. The first measuring point after the addition of sucrose was excluded and an average activity was calculated from the remaining data points.

Data on invertase activity from the experiment with starved ants (workers or larvae) were subjected to analysis of variance (ANOVA). In the feeding experiment, Wilcoxon signed rank test for two dependent variables were applied using glucose-fed ants and sucrose-fed ants obtained from one colony as matched pairs. In addition, a univariate general linear model (GLM) was applied to the entire data set. 'Invertase Activity' was set as variable, 'Species', 'Treatment' (unfed, fed with glucose, or fed with sucrose) and 'Ontogenetic Stage' (workers or larvae) were set as fixed factors, while 'Colony' was a random factor. All statistical tests were conducted using SPSS 14 (SPSS for windows, SPSS Inc., Chicago, USA) or STATISTICA 8.0 (StatSoft, Inc., Tulsa, USA) until otherwise noted.

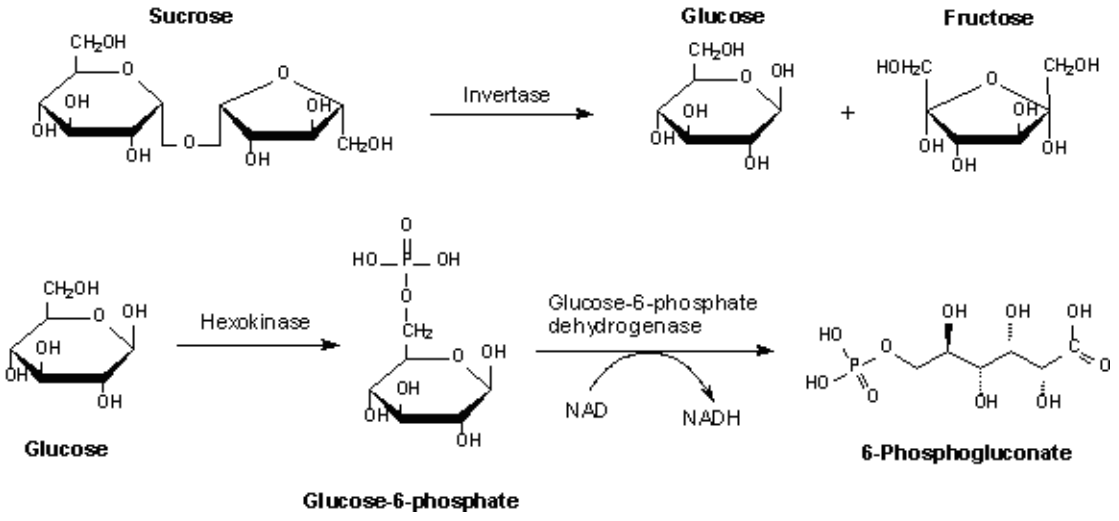


Fig. 2.3.1 Reaction taking place in the extract.
Sucrose is cleaved hydrolytically, if invertase is present in the extract. Subsequently, the released glucose is phosphorylated by hexokinase. The product glucose-6-phosphate is oxidized by glucose-6-phosphat dehydrogenase to 6-phosphogluconate, while NAD is reduced to NADH (from: www.sigmaaldrich.com). NADH production is directly proportional to release of glucose and, thus, to invertase activity.

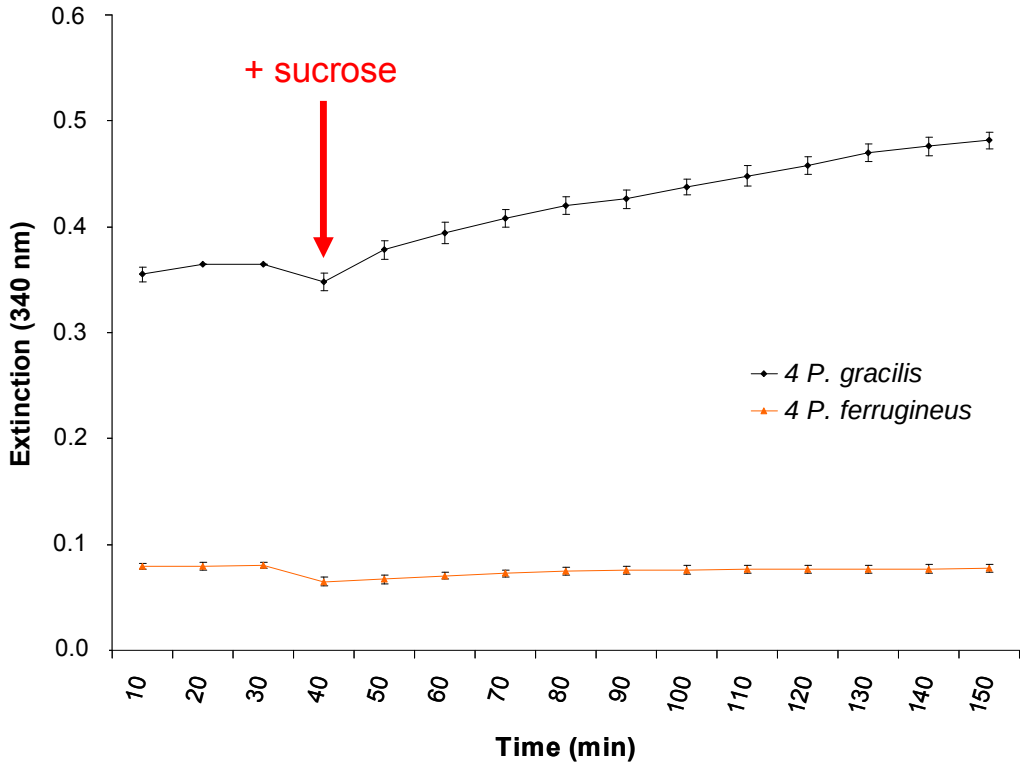


Fig. 2.3.2 Time courses of extinction.
Values for extractions of four workers each from the ant species *Pseudomyrmex ferrugineus* (mutualist) and *P. gracilis* (exploiter) are given. Two replicates per extract were used; means and standard errors of deviation are indicated. The time when sucrose was added is indicated with a red arrow. Sucrose was added at excess.

2.3.4 Standard curve of glucose

To acquire a standard curve defined amounts of glucose (D-Glucose) were quantified. A stock solution of 50 mg glucose in 1 l 50 mM sodium phosphate buffer was prepared three times. Different volumes (2.5; 5.0; 7.5 and 10 µl) were diluted with sodium phosphate buffer to a final volume of 10 µl and transferred into cuvettes. Hundred microliters of 'HK reagent' were added and quantification was conducted under the same experimental conditions as described above. Two replicates per concentration were measured.

The standard curve (MS Excel) of extinction$_{(340\ nm)}$ for pure glucose at different concentrations is shown in Fig. 2.3.3. Regression analysis was conducted under the assumption of a directly proportional relationship and an intersection with the zero point. Glucose release, thus, could be calculated from the extinction$_{(340\ nm)}$ using the equation:

Glucose release [ng] = 465.76 [µg*min]* Δ $E_{(340\ nm)}$ (equation 1)
with Δ $E_{(340\ nm)}$ = difference of extinction at 340 nm

Thus, ant invertase activity could be expressed as ng glucose released per min per mg ant fresh material. The unit [ng glucose μg^{-1} min^{-1}] will be used throughout this study. Means are given with standard error of deviation (SE).

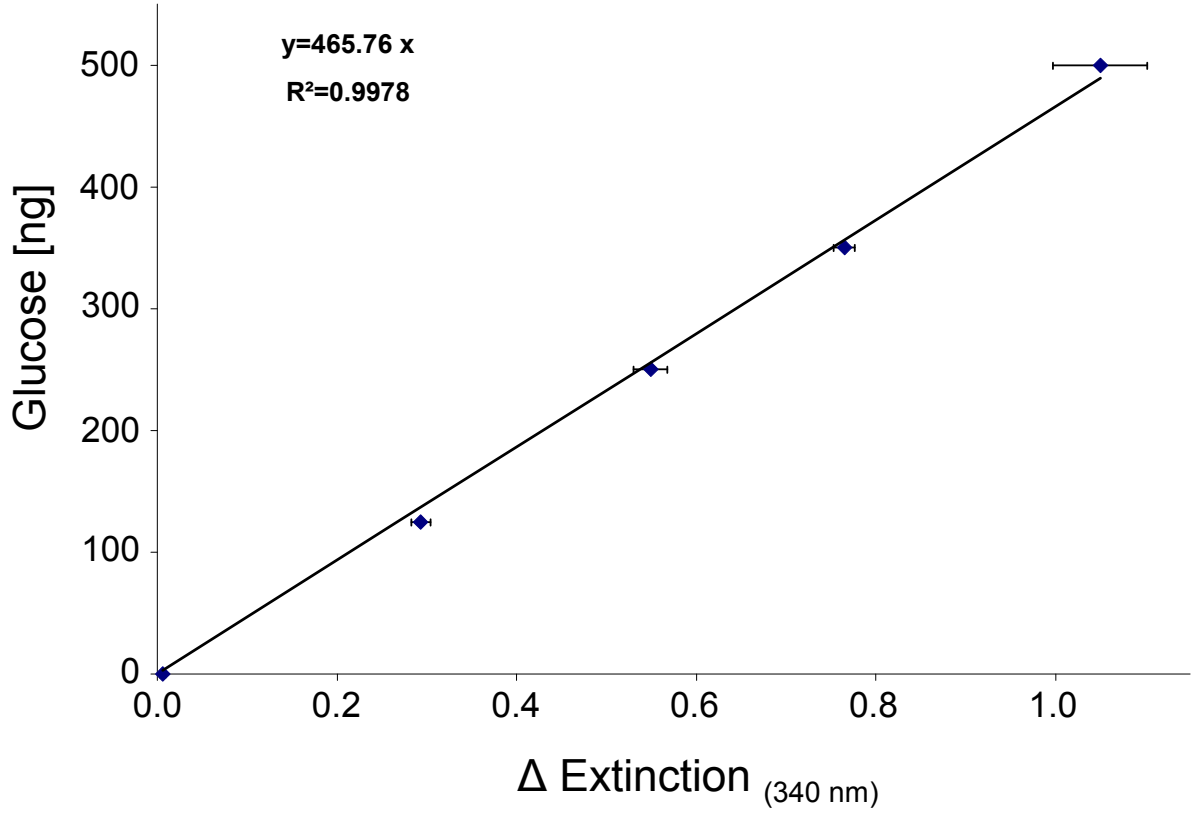


Fig. 2.3.3 Standard curve of glucose.
Means and standard deviation are indicated; reaction volume 110 µl, N=3. Stock solution was prepared three times and diluted until defined amounts of glucose were reached. For each sample two replicates were measured. The equation to calculate glucose release from extinction is indicated with the stability index R^2.

2.4 Behavioral experiments

Behavioral experiments were conducted from August to October 2007. Experiments were conducted at two study sites (site 1 near Puerto Escondido and site 2 near Matias Romero). For each species, two plots with eight trees each were investigated. Plots of the mutualist *Pseudomyrmex ferrugineus* were termed PFER1 and PFER2, while the plots of the parasite *P. gracilis* were PGRA1 and PGRA2. The colonies in each plot were numbered from PFER1a to PFER1h in plot PFER1 etc.; ant individuals were numbered PFER1a-01 and so on. The eight closest trees that were inhabited by the same species were selected. GPS data for each tree was recorded (GPS 60; Garmin, Gräfelfingen, Germany) (Fig. 2.4.1; supplementary Table 1, page 142). In field studies, individual ants from eight host trees per plot were experimentally confronted with other individuals in order to study colony boundaries at the behavioral level. From each acacia tree, forty workers were transferred as follows: Five ants were returned to the same tree to test whether ants react aggressive to any other experimentally transferred ant. Five further individuals were placed on another tree of the same plot inhabited by the same species. Each single ant was observed until it encountered an individual on the tree it was placed on. Before transfer, all ants collected from one acacia were held together in a 250 ml plastic cup sealed with fabric (anti-aphid net). Ant workers were applied individually to branches of acacias using tweezers. After each transfer, tweezers were cleaned with dichloromethane. Each single ant was observed until it encountered an individual on the tree it was placed on. Behavior was either classified as aggressive — when the transferred ant was attacked (chasing, pairwise reciprocal stinging and eventually both opponents falling off the tree) — or as neutral — when no attack took place.

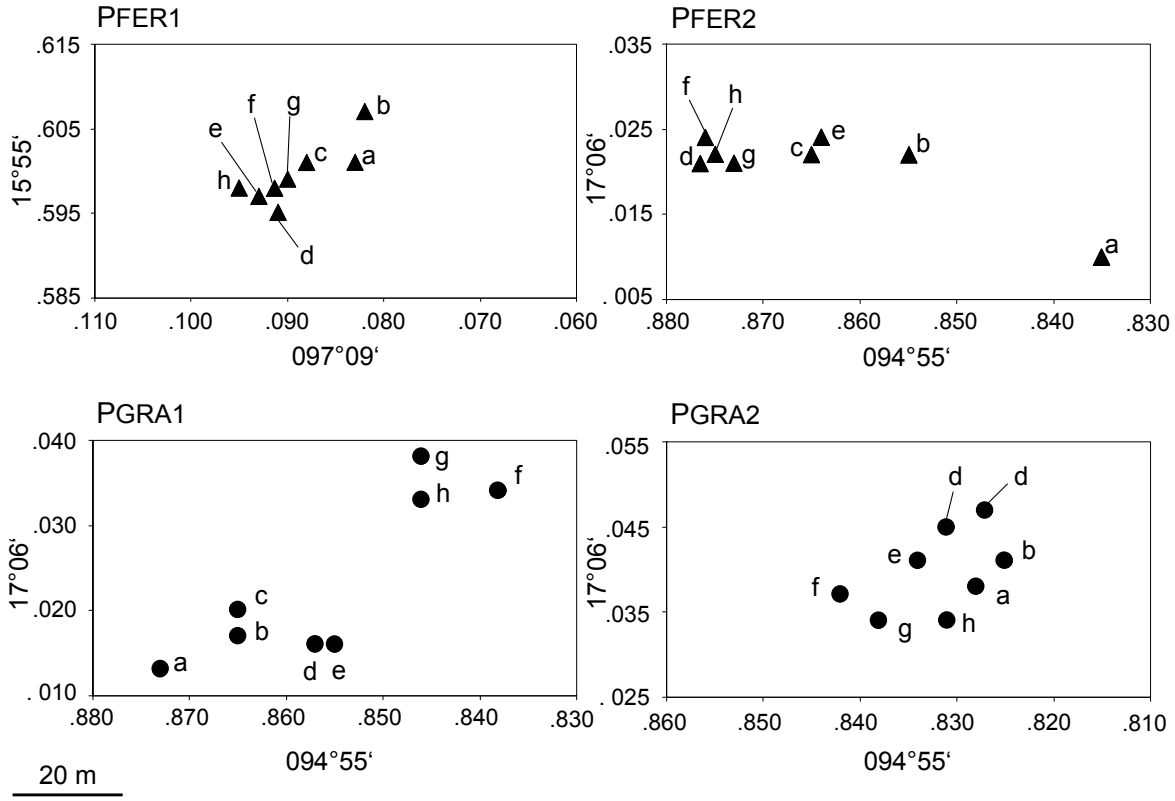


Fig. 2.4.1 Locations of the colonies included in the plots.
Geographic location of each acacia in the four plots is illustrated. Four plots were used to investigate correlations of geographic distance, behavior, chemical data and genetic data of ants. Each plot consisted of eight acacia trees that were either inhabited by the mutualist acacia-ant *Pseudomyrmex ferrugineus* (PFER1 and PFER2) or the parasite *P. gracilis* (PGRA1 and PGRA2). Figures are based on GPS data. Pairwise geographic distances between the colonies of each of the four plots can be found in supplementary Tables 2-5, pages 143-146.

2.5 Colony composition of *Pseudomyrmex peperi*

Individuals of *Pseudomyrmex peperi* derived from two supercolonies (PPEP103 and PPEP132, see Figs. 2.5.1 and 2.5.2 for details) were collected in October 2007. Ants from 20 different acacia shoots were sampled per supercolony (Fig. 2.5.2) by collecting three to seven swollen thorns per shoot and pooling them in Ziploc® bags (Toppits, Minden, Germany). The bags were stored at -20° C for one night. Thorns were then opened and ants transferred to tubes using a funnel and stored in 96% ethanol until needed. The numbers of individuals in the samples collected from each acacia shoot were counted using a binocular microscope. One acacia shoot is referred to one sub-sample throughout the study. Queens, female adults (virgin queens and workers), male adults, female pupae (queen pupae and worker pupae), male pupae, larvae and eggs were differentiated. Queens were identified by their bigger size and strong physogastry, i.e., gasters distended with eggs. Virgin queens

possessed wings and were not physogastric. Workers were identified via their small size and small gasters, males were winged and showed a characteristic morphology with long, slender bodies and long antennae (Fig. 2.5.3).

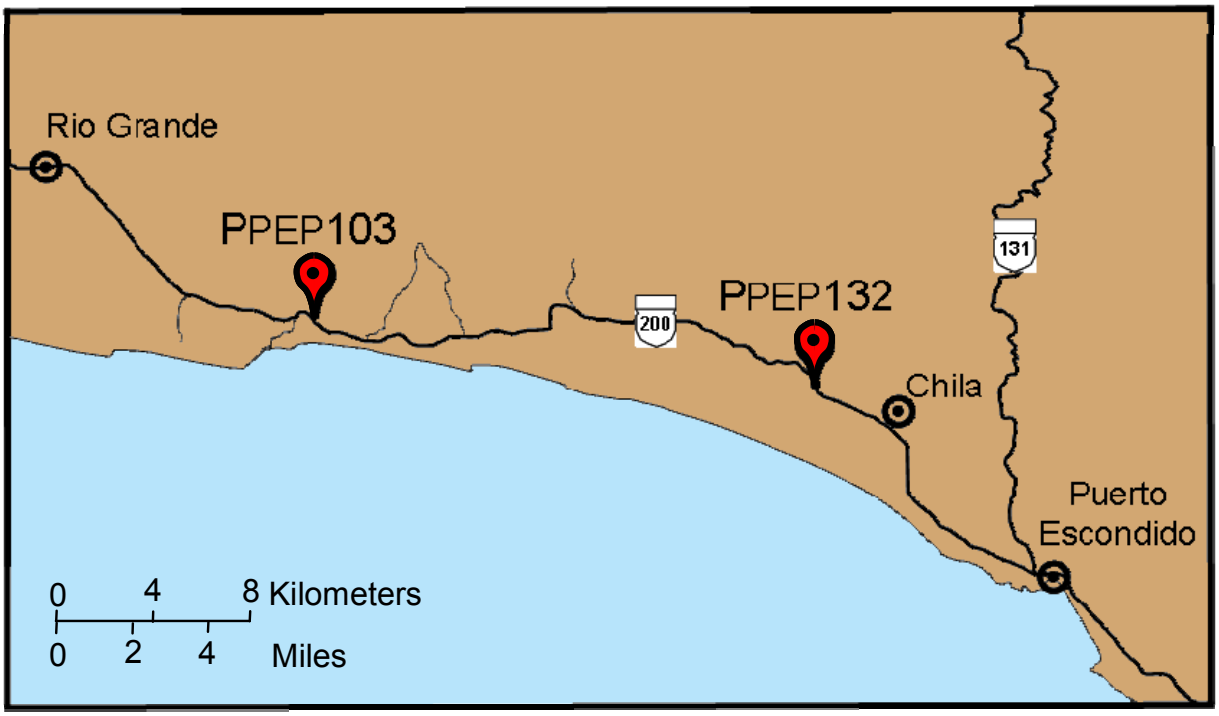


Fig. 2.5.1 Geographic location of *P. peperi* supercolonies.
Illustration of the sites' vicinity. The two study sites (PPEP103 and PPEP132) were located in Oaxaca in South Mexico. Site PPEP103 was about 30 km west of Puerto Escondido, located at km 103 on Highway 200 (N 15°55.809 – 15°55.817; W 97°09.258 – 97°09.267). Site PPEP132 was about 10 km west of the same city located at km 132 on Highway 200 (N 15°57.517 – 15°57.563; W 97°20.653 – 97°20.667).

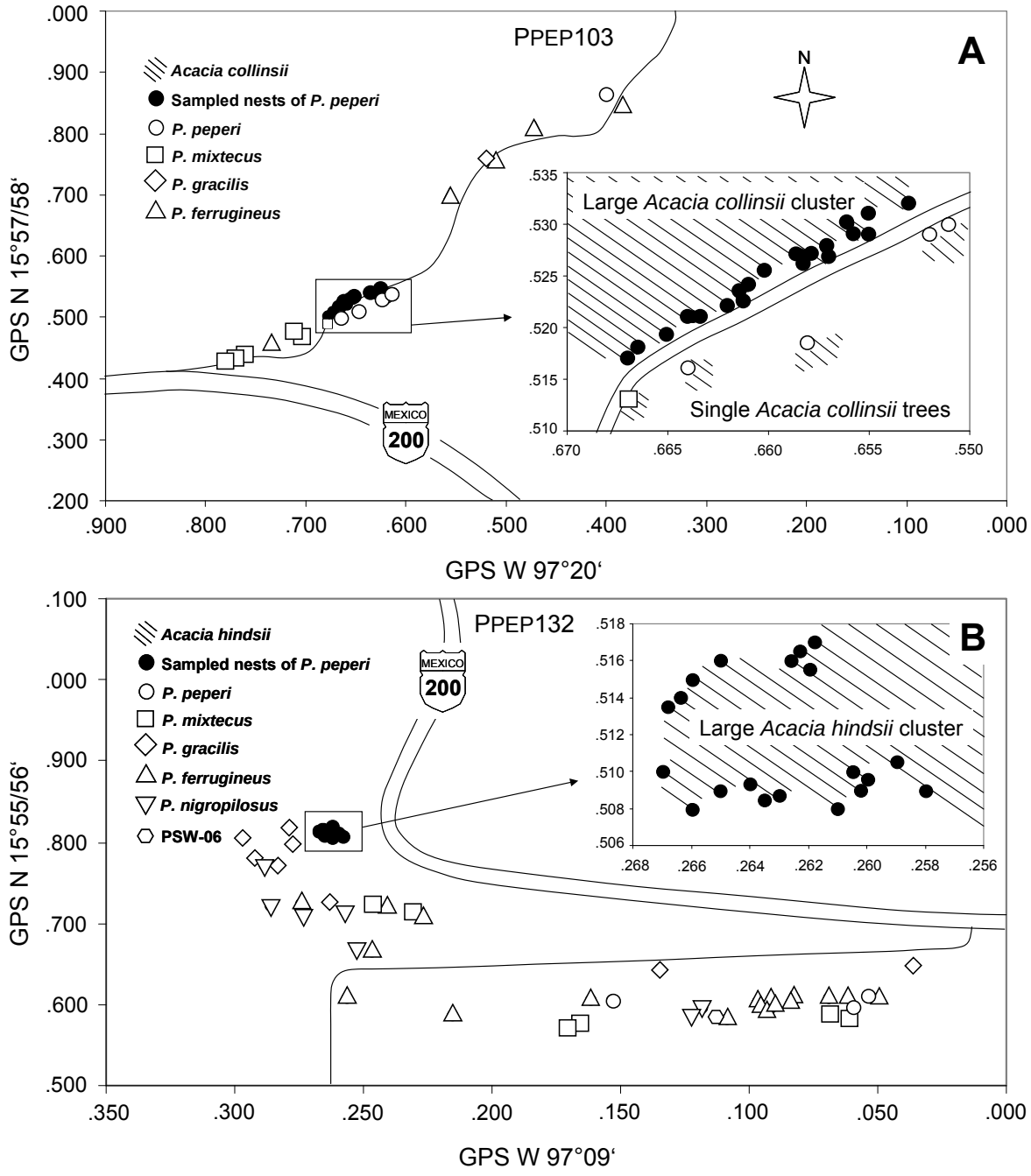


Fig. 2.5.2 Detailed illustrations of *P. peperi* collection sites.
At site A, ants were collected from a large colony (PPEP103) inhabiting an extensive *Acacia collinsii* cluster growing along a field path. This acacia species was the only swollen-thorn acacia occurring at this site. The three acacia-ants *Pseudomyrmex ferrugineus*, *P. mixtecus* and *P. peperi* occurred sympatrically. In addition, the parasitic ant species *Pseudomyrmex gracilis* was found inhabiting one *A. collinsii* tree. At site B, ants were sampled from a colony (PPEP132) inhabiting a large cluster of *Acacia hindsii*. The cluster was surrounded by other acacia individuals and a climbing mimosoid species. Site B was located on a pasture, where the two other myrmecophytes *Acacia cornigera* and *A. collinsii* were also found. In addition to *Pseudomyrmex peperi*, different acacia individuals were colonized by the mutualistic plant-ants *P. ferrugineus* and *P. mixtecus* and in rare cases by the parasitic plant-ants *P. gracilis* and *P. nigropilosus*. The yet undescribed species *P*. spec. PSW-06 was found in a dead twig. Ants of *P. peperi* (black dots) were sampled about every meter along a transect at the edge of inaccessible clusters.

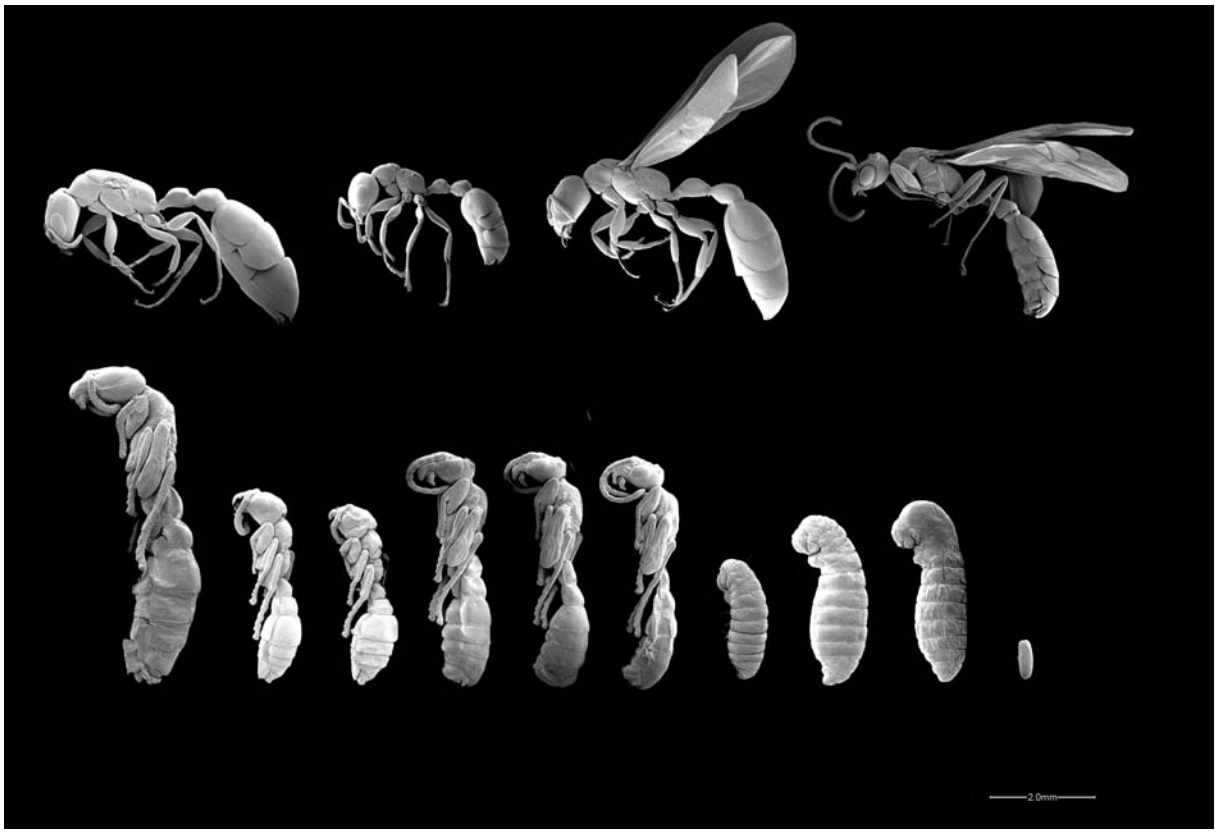


Fig. 2.5.3 REM photographs of different castes of *P. peperi*.
First row: physogastric queen, worker, virgin queen, male; second row: queen pupa, two worker pupae, three male pupae, three larval instars and one egg. Queens are physogastric and show relicts of wings, they are larger in size than workers. Virgin queens possess wings and are not physogastric, but they are larger than workers. Workers are of small size and have no signs of wings. Males have large antennae, small heads, wings and a characteristic habitus. Male pupae are characterized by long antennae and wing discs and are larger than workers. Female pupae have shorter antennae than male pupae. Queen pupae have wing discs. REM photographs by Smail Boukercha and S. Kautz.

2.6 Geographic distance

For all acacias that ants were sampled from, GPS data were recorded (see supplementary Tables 1 and 10, pages 142 and 158). Pairwise distances between trees were calculated with the COORDINATE DISTANCE CALCULATOR (http://boulter.com/gps/distance; Jan-14-2009). The pairwise geographic distances were relevant for the four plots of the mutualist *Pseudomyrmex ferrugineus* and the parasite *P. gracilis*.

2.7 Chemical analyses of cuticular hydrocarbons

Cuticular compounds were extracted from single ants directly after collection in the field. Swollen thorns containing ants were removed from the tree and placed in Ziploc® bags. Up to ten ants per acacia were killed by freezing (-20° C) and then each individual was placed inside the inlet of one GC-Vial and washed with 50 µl of dichloromethane. Dichloromethane was recovered after ten minutes and transferred into glass capillaries (disposable micropipettes with ring mark; Blaubrand® intraMARK, Buddeberg GmbH, Mannheim, Germany). Subsequently, samples were transported to Germany and analyzed on a GC-Trace mass spectrometer (Trace GC Ultra DSQ; Thermo Electron, Austin, TX, USA). The program for separation [SLB™ (5MS, Supelco, Bellefonte, PA, USA), 15 m x 0.25 mm; 0.25 µm film coating] was 70° C initial temperature (1 min), 30° C·min^{-1} to 180° C, then 5° C·min^{-1} to 310° C with He (constant flow 1.5 ml·min^{-1}) as carrier gas. The software XCALIBUR (Thermo Electron) was used for data acquisition. Cuticular substances were identified at the Universität Regensburg by GC-MS using a different GC-MS system (Agilent 6890N gas chromatograph coupled to an Agilent 5973 inert mass selective detector; J&W Scientific, St. Louis, MO, USA). The GC was equipped with an RH-5msþ capillary column (30 m x 0.25 mm x 0.25 mm; J&W Scientific) and the temperature profile was adjusted as described above. Helium was used as carrier gas with a constant flow of 1 ml min^{-1}. A split/splitless injector was used (250° C) with the purge valve opened after 60 sec. The electron impact mass spectra were recorded with an ionization voltage of 70 eV, a source temperature of 230° C and an interface temperature of 315° C. The MSD CHEMSTATION software (J&W Scientific) for Windows was used for data acquisition. To identify n-alkanes and alkenes, the mass spectra were compared with data from a commercial MS library (NIST, Gaithersburg, MD, USA) and methyl and dimethyl alkanes by diagnostic ions and standard MS databases (see above) and by determining Kovats indexes by the method of Carlson et al. (1998). Each peak on the chromatographs corresponds to a compound or a blend of co-eluted compounds with the same retention time. For the statistical analysis, the peak areas of the substances identified by GC-MS were used. The resulting peak areas were standardized to 100% for each individual. Data were transformed to compensate for the non-independence of data according to Reyment (1989). The number of variables was reduced by principal components analysis (PCA) and subsequently, data were analyzed by discriminant analysis (DA) using the

predefined groups 'Host Trees' using STATISTICA 8.0 as described in D'Ettorre and Heinze (2005) as well as in Ugelvig and co-workers (2008).

Principal components analysis is a multivariate statistical technique. Because the number of cuticular hydrocarbons is large and many of them are correlated with each other, principal components analysis was performed to reduce the number of variables to a smaller number of uncorrelated variables, the so-called 'Principal Components'. Each principal component is a linear combination of the original variables. The loading (or weight) of each variable determines its contribution to that principal component. The variability each principal component explains is highest in the first component extracted and decreases with each further component extracted. The discriminant analysis served for classifying individual ant workers into predefined groups, which were the host tree. Thus, discriminanat analysis was not employed for further data reduction but for classification purposes.

2.8 Direct sequencing of DNA regions

Polymerase chain reaction (PCR) and sequencing of gene fragments as well as the application of microsatellite primers was conducted at the Pritzker Laboratory for Molecular Systematics and Evolution in the Field Museum of Natural History in Chicago, IL, USA. Microsatellite primers were developed at the Department of Evolution, Behavior and Genetics, AG Heinze, Universität Regensburg, Germany.

Sequences from both the mitochondrial and the nuclear genome were included. A fragment covering most of the cytochrome oxidase one (mtCOI) gene was sequenced of the mitochondrion. Of the nuclear genome, both protein-coding genes and a ribosomal gene were included: a fragment of the large subunit (28S) ribosomal DNA gene, fragments of the protein-encoding genes abdominal-A (*abd*-A), wingless (*wg*) and long-wavelength rhodopsin (LW *Rh*). Primer sequences are given in Table 2.8.1.

PCR was carried out in 25 µl reaction volume consisting of 2.5 µl 10x PCR buffer (Roche Diagnostics), 3 µl dNTPs (Epicentre Technologies, Madison, WI, USA), 2.5 µl 10x Bovine Serum Albumin (BSA) (New England BioLabs, Ipswich, MA, USA), 1 µl *Taq* Polymerase (New England BioLabs), 1 µl of each primer (10 µM), 9 µl dH_2O and 5 µl of undiluted DNA isolate. Thermal cycling parameters were: initial denaturation for 5 min at 95° C followed by 34 cycles of 95° C for 1 min, 47° C

(mtCOI) or 54° C (nu genes) for 1 min, 72° C for 2 min and a final elongation for 10 min at 72° C, holding temperature was set at 4° C. Amplification products were viewed on 1% agarose gels stained with ethidium bromide and subsequently purified using GELASE enzyme (Epicentre Technologies).

Fragments were sequenced using the Big Dye Terminator reaction kit Version 3.1 (Applied Biosystems, Forster City, CA, USA). Sequencing and PCR amplification were performed using the same sets of primers. Cycle sequencing parameter were: initial denaturation for 1 min at 96° C followed by 32 cycles of 96° C for 15 sec, 50° C for 10 sec, 60° C for 4 min. Sequence products were precipitated with 10 µl sterile dH_2O, 2 µl of 3 M NaOAc and 50 µl of 95% ethanol before loading on an ABI 3730 (Applied Biosystems) automatic sequencer. Sixty-four of 159 sequences were generated in the Ward lab, according to the procedures given in Ward and Downie (2005).

Table 2.8.1 Primers used in the present study.
Positions correspond to those in the following GENBANK sequences: Apis, *Apis mellifera* mitochondrial genome (L06178) (Crozier & Crozier 1993); D. mel., *Drosophila melanogaster* (M21017); Myr., *Myrmica rubra* (AF332515); Phe., *Pheidole morrisi* (AY101369.1).

Primer	Sequence (5' to 3')	Position	Source
28S-3318F	CCCCCTGAATTTAAGCATAT	D. mel 3318-3337	(Schmitz & Moritz 1994)
28S-3706R	GGTTTACCCCTGAACGGTT	D. mel. 3706-3686	(Ward & Downie 2005)
28S-3665F	AGAGAGAGTTCAAGAGTACGTG	D. mel. 3665-3686	(Belshaw & Quicke 1997)
28S-4068R	TTGGTCCGTGTTTCAAGACGGG	D. mel. 4068-4047	(Belshaw & Quicke 1997)
Wg578F	TGCACNGTGAARACYTGCTGGATGCG	Phe. 578-603	(Ward & Downie 2005)
Wg1032R	ACYTCGCAGCACCARTGGAA	Phe. 1032-1013	(Abouheif & Wray 2002)
LR143F	GACAAAGTKCCACCRGARATGCT	Apis 143-165	(Ward & Downie 2005)
LR639ER	YTTACCGRTTCCATCCRAACA	Apis ~639-624	(Ward & Downie 2005)
AA1182F	CCGGCGATATGAGTACGAAATTC	Myr. 1182-1204	(Ward & Downie 2005)
AA1824R	TAGAAYTGTGCCGCCGCTGCCAT	Myr. 1824-1802	(Ward & Downie 2005)
COI-LCO1490	GGTCAACAAATCATAAAGATATTGG	Apis 1810-1834	(Folmer *et al.* 1994)
COI-HCO2198	TAAACTTCAGGGTGACCAAAAAATC	Apis 2518-2493	(Folmer *et al.* 1994)
COI-Ben3R	GCWACWACRTAATAKGTATCATG	Apis 2911-2889	(Brady *et al.* 2000)
COI-Ben4R	GCAATWACATARTARGTGTCATG	Apis 2911-2889	(Brady *et al.* 2000)
COI-Jerry	CAACATTTATTTTGATTTTTTGG	Apis 2481-2503	(Simon *et al.* 1994)
COI-Pat	TTCAATGCACTTATTCTGCCATATTA	Apis 3382-3357	(Simon *et al.* 1994)

2.8.1 Sequence alignment

ABI traces were assembled with SEQMAN 4.03 (DNAStar, Madison, WI, USA) and manually adjusted. Only unambiguous sequences without double peaks were included in the study. The identity of sequences was verified using BLAST search (Altschul *et al.* 1997). Sequences were aligned using CLUSTAL W (Thompson *et al.* 1994) as implemented in BIOEDIT 7.0.0 (Hall 1999). Alignment parameters were default. Gaps and length variants are not expected in coding mitochondrial genes as mtCOI and sequences with gaps in the corresponding alignment were considered to be a putative pseudogene and excluded from the analysis. The protein-encoding nuclear genes showed little variation in length with the exception of LW *Rh*, which possessed an intron. The intron was removed from the data set before analysis. The alignment of the 28S sequences was more difficult due to some hypervariable regions towards the end of the alignment, which was excluded before data analysis. In the combined data set, only specimens were included, of which all five gene fragments could be generated with the exception of *Pseudomyrmex perboscii*, of which *abd*-A is lacking and coded with 'N' in the alignment.

2.8.2 Phylogenetic analyses

For phylogenetic analyses, both a Bayesian approach and a Maximum Likelihood (ML) analysis were used. Bayesian methods allow efficient analysis of complex nucleotide substitution models in a parametric statistical framework (Huelsenbeck *et al.* 2001; Larget & Simon 1999) and include estimation of uncertainty (Huelsenbeck *et al.* 2000). Posterior probabilities and bootstrap support values generated from Maximum Likelihood analysis and Maximum Parsimony analyses have been demonstrated to differ (Alfaro *et al.* 2003; Simmons *et al.* 2004; Suzuki *et al.* 2002; Wilcox *et al.* 2002). Bayesian support values resulting from likelihood analyses appear to be overestimates in certain cases, especially when short branches are involved. In contrast, bootstrap values are commonly underestimates and can be viewed as helpful lower bounds of support values. Here, a conservative perspective was adopted and only clades having a posterior probability of at least 0.95 and bootstrap support equal to or above 75% were considered well-supported.

Congruence between the data sets was assessed by comparing bootstrap support of clades above 70% for each locus (Lutzoni *et al.* 2004).

2.8.3 Bayesian analyses

The Bayesian (B/MCMC) analyses were performed using MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001). Posterior probabilities were approximated by sampling the trees using a Markov chain Monte Carlo (MCMC) method. For all analyses the sequences were tested for the most appropriate model of DNA substitution analyses by the program MODELTEST version 3.7 (Posada & Crandall 1998) using LRT. Different models were determined as the most appropriate Maximum Likelihood model of evolution for the data sets: GTR+I+ Γ (mtCOI, 28S), HKY (*wg*), HKY+Γ (LW *Rh*) and GTR+ Γ (*abd*-A). In the combined analysis, the data set was partitioned into 13 parts (1^{st}, 2^{nd}, 3^{rd} codon positions of mtCOI, 1^{st}, 2^{nd}, 3^{rd} codon positions of *abd*-A, 1^{st}, 2^{nd}, 3^{rd} codon positions of LW *Rh*, 1^{st}, 2^{nd}, 3^{rd} codon positions of *wg* and 28S rDNA). For each of the 13 partitions, MRBAYES estimated the proportion of invariant sites, the gamma distribution shape parameter, base frequencies and the substitution rates (GTR model) or transition/transversion ratio (HKY model). Each partition was allowed to have its own model parameters as proposed by Nylander et al. (2004). No molecular clock was assumed. A run with 4,000,000 generations starting with a random tree and employing 12 simultaneous chains was executed. Every 100^{th} tree was saved into a file. The first 300,000 generations (i.e., the first 3000 trees) were deleted as the 'burn-in' of the chain. The log-likelihood scores of sample points were plotted against generation time using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id) to ensure that stationarity was reached after the first 300,000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist 2001). Of the remaining 74,000 trees (37,000 from each of the parallel runs) a majority rule consensus tree with average branch length was calculated using the 'sumt' option of MRBAYES. Posterior probabilities were obtained for each clade. Phylogenetic trees were drawn using TREEVIEW (Page 1996).

2.8.4 Maximum Likelihood analyses

The Maximum Likelihood analyses were performed with GARLI Version 0.951 (Zwickl 2006) employing the models as determined by MODELTEST. For the combined analysis, the model GTR+I+Γ was determined to fit the data best since running partitions is not possible using GARLI. Bootstrap support was based on 2,000 replications in the combined analysis and 1,000 in each single gene analysis.

2.8.5 Alternative hypothesis testing

To test whether the data are sufficient to reject a monophyly of *Pseudomyrmex* species with parasitic or mutualistic relationships to acacias, alternative hypothesis testing was employed. To test the hypothesis, the combined five gene fragments data set was analyzed using two different methods: First, the Shimodaira-Hasegawa (SH) test (Shimodaira & Hasegawa 1999) and second an expected likelihood weight (ELW) test following Strimmer & Rambaut (2002). The tests were performed using TREE-PUZZLE 5.2 (Schmidt *et al.* 2002) on a sample of 200 unique trees, the best trees agreeing with the null hypotheses and the unconstrained ML tree. These trees were inferred in TREE-PUZZLE employing the GTR+I+Γ nucleotide substitution model.

2.8.6 Ancestral states reconstruction 1

Mutualistic vs. non-mutualistic and invertase activity ancestral states within the acacia-ants were reconstructed based on the combined data set phylogeny. Three character states representing all possible associations among acacia-ants (0=generalists, 1=parasites and 2=mutualists) and two character states representing the possible invertase activity states (0=no invertase activity, 1=invertase activity) were considered potential ancestral states. Ancestral states were reconstructed with maximum likelihood as the optimality criterion (Pagel 1994) on 1000 trees sampled with B/MCMC (as described above) using the Trace Character Over Trees option in MESQUITE 0.995 (Maddison & Maddison 2007). Using a likelihood ratio test, the asymmetric two-parameter model was selected for this analysis. Only ancestral states reconstructed with raw likelihood scores greater than 2.0 (i.e., the default setting T=2.0 in MESQUITE), corresponding to a conservative approximation of

proportional likelihood values >0.95 in the current analysis, were considered to be significant following Edwards (1972).

2.8.7 Ancestral states reconstruction 2

To infer whether polygyny in *Pseudomyrmex peperi* is ancestral or derived within mutualistic acacia-ants of the *P. ferrugineus* group, an ancestral states reconstruction was employed (Pagel 1999b). All taxa from the *P. ferrugineus* group, of which sequence data are available from this study, were included. The colony structure of all *P. ferrugineus* group taxa is described in Ward (1993). Of the ten species that belong to this species group, seven could be included in the present study. Taxa from other species groups for which the colony structure is known based on own fieldwork were also included. Nine to 20 colonies of the species *P. gracilis*, *P. nigropilosus* (both *gracilis* group), *P. salvini* and the undescribed *P*. spec. PSW-06 were analyzed and more than one queen per nest hence concluding monogyny (data not shown) were never found. Monogyny and polygyny ancestral states among the taxa were reconstructed based a five gene fragments phylogeny (mtCOI, *wg*, LW *Rh*, *abd*-A, 28S rDNA, for a total of 3313 base pairs). Two character states representing the two possible colony forms (coded as 1=monogyny and 0=polygyny) were considered as the potential ancestral states. Ancestral states were reconstructed as described above.

2.9 Development of microsatellite primers

2.9.1 Extraction of DNA

Total genomic DNA for the development of microsatellite primers was extracted following a CTAB extraction protocol by Sambrook and Russell (2001). Six e-cups (each containing six ants of one colony) were cooled in liquid nitrogen and throughoutly disrupted with a micro pestle. Pre-warmed (65° C, 500 µl) CTAB solution (0.75 M NaCl, 50 mM Tris/HCl pH 8.0, 10 mM EDTA, 1% hexadecyltrimethyl-ammoniumbromid) was added and the sample was incubated at 65° C. After 1 h, 5 µl of Proteinase K (MBI Fermentas, St. Leon-Rot, Germany) were added and the mixture was incubated over night at 55° C. A chloroform:isoamyl alcohol (24:1) precipitation was conducted, followed by precipitation with 3 M NaAc, pH 5.2 and ice-cold isopropanol. Samples were incubated at -20° C for 1 h, centrifuged at 15,000 g for 15 min (Centrifuge 5417 R, Eppendorf, Hamburg, Germany) and then washed with ethanol. Genomic DNA of all 36 ants was dissolved and combined in 25 µl TE buffer (recipe 1). For testing and applying microsatellites, DNA was isolated from individual ants following the same protocol, but individual ants were dissolved in 40 µl TE buffer. Success of DNA isolation was confirmed by loading 1 µl of genomic DNA onto a 0.8% TBE (recipe 2) agarose gel (recipe 3; Fig. 2.9.1).

Recipe 1: TE buffer
10 mM Tris-HCl, pH 8.0
1 mM EDTA
autoclave

Recipe 2: TBE buffer (10x) solution
Tris base 107.81 g l^{-1} (0.89 M); autoclave
EDTA 5.84 g l^{-1} (0.02 M) pH 8.0; autoclave
Boric acid 55.0 g l^{-1} (0.89 M)

Recipe 3: agarose gel

50 ml 1x TBE buffer

0.8 g agarose

microwave, allow to cool and then add

5 µl ethidium bromide

pour into gel tray

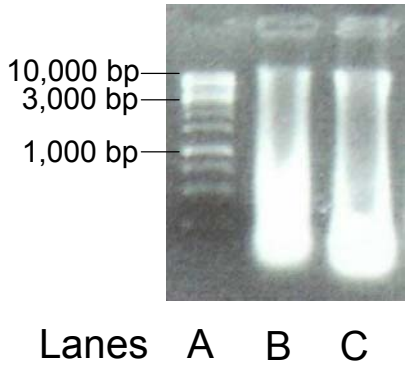


Fig. 2.9.1 Extracted DNA.
Extracted DNA (1 µl) was loaded onto an agarose gel (0.8% TBE) to view whether DNA extraction was successful. Three lanes are shown in this picture. Lane A: 5 µl of ladder (1 kb; MBI Fermentas) were used as size standard, fragments with size of 1 kb, 3 kb and 10 kb are indicated. Lane B: 1 µl of extracted DNA derived from 36 *Pseudomyrmex ferrugineus* workers. Lane C: 1 µl of extracted DNA derived from 36 *P. gracilis* workers. Photo by S. Kautz.

2.9.2 Restriction with Tsp509I

DNA was restricted using the Tsp509I (10 U μl^{-1}) restriction enzyme (New England Biolabs). The reaction consisted of 25 µl genomic DNA (ca. 50-100 µg), 10 µl restriction enzyme, 15 µl restriction buffer (10x Tsp-buffer; New England Biolabs) and filled up with PCR-water to 150 µl. After 5 h of incubation at 65° C, the restricted product was viewed on a 1.5 % TBE agarose gel (Fig. 2.9.2). Fragments ranging from 150 to 400 bp in length were expected. The restricted products were cleaned using phenol:chloroform:isoamyl alcohol (25:24:1), followed by precipitation with 3 M NaAc (10^{-1} of reaction volume) and ethanol and washed with ethanol before resuspension in 25 µl PCR-water.

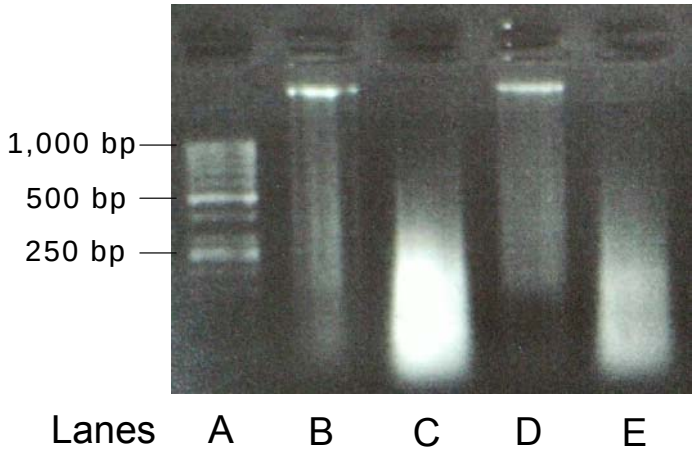


Fig. 2.9.2 Restricted DNA.
Restricted DNA and extracted DNA (1 µl each) were loaded onto an agarose gel (1.5% TBE) to view whether DNA restriction and extraction of individual workers were successful. Five lanes are shown in this picture. Lane A: 5 µl of ladder (50 bp; MBI Fermentas) were used as size standard, fragments with size of 250 bp, 500 bp and 1000 bp are indicated. Lane B: 1 µl of extracted DNA derived from one *Pseudomyrmex ferrugineus* worker. Lane C: 1 µl of restricted DNA derived from 36 *P. ferrugineus* workers. Lane D: 1 µl of extracted DNA derived from one *Pseudomyrmex gracilis* worker. Lane E: 1 µl of restricted DNA derived from 36 *P. gracilis* workers. Photo by S. Kautz.

2.9.3 Ligation of adaptors

Adaptors were prepared immediately before ligation to the restricted product. Preparation was carried out in a total volume of 50 µl containing 39.6 µl TE buffer (pH 8.0), 0.4 µl NaCl (5 M), 5.0 µl Tsp adaptor short and 5.0 µl Tsp adaptor long [500 µM each, MWG Biotech, Ebersberg, Germany; Tsp AD short and Tsp AD long (Tenzer *et al.* 1999)]. A thermotreatment was applied using a thermocycler (Whatman-Biometra, Göttingen, Germany). Conditions were 3 min at 95° C, 2 min at 65° C, 2 min at 45° C and 2 min at 25° C. Holding temperature was set at 4° C.

Adaptor ligation was conducted at 16° C for 14 h in a thermocycler. The reaction contained 10 µl 10x ligase buffer (New England Biolabs), 7 µl T4 DNA ligase (New England Biolabs, 5 U μl^{-1}), 10 µl of the adaptor that was prepared immediately before, 12 µl of the restricted DNA and filled up to 100 µl with PCR-water. After adaptor ligation, the product was purified using millipore filters (Ultrafree-4 spinning columns, Millipore, Billerica, MA, USA). The purified product was used for the consecutive steps.

2.9.4 Pre-selective PCR

A pre-selective PCR was carried out to amplify the restricted and ligated DNA fragments. The purified product (s.o.) was diluted (1:10) with PCR-water and 32 reactions were carried out in a total reaction volume of 25 µl. Each reaction contained 2.5 µl of 10x *Taq* buffer (containing 100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% Noidet P40; MBI Fermentas), 2.5 µl of dNTPs (each 2.5 mM; MBI Fermentas), 2.5 µl $MgCl_2$ (25 mM; MBI Fermentas), Tsp AD short as primer (10 µM; MWG Biotech), 0.25 µl *Taq* (5 U μl^{-1}; MBI Fermentas), 9.75 µl of PCR-water and 5.0 µl of the diluted (1:10) product. Amplification consisted of 20 cycles of 1 min at 93° C, 1 min at 55° C and 1 min at 72° C, preceded by 5 min at 72° C. Holding temperature was set at 4° C. To check for success of amplification, 5 µl of every 8^{th} sample (4 samples in total) were loaded onto a 1.5% TBE agarose gel. A DNA 'smear' ranging from 150 to 400 bp was expected and could be observed (Fig. 2.9.3). PCR-products of 32 samples were pooled and purified using millipore filters.

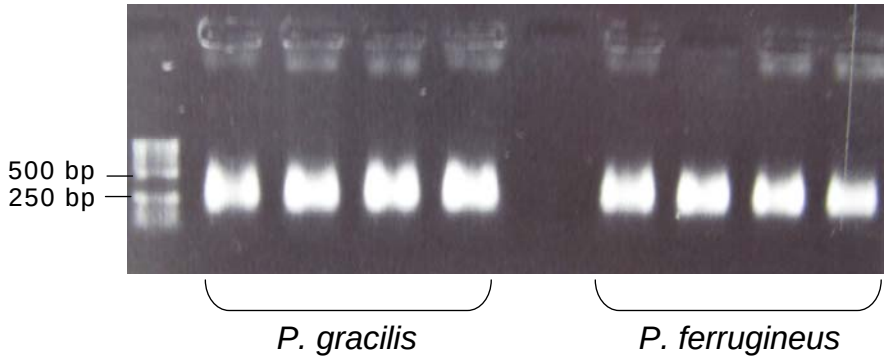


Fig. 2.9.3 Products after pre-selective PCR.
After pre-selective PCR, products were loaded onto an agarose gel (1.5% TBE) to view whether amplification was successful. In the first lane, 5 µl of ladder (50 bp; MBI Fermentas) were used as size standard, fragments with size of 250 bp and 500 bp are indicated. Four lanes show the products derived from amplification of restricted *P. gracilis* DNA, while the other four lanes show the products derived from amplification of restricted *P. ferrugineus* DNA. Photo by S. Kautz.

2.9.5 Ligation of magnetic beads to oligo repeats

In the present study, I exclusively screened for GA repeats in the genome of *Pseudomyrmex ferrugineus*, since they seem to be the most common repetitive motives in ants (A. Trindl, pers. comm.). Magnetic beads (1 mg in 100 µl, DYNABEADS M-280, Steptavidine; Dynal, Oslo) were washed three times with an equal amount

(100 µl) of PBS (pH 7.4) containing 0.1% BSA (recipes 4 and 5), washed once with 1x BW buffer (recipes 6 and 7) and re-suspended in 100 µl 2x BW buffer (recipe 6). All washing steps were conducted using a magnetic block (Magnetic particle concentrator, MPC-S, Dynal; Fig. 2.9.4). To enrich repeat motifs, $(GA)_{13}$ biotinilated probes were linked to streptavidin-coated magnetic beads by incubation at RT for 1 h under rotation in a hybridization oven (HB-1000 Hybridizer; peQLab, Erlangen, Germany).

Recipe 4: PBS buffer (10x)

80 g NaCl
2 g KCl
14.4 g $Na_2HPO_4 * 2H_2O$
2 g KH_2PO_4
pH 7.4
add to 500 ml with ddH_2O, autoclave and add evaporated water

Recipe 5: PBS with BSA

PBS (1:10 dilution with ddH_2O)	1980 µl
BSA (10 µg $µl^{-1}$)	20 µl
total	2000 µl

Recipe 6: 2x BW buffer

1 M Tris-HCl (pH 7.5)	20 µl
0.5 M EDTA (pH 8.0)	4 µl
5 M NaCl	800 µl
ddH_20	1176 µl
total	2000 µl

Recipe 7: 1x BW buffer

2x BW buffer 1:2 dilution (1000 µl BW buffer + 1000 µl ddH_2O)

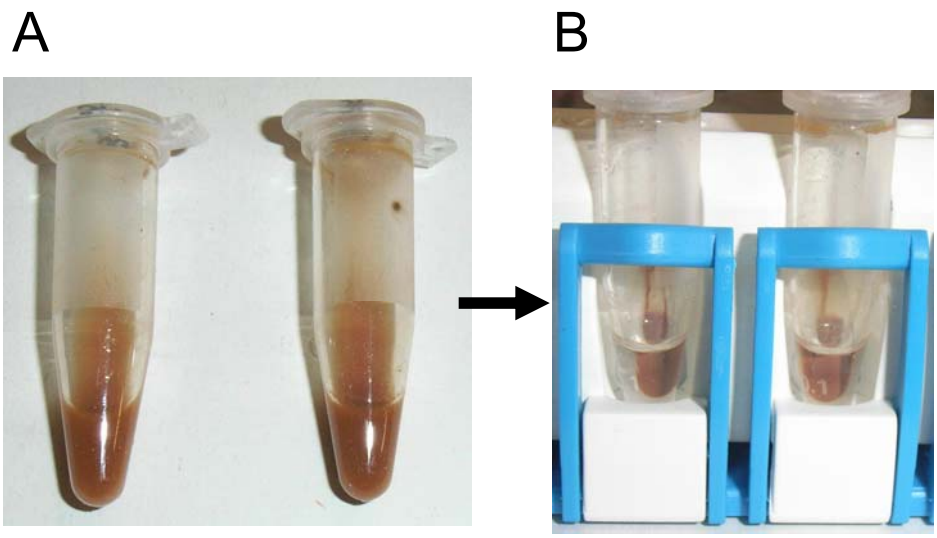


Fig. 2.9.4 Magnetic bead solution and magnetic block.
Magnetic beads were washed several times using different buffer solutions. After each solution had been added, the tubes containing magnetic beads (A) were placed into the magnetic block (B). In this block, the magnetic beads were pulled towards the wall of the tubes and the solution could be removed without removing the magnetic beads. Photo courtesy of V. Schmid.

2.9.6 Hybridization of magnetic beads with DNA

Beads that were ligated to biotinilated $(GA)_{13}$ oligo repeats, were washed twice with 200 µl 1x BW buffer, once with 200 µl 5x SSC/0.1% SDS (recipes 8-10) and dissolved in 150 µl pre-warmed (65° C) 10x SSC/0.2% SDS solution. The mix was kept in a heat block (65° C). In the meantime, 90 µl of the PCR-product were combined with 60 µl of ddH_2O and denatured for 5 min at 95° C using a heat block. To the denatured DNA, 150 µl of bead solution were added. After incubation at 65° C for 5 h while rotating in a hybridization oven, the reaction was washed twice for 5 min at RT with 400 µl 2x SSC/0.1% SDS and once for 10 min at 65° C (pre-warmed solution and magnetic block) in 400 µl 2x SSC/0.1% SDS and re-dissolved in 200 µl TE buffer. This procedure served to fish for GA repeats in the genome of *P. ferrugineus.* The solution was viewed on a 1.5 % agarose gel (Fig. 2.9.5) to test whether DNA was still present. Since this was the case, the procedure was continued.

Recipe 8: 20*SSC

175.3 g NaCl

88.2 sodiumcitrate (100.51 g if sodiumcitrate dihydrate: 0.34 M)

add to 1 l with ddH_2O

pH 7.0 (adjust with NaOH)

Recipe 9: 10% SDS

5 g SDS add to 50 ml with ddH_2O; do not autoclave

microwave to dissolve

Recipe 10: SSC and SDS

10*SSC/0.2% SDS (5 ml 10x SSC + 100 µl 10% SDS)

5*SSC/0.1% SDS (5 ml 5x SSC + 50 µl 10% SDS)

2*SSC/0.1% SDS (5 ml 2x SSC + 50 µl 10% SDS)

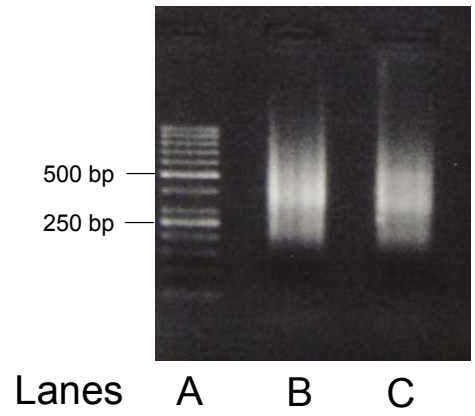


Fig. 2.9.5 Bead solution loaded onto an agarose gel.
The solution containing magnetic beads hybridized with DNA was viewed on a 1.5% TBE agarose gel to test whether hybridization was successful. Lane A: 5 µl of ladder (50 bp; MBI Fermentas) were used as size standard, fragments with size of 250 bp and 500 bp are indicated. Lane B shows the bead solution hybridized to *P. gracilis*. Lane C shows the bead solution hybridized to *P. ferrugineus*. Photo by S. Kautz.

2.9.7 Selective PCR

A selective PCR was carried out using the GA enriched DNA fraction as template. Two 25 µl samples were prepared containing 2.5 µl 10x *Taq* buffer, 2.5 µl of dNTPs (each 2.5 mM; MBI Fermentas), 2.5 µl $MgCl_2$ (25 mM; MBI Fermentas), Tsp AD short as primer (10 µM), 0.25 µl *Taq* (5 U μl^{-1}; MBI Fermentas), 13.75 µl of PCR-water and

1.0 µl of the DNA ligated to beads (GA enriched DNA fraction). Amplification consisted of 30 cycles of 30 sec at 95° C, 30 sec at 55° C and 30 min at 72° C, preceded by an initial denaturation of 5 min at 95° C, followed by a final elongation step of 10 min at 72° C. Holding temperature was set at 4° C. Successful amplification of fragments ranging from 150 to 400 bp was viewed on a 1.5 % TBE agarose gel.

2.9.8 Cloning

A library of the GA enriched DNA fraction of *P. ferrugineus* was established using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). For ligation, the PCR-product was used immediately after selective PCR. If PCR-products were older, the 3'-A overhang on the PCR-product would degrade and ligation would be less efficient. All steps were conducted on ice. The ligation mix contained 1 µl 10x ligation buffer, 2 µl of vector, 0.5 µl of T4 DNA ligase (all Invitrogen) and water up to a final volume of 10 µl. Three concentrations of PCR-product (1.0 µl, 2.0 µl and 3 µl) and one negative control containing water only were included to optimize the ligation reaction. The PCR-product was introduced into the ligation mix and gently mixed with a pipette tip. The ligation mix was incubated at 14° C for 14 h using a thermocycler.

Transformation of the ligated product was conducted using the *E. coli* strand XL-1-Blue kindly provided by the AG Schneuwly (Entwicklungsbiologie, Universität Regensburg). The ligation product (1 µl and 3 µl of each ligation product) was added to the competent cells and mixed gently with the pipette tip. After incubation on ice for 20 min, the cells were heat-shocked in a heat block for 30 sec at 42° C and were placed on ice immediately for 5 min. 400 µl of LB media (without ampicillin, see recipe 11) were added using sterile technique and cells were incubated shaking for 1 h in the hybridization oven at 37° C. X-Gal (40 µl, 40 mg ml^{-1} DMF, Epicentre Technologies) was spread on agar plates (recipe 12) and allowed to dry. Consecutively, the transformation product was spread on the agar-plates using sterile technique. Two replicates per sample with 10 µl and 90 µl of bacterial solution, respectively, were prepared. The plates were incubated over night at 37° C followed by incubation at 4° C for 2 h.

Recipe 11: Liquid LB (Luria-Bertani) medium (with and without ampicillin)
5 g tryptone
2.5 g yeast extract
5 g NaCl

The medium was filled up to 500 ml with ddH_2O, pH was adjusted to 7.0 using HCl. After autoclaving the media, 50 ml were poured into an extra tube. The remaining solution was allowed to cool down to 50° C before 450 µl of ampicillin (100 mg ml^{-1}) were added. The media contained a final concentration of 100 µg ml^{-1} ampicillin.

Recipe 12: Agar plates LB medium with ampicillin (for ca. 20 plates)
5 g tryptone
2.5 g yeast extract
5 g NaCl
7.5 g agar

The medium was filled up to 500 ml with double distilled water, pH was adjusted to 7.0 with HCl. After autoclaving the medium was allowed to cool down to 50° C and then 500 µl of ampicillin (100 mg ml^{-1}) were added. The medium contained a final concentration of 100 µg ml^{-1} ampicillin.

2.9.9 Blue and white screening and colony picking

Micro titer plates (96-well) were prepared. Each slot was filled with 150 µl LB ampicillin solution containing 30% glycerol. White colonies were picked with a yellow pipette tip each and transferred into one slot of the micro titer plate. The plate was incubated for 1 h at 37° C while shaking (Certomat-R, Braun Biotech international, Melsungen, Germany; 210 rpm) to allow bacterial growth. A second micro titer plate was prepared for replica with 150 µl of LB ampicillin (no glycerol) into each slot. A sterilized hedgehog was placed in the LB ampicillin glycerol plate and then transferred into the LB ampicillin plate for inoculation. The LB ampicillin plate was incubated over night at 37° C (210 rpm) and afterwards stored at 4° C until further needed. The original LB ampicillin glycerol plate was stored at -70° C in the freezer.

2.9.10 Dot blotting

A dot blotting procedure was performed to verify the presence of GA repeats in individual clones. The Bio Dot SF apparatus (BioRad, Hercules, CA, USA) was used for hybridization. A whole PCR micro titer plate (96 samples) was prepared. Two-hundred microliters of 0.4 M NaOH containing 10 mM l^{-1} EDTA were pipetted into each well and 10 µl of the sample from the LB ampicillin micro titer plate were added. The plate was incubated at 95° C for 10 min (thermocycler) for denaturation of DNA followed by incubation on ice for some minutes (boiling prep).

The membrane (Hybond^{TM}-N^{+} membrane; GE Healthcare, UK) was pre-wetted in 6x SSC and one of three filter papers was pre-wetted in PCR-water. The Bio Dot SF apparatus was assembled according to the manuals instructions (gasket support plate, sealing gasket, three layers of filter paper, membrane). A vacuum was applied and then the screws that hold the apparatus together were re-tightened. The membrane was re-hydrated by pipetting 400 µl sterile water into each dot slot. Then, 150 µl of the denatured DNA were added by pipetting the solution into the center of the slot. The sample was pulled through by applying vacuum. After the sample was filtered through, 300 µl of 0.4 M NaOH were added to each well and a vacuum was applied. Consecutively, 300 µl of 2x SSC were added and a vacuum was applied. Afterwards, the Bio Dot SF apparatus was disassembled, the blotted membrane was removed and rinsed with 2x SSC. The membrane was placed onto a paper towel and incubated at 80° C for 1 h (oven). Membrane were stored in plastic wrap at 4° C.

2.9.11 Hybridization of dot blots

Hybridization buffer, primary wash buffer and secondary wash buffer (recipes 13-15) were prepared. The blotted membranes were placed into hybridization tubes and 15 ml hybridization buffer were added to each tube. Pre-hybridization was conducted at 60° C for 1 h. Fluorescin labeled oligo nucleotide probes (15 µl of a 100 µM stock solution, MWG Biotech) were added to the hybridization buffer and incubation was conducted over night. Membranes were washed the next day. The hybridization buffer was removed and 50 ml of primary wash buffer were added to each tube. After rotating for 5 min at RT, primary buffer was removed, the washing step was repeated and the buffer was discarded. Consecutively, pre-warmed (60° C) secondary wash

buffer was added and membranes were incubated for 15 min at 60° C. Afterwards, secondary wash buffer was discarded.

Recipe 13: Hybridization buffer
0.05 g hybridization buffer component (GE Healthcare)
0.01 g SDS
2.5 ml liquid block (GE Healthcare)
add to 50 ml using 5x SSC

Recipe 14: Primary wash buffer (5x SSC /0.1% SDS); 200 ml
50 ml 20x SSC
148 ml H_2O
2 ml 10% SDS
add to 200 ml using ddH_2O

Recipe 15: Secondary wash buffer (1x SSC/0.1% SDS); 200 ml
10 ml 20x SSC
188 ml H_2O
2 ml 10% SDS
add to 200 ml using ddH_2O

2.9.12 Membrane blocking and detection

The membranes were incubated in liquid block (RPN 3601, GE Healthcare) and buffer A (recipes 16 and 17) in the ratio 1:10 for 90 min at RT (rotation). Consecutively, membranes were incubated with anti-fluorescin-AP solution (recipe 18) for 1 h while rotating. Three washing steps with Tween solution (recipe 19) were performed for 10 min, respectively. Excess wash buffer was drained off and membranes were placed on a sheet of tough plastic foil with the sample site down. Detection reagent (CPD-Star Detection Reagent, RPN 3682, GE Healthcare) was pipetted onto blots and incubation took place for 1 min. A peace of carton in the size of the membranes was wrapped in plastic foil; each membrane was placed on one peace of wrapped carton, wrapped in plastic foil again and placed in a film cassette with the sample site up. In the dark room, a sheet of Hyperfilm-MP (Amersham

Hyperfilm ECL, GE Healthcare) was placed on top of the membrane, the cassette was closed and the film was exposed to the membranes for 3 sec. The film was developed: 2 min in developing solution, rinsing in water, 2 min in fixation solution, throughout washing with water and let dry. The positive reaction of 247 out of 355 dots could be confirmed (Fig. 2.9.6).

Recipe 16: buffer A

100 mM Tris-HCl

300 mM NaCl, pH 9.5

Recipe 17: buffer A/ liquid block

69.3 ml buffer A

7.7 ml liquid block

Recipe 17: AP-Mix

12 µl Anti-fluorescin AP (11 426 338 910, Roche Diagnostics)

54 ml buffer A

270 µg BSA

Recipe 18: buffer A/Tween 20

100 ml buffer A

0.3 ml Tween 20 (20605, USB)

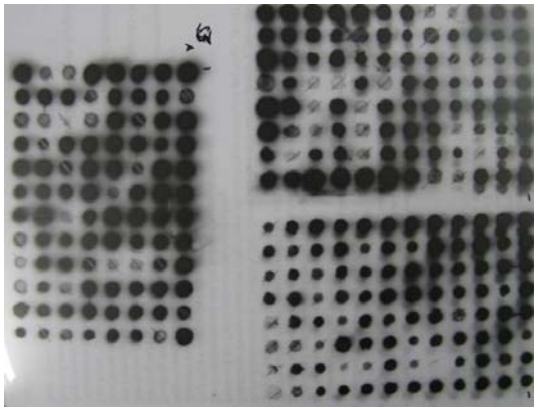


Fig. 2.9.6 Developed film with dot blots.
A positive reaction (indicated by dark dots) of 247 out of 355 dots could be confirmed for *P. ferrugineus* (a total of 288 dots are shown here). Photo by S. Kautz.

2.9.13 Preparation of plasmids

Positive clones were selected and 15 µl of the bacterial solution from the micro titer plates without glycerol were transferred into 15 ml screw cap tubes filled with 3 ml of LB ampicillin media. Samples were grown over night at 37° C while shaking. The Nucleo Spin Plasmid kit (Macherey & Nagel, Düren, Germany) was used for plasmid preparation. Of each sample, two 1.5 ml aliquots were prepared and centrifuged for 30 sec at 11.000 g. The two bacterial pellets of each sample were combined in 250 ml A1 buffer containing RNAse (supplied). All procedures were conducted following the manual's instructions and the additional wash step using buffer AW (supplied) was included. The final elution was conducted with 50 µl PCR-water instead of elution buffer. Plasmid purification was viewed on 0.8% TBE agarose gels (Fig. 2.9.7). Plasmids were stored at 4° C until further use.

2.9.14 Cycle sequencing

A modified and automated dideoxynucleotide chain-termination method (Sanger *et al.* 1977) was employed during cycle-sequencing. Fragments were sequenced on a thermocycler using the Big Dye Terminator reaction kit version 1.1 (Applied Biosystems). PCR-Products were sequenced with the primers M13reverse and T7 (Invitrogen). The final sequencing volume was 20 µl consisting of 3.0 µl BigDye buffer, 2.0 µl BigDye Terminator Version 1.1, 1.0 µl primer (10 µM) and 3.0 µl of the prepared plasmid product. The utilized cycle sequencing program consisted of 30 cycles of 96 for 10 sec, 50° C (M13reverse) or 55° C (T7) for 8 sec and 4 min at 60° C. BigDye contains fluorescing dNTPs and should not be exposed to light. To prevent damage, tubes and racks were wrapped in aluminum foil and kept on ice.

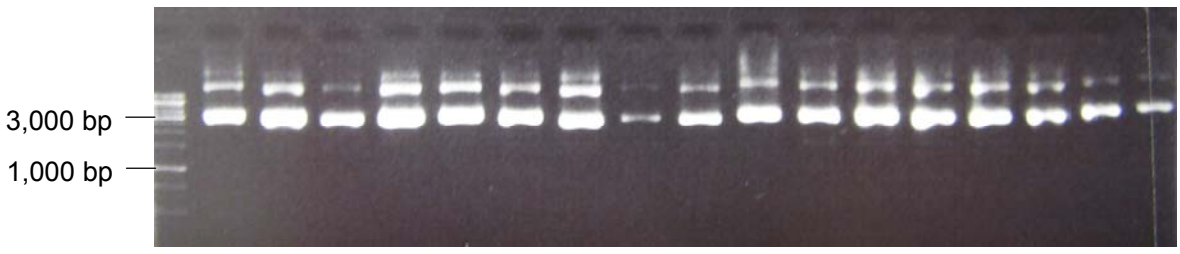


Fig. 2.9.7 Purified plasmids loaded onto an agarose gel.
Plasmid purification was viewed on 0.8% TBE agarose gels. Ladder (1 kb; MBI Fermentas) was used as size standard. Photo by S. Kautz.

2.9.15 Precipitation

In order to remove excess fluorescing dideoxynucleotides the cycle sequencing products have to be purified. In the current study cycle sequencing products were precipitated using NaOAc (3 M, pH 4.8) and ethanol. To each sample 10 µl of 3M NaOAc and 80 µl of water were added and gently mixed. Consecutively, 250 µl of ethanol (100% RT, p.A.) were added and the solution was mixed. E-cups were centrifuged at 14,000 g for 15 min at RT. Immediately after centrifugation the supernatant was discarded carefully not to disturb the pellet that was formed on the bottom. To each cup 100 µl of 70% ethanol were added. Cups were inverted a few times for mixing before centrifugation at 14,000 g for 5 min (RT). E-cups were spun at 2,000-3,000 g for 15 min at 4° C. Ethanol was removed carefully and samples were incubated at 50° C until ethanol had evaporated completely. Samples were re-suspended in 20 µl PCR-water.

2.9.16 Electropherograms and primer development

Purified products were loaded on an ABI Prism 310 Genetic Analyzer used with a 310 Genetic Analyses Capillary 47 cm and POP4-Polymer (Applied Biosystems). The fluorescent labeled samples are injected into glass capillaries that are filled with a stationary polymer. When a voltage is applied the DNA fragments that carry a negative charge migrate towards the other end of the capillaries. Shorter fragments migrate faster than longer ones. At the end of the capillaries, the fragments are separated according to their length. By a moving polymer that flows over the end of the capillaries, they enter an analyzing glass cuvette. In this glass cuvette, a laser activates the labels and causes the dye molecules to fluoresce. Fluorescent radiation is detected by a charged coupled device camera and converted into an electronic signal, which is transferred into a computer workstation. The result is an electropherogram, in which each peak represents a single nucleotide. Each color corresponds to one of the four bases. Sequences were assembled and edited in SEQUENCING ANALYSIS 3.4.1 (Applied Biosystems) and visually checked for microsatellites. Repeat motifs were identified in 87 clones and primers flanking the core microsatellite repeats were designed and tested for 44 loci. Primers were designed manually in cases, where repeat motifs were homogenous and where the

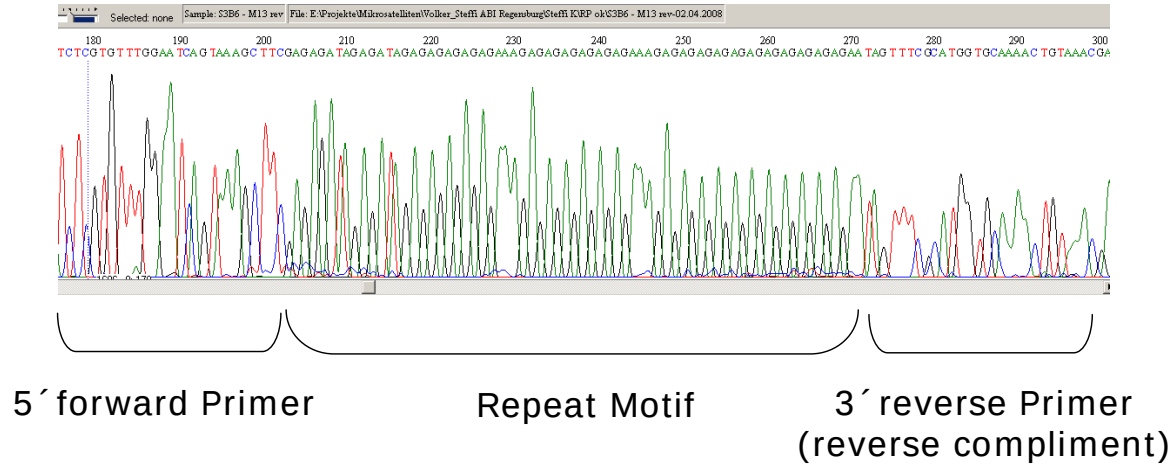


Fig. 2.9.8 Repeat motif.
Purified plasmids were sequenced and loaded onto an ABI Prism 310 Genetic Analyzer. Sequences were viewed using the program SEQUENCING ANALYSIS 3.4.1 and visually checked for repeat motifs. In the displayed example, a (GA) repeat motif is detected. The repeat motif is $(GA)_3TA(GA)_2TA(GA)_6AA(GA)_7AA(GA)_{12}$. The flanking regions allowed for primer design. The reverse primer has to be designed in the reverse complement of the sequence that is displayed.

flanking regions allowed primer design. Criteria for primer development were balanced proportion of all four bases, a size ranging from 16 to 24 bases and not more than 50% GC content in the primer region (Fig. 2.9.8). Primers were ordered from MWG Biotech.

2.9.17 PCR amplification

For all primers, PCR amplification was performed in 20 µl reactions containing 1-50 ng DNA template, 0.5 U *Taq* polymerase (MBI Fermentas), 0.5 µM of each forward and reverse primer (MWG Biotech), 1x *Taq* buffer (MBI Fermentas, content see above), 1x Enhancer (peQLab), 2 mM $MgCl_2$, 250 µM of each dNTP (MBI Fermentas) using a T-Gradient thermocycler (Whatman-Biometra). Cycling conditions were 4 min at 94° C, 35 cycles of 1 min 15 sec at 95° C, 1 min at 45° C to 60° C (using a gradient), 45 sec at 72° C and a final extension of 10 min at 72° C. In cases of successful PCR with four individuals (Fig. 2.9.9), the PCR was repeated with the forward primer 5'-labeled with 6-FAM, TET or HEX (MWG Biotech). The labeled products were diluted with water, mixed with Genescan-500 (Tamra) size standard and scored on an ABI Prism 310 Genetic Analyzer used with a 310 Genetic Analyses Capillary 47 cm and POP4-Polymer. Loci were genotyped using GENESCAN® 3.1 (Applied Biosystems) (Fig. 2.9.10). To assess variability of the

microsatellites, DNA was extracted from individual ants from two different populations, located close to Puerto Escondido at the Pacific coast in South Mexico (15°55´ N and 097°09´ W) and close to Matias Romero in the Isthmus of Tehuantepec (17°06´ N and 094°55´ W). Each primer pair was tested on 22 individuals of *P. ferrugineus* with each eleven individuals derived from eleven colonies per population (i.e., one individual per colony) and on 20 individuals (three colonies) of *P. mixtecus* and 24 (five colonies) of *P. peperi*, respectively. For the latter two species, only samples from Puerto Escondido were tested. In cases of failure of PCR amplification, doubling DNA content always led to successful PCR. In one case (population Puerto Escondido, locus Psfe19), DNA ran out.

The development of microsatellite primers was conducted in collaboration with V. Schmid (Universität Regensburg). Primers for *P. ferrugineus* are published in Kautz and *et al*. (2009b). Primers for *P. gracilis* were developed using the same procedures as described for *P. ferrugineus* and are published in Schmid *et al* (2009).

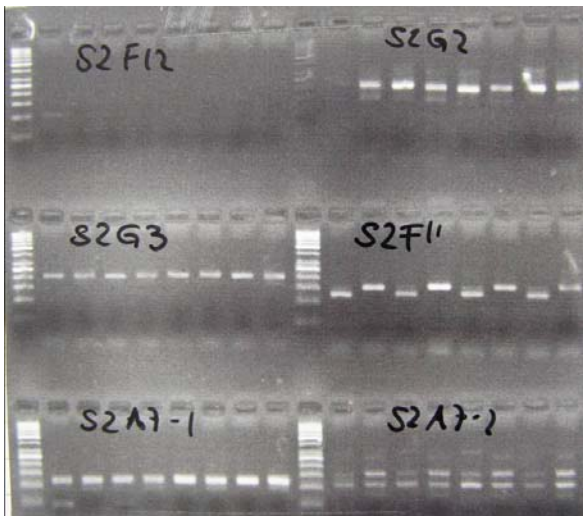


Fig. 2.9.9 PCR-products using microsatellite primers.
PCR-products were amplified using microsatellite primers developed in this study. Each new primer pair was tested on two individuals using a temperature gradient with four different temperatures (45° C, 50° C, 55° C, 60° C; from left to right for each primer pair). Products were viewed on 0.8% TBE agarose gel and a 50 bp ladder (MBI Fermentas) was used as size standard. On the agarose gel shown here, six different primer pairs were checked. Primer pair S2F12 did not yield any product, while all other primer pairs amplified successfully. However, primer pair S2A7-2 led to amplification of more than two bands indicating unspecific annealing and was not considered in further steps. Primer pair S2F11 showed polymorphism of the amplified products between the two individuals tested here. Photo by S. Kautz.

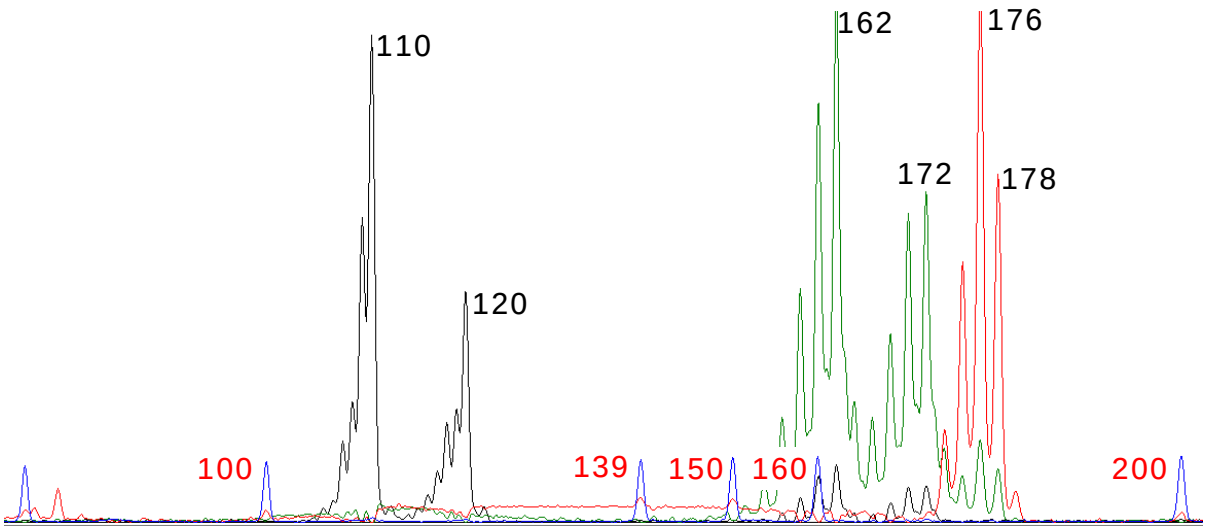


Fig. 2.9.10 Labelled PCR-products scored on an ABI Prism 310 Genetic Analyzer.
Labelled PCR-products were mixed with Genescan-500 (Tamra, displayed in blue) size standard and scored on an ABI Prism 310 Genetic Analyzer. In the run displayed here, three labels were combined: 6-FAM (red), TET (green) and HEX (black). The sizes of the respective products were calibrated using the size standard and are displayed in this picture.

2.10 Application of microsatellite primers

2.10.1 DNA isolation

Aim of this study was to compare the pattern of variation of the cuticular profile with that of neutral genetic markers for the worker force of each acacia and not to investigate a strict correspondence at the individual level. Thus, different individuals were used for cuticular hydrocarbon analysis, for behavioral trials and for DNA isolation. DNA for microsatellite analyses of the mutualist *Pseudomyrmex ferrugineus* and the parasite *P. gracilis* was isolated from six workers of each of 32 colonies totaling 192 workers. DNA for microsatellite analyses of the polygynous acacia-mutualist *P. peperi* was isolated from 76 workers, 75 males, 37 virgin queens and 80 queens derived from the two supercolonies mentioned above following a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Sambrook & Russell 2001) or using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following protocol B for insects. Gasters of queens were discarded before DNA extraction. The final elution of DNA was performed with sterile water instead of the AE buffer. PCR was carried out in 10 µl reactions consisting of 1.0 µl 10x PCR buffer (Roche Diagnostics), 0.6 µl dNTPs (Epicentre Technologies), 2.0 µl 10x BSA (New England BioLabs), 0.1 µl *Taq* Polymerase (Roche Diagnostics), 0.4 µl of each primer (10 µM), 3.5 µl dH_2O and

2.0 µl of undiluted DNA isolate. Thermal cycling parameters were: initial denaturation for 4 min at 94° C followed by 34 cycles of 94° C for 45 sec, 55° C for 30 sec, 72° C for 45 sec and a final elongation for 10 min at 72° C, holding temperature was set at 4° C. The forward primer was 5'-labelled with 5-Fam (blue), Vic (green), Ned (yellow), or Pet (red) and four colors were combined in each run. Samples were scored on an ABI 3730 with 9.7 µl HiDi formamide and 0.3 µl LIZ 500 ladder (Applied Biosystems) and 0.6 µl of each of the four products. Loci were genotyped using the GENEMAPPER 3.7 software (Applied Biosystems). For *P. ferrugineus*, the twelve primer pairs Psfe06-Psfe08 and Psfe13-Psfe21 were employed, while eleven primer pairs were used for *P. gracilis* (Psgr03-Psgr07 and Psgr09-Psgr12). The eight loci Psfe14-Psfe21 were used for genetic analyses of *P. peperi*.

2.10.2 Genetic analyses

Null alleles was tested for using MICROCHECKER (van Oosterhout *et al.* 2004), which uses a Monte Carlo simulation method to estimate deviations from expected homozygotes and heterozygotes based on the Hardy-Weinberg theory of equilibrium and the frequency of any null alleles. However, since inbreeding would also lead to an excess of homozygotes and violate the Hardy-Weinberg equilibrium, results obtained from males were also used to test for null alleles. If there were null alleles present, they would be blank in males, since they are haploid. The number of alleles, allele frequencies, observed heterozygosity and expected heterozygosity at each microsatellite locus for each plot of *Pseudomyrmex ferrugineus* and *P. gracilis* as well as for both supercolonies of *P. peperi* were calculated using the online version of the GENEPOP software (Raymond & Rousset 1995). Genotype proportions in the two populations were tested for conformity to Hardy-Weinberg expectations (HWE) using exact tests as implemented in GENEPOP. Input files were converted using CONVERT (Glaubitz 2004).

Analysis of molecular variance (AMOVA) as implemented in ARLEQUIN 3.1 (Excoffier *et al.* 2005) was used to describe population genetic structure. This test partitions the total genetic variance and calculates fixation indices for each level of variance (Excoffier *et al.* 1992). In the study of *Pseudomyrmex ferrugineus* and *P. gracilis*, two hierarchical levels (among workers of one acacia as well as between workers of the eight acacias in each plot) were defined to test for genetic structure.

For this test in *P. peperi*, all female genotypes of each supercolony were combined, i.e., queens and female progeny. The distribution of genetic variation was examined at four hierarchical levels: among supercolonies, among sub-samples (individuals derived from one acacia shoot) within supercolonies, among individuals within sub-samples (i.e., among individuals derived from one acacia shoot), among individuals within sub-samples (individuals derived from one acacia shoot) and among all individuals.

Parentage analysis were conducted to estimate the number of sib groups within each plot of *Pseudomyrmex ferrugineus* and *P. gracilis* based on maximum likelihood as implemented in COLONY version 1.2 (Wang 2004). This approach uses group likelihood ratios based on multilocus gene arrays to partition individuals into full-sib and half-sib families for haplodiploid species. Without prior knowledge of the rate of allelic dropouts or other sources of typing errors, a realistic error rate of 0.01 for all loci was assumed. First, only full-sib families were assumed and no half-sib relationships were assumed. This scenario corresponds to singly mated queens. Second, full-sib families were allowed to be nested in half-sib families to test for multiply mated queens (polyandry).

Pairwise relatedness of workers derived from one acacia in the plots of *Pseudomyrmex ferrugineus* and *P. gracilis* was estimated using the program KINSHIP 1.1.2 (Goodnight & Queller 1999).

2.10.3 Correlations of geography, behavior, chemistry and genetics

A Mantel test serves to measure the associations between the elements of two matrices by taking into account the autocorrelation that exists between the elements of each matrix (Excoffier & Heckel 2006). Here, Mantel test was used to test for pairwise significant associations between geographic, behavioral, chemical and genetic distances within each experimental plot. The workers collected from each acacia were the group unit in the statistical design. Partial Mantel correlation tests using distance matrices from geographic (in meters), behavioral (as proportion of aggressive interactions), chemical (as Mahalanobis distances received from the discriminant analysis) and genetic distances (as pairwise F_{ST}) were carried out in ARLEQUIN ver 3.11 (Excoffier *et al.* 2005) using 2000 permutations. Two-tailed *P*-values are reported.

3 Results

3.1 Invertase activity

3.1.1 Invertase activity in workers and larvae

Invertase activity in workers was highly significantly affected by 'Species' (ANOVA: $F_{\text{invertase activity }(4,45)}$=12.106, P<0.001). The exploiters, *Pseudomyrmex gracilis* and *P. nigropilosu*s (starved, i.e., directly quantified after collection in the field), showed high invertase activities of 89±19 and 107±20 ng (mean±SE) of glucose released per µg ant fresh weight and min, which was significantly higher than the activity in the mutualists *P. ferrugineus*, *P. mixtecus* and *P. peperi* (P<0.05 according to Least Significant Difference (LSD) post hoc test, see Fig. 3.1.1). Activity in the mutualists ranged from only 15±2 to 18±7 ng of glucose released μg^{-1} min^{-1} (Fig. 3.1.1). In contrast, unfed larvae of all species showed high invertase activities, which were up to four times higher than in workers. The difference between workers and larvae was significant for all species (P<0.05 according to Wilcoxon signed rank test conducted separately for all individual species, N=10 for each species).

The factor 'Species' significantly affected larval invertase activity (ANOVA: $F_{\text{invertase activity }(4,45)}$ =2.962, P=0.03). Larval invertase activity of the exploiters averaged to 281±57 ng and 407±74 ng of glucose released μg^{-1} min^{-1}. It tended to be slightly lower in two of the mutualists (*P. ferrugineus* and *P. mixtecus*: 224±22 ng of glucose μg^{-1} min^{-1}), while larval invertase activity of the mutualist *P. peperi* was high (377±42 ng of glucose released μg^{-1} min^{-1}). Significant differences in larval invertase activity were found between *P. ferrugineus* and *P. mixtecus* compared to *P. nigropilosus* and *P. peperi*, with *P. gracilis* showing intermediate activity according to the LSD post hoc test (P<0.05, see Fig. 3.1.1).

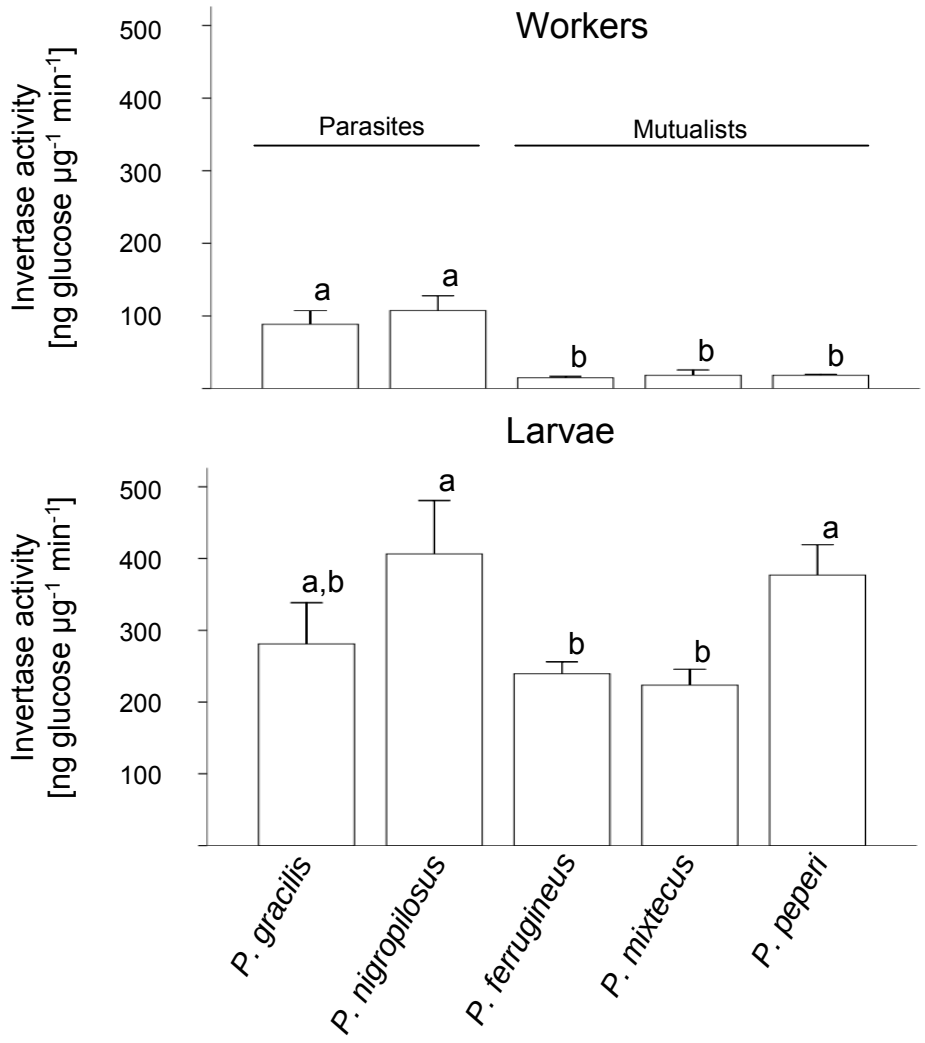


Fig. 3.1.1 Invertase activity in ants directly collected in the field.
Invertase activity was quantified after collection in the field and display enzymatic activities of ants in their natural habitat. Bars represent means+SE. 'Species' was a significant source of variance ($P<0.001$, univariate ANOVA) and species labeled with different letters are significantly different ($P<0.05$, LSD post hoc analysis). N=10 colonies per species.

3.1.2 Substrate induction of invertase

Enzymatic activity in workers of the generalist species *P. salvini* and of the two exploiters *P. gracilis* and *P. nigropilosus* increased significantly in response to feeding on sucrose as compared to glucose-containing diet (for all three species: $P<0.05$ with N=10; Wilcoxon signed rank test). In generalist workers, feeding on sucrose increased invertase activity on average by the factor 2.5, while the exploiters showed on average a three-fold increase. In contrast to generalists and exploiters, workers of the mutualists showed no induction in response to sucrose diet ($P>0.05$ according to Wilcoxon signed rank test for all three species).

Investigating the larvae revealed a different picture, as feeding on sucrose induced invertase activity in larvae of all species tested (for all species except *P.* spec. PSW-06: P<0.05 according Wilcoxon signed rank test with N=10; Fig.3.1.2; *P.* spec. PSW-06 could not be tested due to N=4). However, the relative increase in enzymatic activity was lowest for mutualists (average increase factor of 1.7), followed by generalists (2.0) and exploiters (3.1). A univariate GLM was applied to the entire data set to test for the effects of 'Species', 'Treatment', 'Ontogenetic Stage' (all fixed factors) and 'Colony' (random factor) on invertase activity. The effects of 'Species', 'Treatment' and 'Ontogenetic Stage' were highly significant (P<0.001), while the effect of 'Colony' was not (P=0.469; see Table 3.1.1).

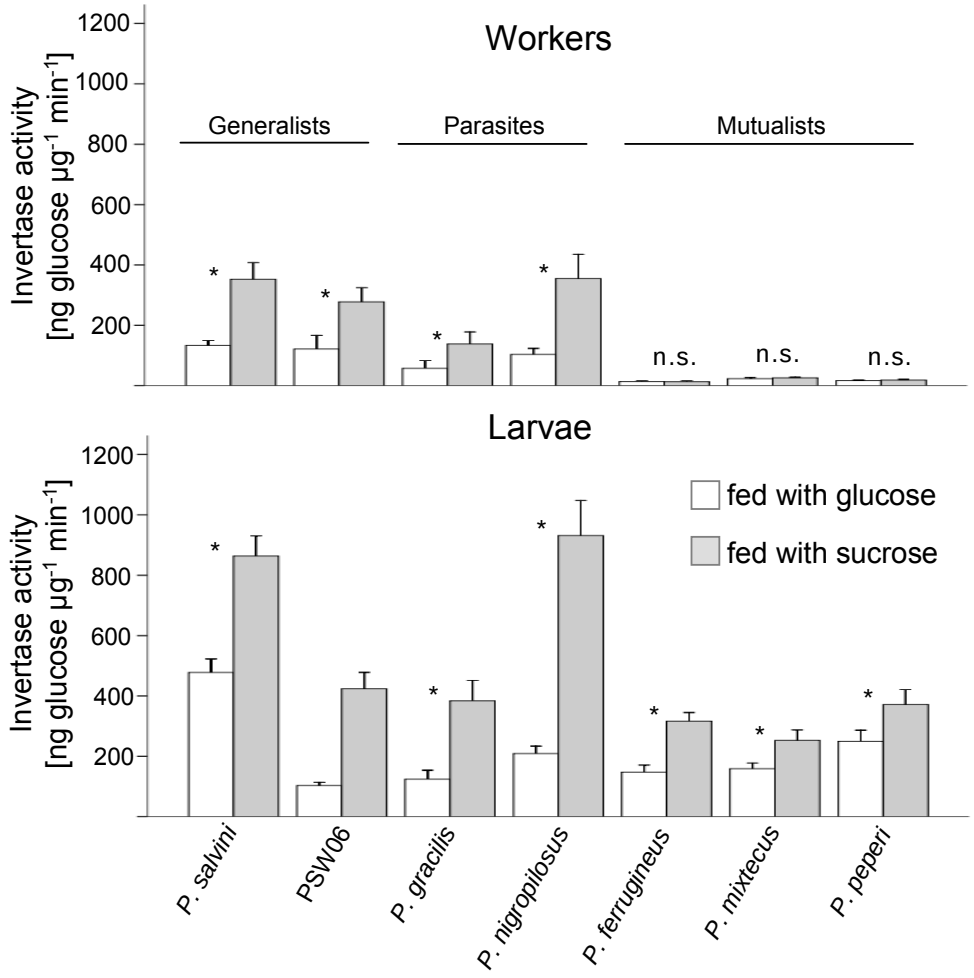


Fig. 3.1.2 Invertase activity in ants that were fed.
Ants were offered glucose or sucrose solution (10% w/v) for five days prior to quantification of invertase activity. Bars are means+SE. Asterisks (*) indicate that values of invertase activity differ significantly (P<0.05 according to Wilcoxon signed rank test) between sucrose-fed ants and glucose-fed ants. n.s. not significant. N=10 colonies per species except *P.* spec. PSW-06 where N=4.

Table 3.1.1 Results of GLM.
Effects of 'Species', 'Treatment', 'Ontogenetic Stage', 'Colony' on invertase activity in ants. Results obtained using the GLM (general linear model) for analysis of variance after a univariate design with invertase activity as variable. 'Species', 'Treatment', 'Ontogenetic Stage' were set as fixed factors, 'Colony' as random factor.

Source		SS	df	F	P
Intercept	Hypothesis	14488739.435	1	529.234	<0.001
	Error	383113.172	15.7		
Species	Hypothesis	4452165.306	6	742027.551	<0.001
	Error	8444084.282	337		
Treatment	Hypothesis	2460340.828	2	49.096	<0.001
	Error	8444084.282	337		
Ontogenetic stage	Hypothesis	5862376.715	1	233.965	<0.001
	Error	8444084.282	337		
Colony	Hypothesis	217547.854	9	0.965	0.469
	Error	8444084.282	337		

3.2 Behavioral trials

Behavior of resident ant individuals varied when they encountered experimentally introduced ant individuals. When ants of one acacia were collected and then replaced onto the original tree, encounter was neutral in all cases for both species (N=160 tests; Fig. 3.2.1). In the between-tree tests, neutral behavior was detected in all replicates of 10.7% of the pairwise combinations (for both species total N=1120 tests; Fig. 3.2.1). In 59.8% of the pairwise combinations, behavior was always aggressive. In the remaining 29.5% of pairwise combinations, behavior of workers was classified as neutral in a portion of the five replicates per combination and aggressive in the other portion. Aggression was shown in some replicates, but not in others. In both plots, *Pseudomyrmex ferrugineus* showed lower aggression (59% and 72% of all encounters in plots PFER1 and PFER2, respectively, were aggressive; N=320 replicates per plot) than *P. gracilis* (73% and 80% of all encounters in plots PGRA1 and PGRA2, respectively). Even though, no differentiation was made within the category 'aggressive', differences were observed in the degree of aggressiveness. In plot PFER1, response time was extremely long, sometimes reaching almost 5 minutes. In the other three plots, response time was usually short (<30 sec). Aggression of *P. gracilis* seemed more severe than of *P. ferrugineus*, aggressive encounters often showed extreme escalation on both sides, of the resident individual and the intruder.

PFER1

	a	b	c	d	e	f	g	h
a	0/5	5/0	4/1	1/4	0/5	5/0	5/0	5/0
b	5/0	0/5	5/0	5/0	5/0	0/5	2/3	5/0
c	5/0	5/0	0/5	3/2	1/4	5/0	3/2	5/0
d	2/3	5/0	2/3	0/5	0/5	5/0	5/0	4/1
e	0/5	5/0	1/4	0/5	0/5	1/4	5/0	4/1
f	2/3	5/0	5/0	5/0	5/0	0/5	0/5	2/3
g	0/5	5/0	2/3	5/0	5/0	0/5	0/5	3/2
h	5/0	5/0	5/0	5/0	5/0	3/2	0/5	0/5

PFER2

	a	b	c	d	e	f	g	h
a	0/5	3/2	5/0	5/0	5/0	5/0	5/0	0/5
b	5/0	0/5	5/0	5/0	5/0	5/0	5/0	5/0
c	5/0	5/0	0/5	5/0	0/5	5/0	0/5	5/0
d	5/0	5/0	5/0	0/5	5/0	5/0	0/5	5/0
e	5/0	1/4	0/5	5/0	0/5	4/1	5/0	5/0
f	5/0	3/2	5/0	5/0	5/0	0/5	5/0	0/5
g	5/0	5/0	5/0	0/5	5/0	5/0	0/5	5/0
h	4/1	5/0	5/0	5/0	5/0	0/5	5/0	0/5

PGRA1

	a	b	c	d	e	f	g	h
a	0/5	3/2	5/0	5/0	3/2	3/2	5/0	5/0
b	5/0	0/5	0/5	5/0	5/0	4/1	5/0	4/1
c	5/0	0/5	0/5	5/0	4/1	5/0	4/1	5/0
d	5/0	5/0	5/0	0/5	0/5	4/1	5/0	5/0
e	5/0	5/0	5/0	0/5	0/5	4/1	5/0	5/0
f	5/0	5/0	5/0	5/0	5/0	0/5	3/2	2/3
g	5/0	5/0	5/0	5/0	5/0	5/0	0/5	0/5
h	5/0	5/0	5/0	5/0	5/0	5/0	0/5	0/5

PGRA2

	a	b	c	d	e	f	g	h
a	0/5	5/0	5/0	5/0	5/0	5/0	5/0	0/5
b	5/0	0/5	5/0	5/0	5/0	5/0	5/0	4/1
c	5/0	5/0	0/5	5/0	5/0	5/0	0/5	5/0
d	5/0	5/0	5/0	0/5	5/0	5/0	5/0	5/0
e	5/0	5/0	5/0	5/0	0/5	4/1	5/0	5/0
f	5/0	5/0	5/0	5/0	5/0	0/5	5/0	5/0
g	5/0	5/0	0/5	5/0	5/0	5/0	0/5	5/0
h	0/5	3/2	5/0	5/0	5/0	5/0	5/0	0/5

Fig. 3.2.1 Behavioral responses of ants towards conspecifics.
The behavioral response of resident ants towards ants that were experimentally placed on the host tree was recorded. Five ants each (row) were placed onto another tree (column) and the behavior of ants encountering each other was recorded as being aggressive/neutral. White cells indicate that at least 4 of 5 encounters were aggressive, black cells indicate that at least 4 of 5 encounters were neutral and striped cells indicate 2 or 3 of 5 encounters as aggressive. Behavioral distances are given in supplementary Tables 2-5 (pages 143-146).

3.3 Cuticular compounds

Gas chromatography-mass spectrometry (GC-MS) analysis of cuticular compounds identified a total of 19 cuticular compounds for *P. ferrugineus* and 26 for *P. gracilis* (Table 3.3.1; Fig. 3.3.1). The compounds varied in chain length between C27 and C37. Although some straight-chain n-alkanes and n-alkenes were present, the majority of the compounds were methyl-branched alkanes (mono- and dimethyl).

In plot Pfer1, 19 hydrocarbons were subjected to a principal components analysis (PCA). Using eigenvalues >1.0 four factors were extracted in plot PFER1, which explained 73% of the total variance. Discriminant analysis (DA) based on these four factors extracted and using ‘Host Tree’ as grouping variable showed significant

differences of cuticular profiles between the ants derived from different acacias (Fig. 3.3.2; Wilks' lambda: 0.1119743; $F_{28,210}$=6.28; P<0.001) in plot PFER1. In 23 of 28 pairwise comparisons (82%) Mahalanobis distances were significant (supplementary Table 2, page 143). In STATISTICA 8.0, it is possible to calculate posterior probabilities of each individual ant being assigned to each of the groups. In the present study, one group comprised all ants collected from one acacia individual. The posterior probability is greater the closer the individual is to the group centroid in a two-dimensional space. Posterior probabilities of individuals being assigned to the group of ants collected from their original acacia host were 62% with a total of 26 out of 69 individuals misclassified (supplementary Table 6). In plot PFER2, four factors extracted in a PCA explained 75% of the variance. According to DA, ants of the same acacia possessed characteristic cuticular profiles (Fig. 3.3.2; Wilks' lambda: 0.0039461; $F_{35,263}$=20.52; P<0.001) and all members of each acacia always clustered closely. All pairwise Mahalanobis distances were significant (supplementary Table 3, page 144). The portion of ant individuals being correctly assigned to their original acacia was 85% with eleven out of 74 individuals being misclassified (supplementary Table 7, page 149).

In plot PGRA1, 26 hydrocarbons were subjected to a PCA and six factors (explaining 83% of the total variance) were extracted. Using 'Host Tree' as grouping variable revealed that the individuals sampled from each acacia always clustered very closely and that individuals derived from the same host possessed characteristic cuticular profiles (Fig. 3.3.2; Wilks' lambda: 0.0006609; $F_{42,308}$=27.63; P<0.001). Mahalanobis distances were significant in 26 of 28 pairwise comparisons (93%), in which all ants from one host tree functioned as group (supplementary Table 4, page 145). Posterior probabilities of individuals being assigned to their original group (i.e., host tree) were 87% with a total of ten out of 78 individuals misclassified (supplementary Table 8). In plot PGRA2, five factors extracted explained 82% of the total variance. Individuals collected from the same tree always clustered very closely and colonies were distinct (Fig. 3.3.2; Wilks' lambda: 0.0009723; $F_{35,263}$=31.59; P<0.001). All pairwise Mahalanobis distances were significant (supplementary Table 5). Correct assignments were 82% with 13 out of 74 individuals misclassified (supplementary Table 9, page 155).

Table 3.3.1 Cuticular compounds for *P. ferrugineus* and *P. gracilis.*
Peak number (see profile Fig. 3.3.1), retention time, short and full name of the 32 cuticular hydrocarbon compounds in the profiles of *P. ferrugineus* and *P. gracilis*. The presence of each compound in the profiles of *P. ferrugineus* and *P. gracilis* is indicated by an **x**.

Peak number	RT Time	Short compound name	Full compound name	PFER	PGRA
1	15.15	4meC26	4-methylhexacosane		**x**
2	15.29	C27:1	Δx-heptacosene		**x**
3	15.63	C27	heptacosane	**x**	**x**
4	16.06	13meC27	13-methylheptacosane		**x**
5	16.41	11,15dimeC27	11, 15-dimethylheptacosane		**x**
6	16.62	3meC27	3-methylheptacosane		**x**
7	16.98	C28	octacosane		**x**
8	17.30	14meC28	14-methyloctacosane		**x**
		13meC28	13-methyloctacosane		**x**
		12meC28	12-methyloctacosane		**x**
9	17.77	4meC28	4-methyloctacosane	**x**	**x**
		3meC28	3-methyloctacosane	**x**	**x**
10	18.07	C29:1	Δx-nonacosene	**x**	
11	18.27	C29	nonacosane	**x**	**x**
12	18.65	15 meC29	15-methylnonacosane		**x**
		13 meC29	13-methylnonacosane		**x**
		11meC29	11-methylnonacosane		**x**
13	19.03	11,15dimeC29	11, 15-dimethylnonacosane		**x**
		13,15dimeC29	13, 15-dimethylnonacosane		**x**
		5meC29	5-methylnonacosane		**x**
14	19.21	3meC29	3-methylnonacosane	**x**	**x**
15	19.55	3,xdimeC29	3, x-dimethylnonacosane		**x**
		C30	triacontane	**x**	**x**
16	19.85	14meC30	14-methyltriacontane		**x**
		12meC30	12-methyltriacontane		**x**
17	20.32	4meC30	4-methyltriacontane	**x**	**x**
18	20.45	3meC30	3-methyltriacontane	**x**	**x**
19	20.58	C31:1	Δx-hentriacontene		**x**
20	20.81	C31	hentriacontane	**x**	
21	21.14	15meC31	15-methylhentriacontane		**x**
		13meC31	13-methylhentriacontane		**x**
		11meC31	11-methylhentriacontane		**x**
22	21.44	11,15dimeC31	11, 15-dimethylhentriacontane		**x**
23	21.67	3meC31	3-methylhentriacontane	**x**	**x**
24	23.00	x, y-dimeC32	x, y-dimethyldotriacontane	**x**	
25	23.22	14meC32	14-methyldotriacontane		**x**
		12meC32	12-methyldotriacontane		**x**
26	23.50	15meC33	15- methyltritriacontane	**x**	
		13meC33	13-methyltritriacontane	**x**	**x**
		11meC33	11-methyltritriacontane	**x**	**x**
27	23.79	11,15dimeC33	11, 15-dimethyltritriacontane	**x**	**x**
		11,12dimeC33	11, 12-dimethyltritriacontane	**x**	
28	24.08	unID	unidentified compound	**x**	
29	25.71	17meC35	17-methylpentatriacontane	**x**	**x**
		15meC35	15-methylpentatriacontane	**x**	**x**
		13meC35	13-methylpentatriacontane	**x**	**x**
		11meC35	11-methylpentatriacontane	**x**	**x**
30	26.03	11,15dimeC35	11, 15-dimethylpentatriacontane	**x**	**x**
31	26.33	unID	unidentified compound	**x**	
32	28.15	11,xdimeC37	11, x-dimethylheptatriacontane	**x**	

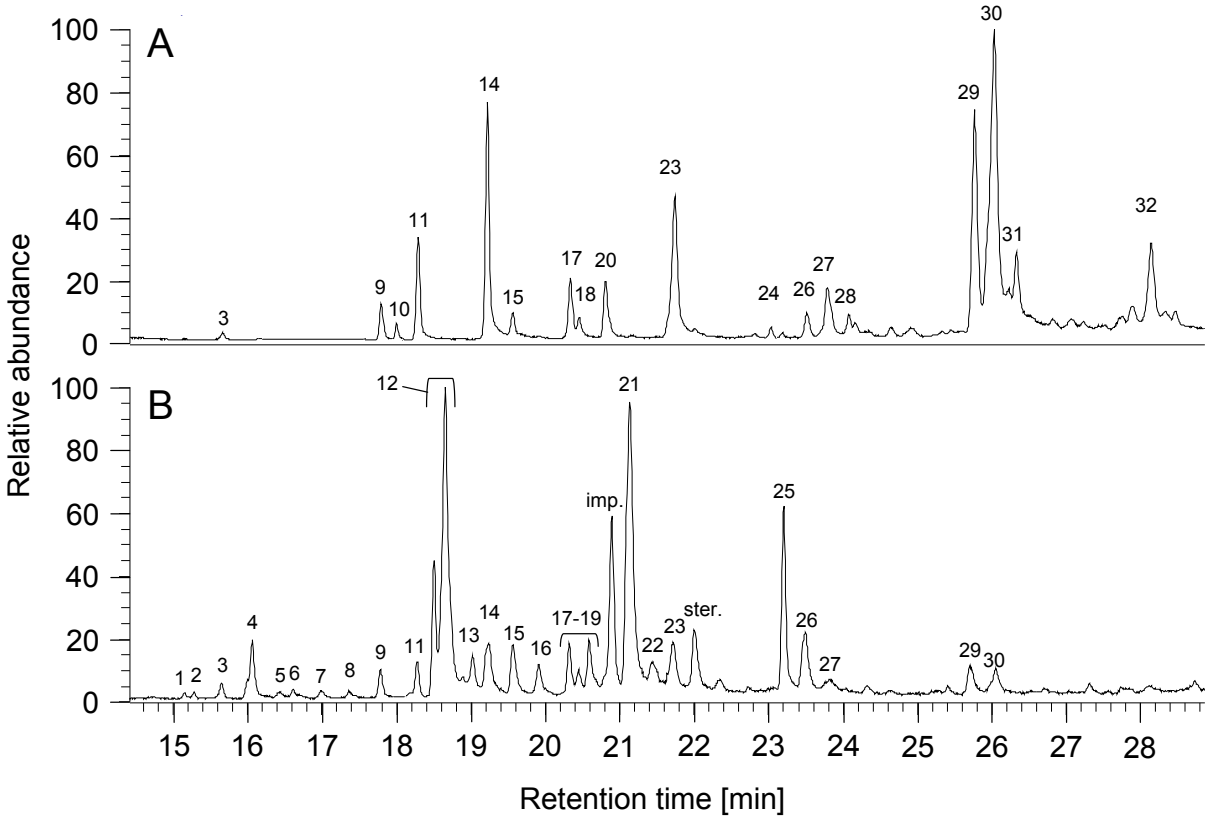


Fig. 3.3.1 Cuticular hydrocarbon profiles of *P. ferrugineus* and *P. gracilis*.
A total of 32 hydrocarbon peaks were detected, of which 19 were present in the profile of *P. ferrugineus* (A) and 26 in the profile of *P. gracilis* (B). Peak numbers correspond to the following compounds: 1: 4meC26; 2: C27:1; 3: C27; 4: 13meC27; 5: 11,15dimeC27; 6: 3meC27; 7: C28; 8: 14meC28, 13meC28, 12meC28; 9: 4meC28, 3meC28; 10: C29:1; 11: C29; 12: 15 meC29, 13 meC29, 11meC29; 13: 11,15dimeC29, 13,15dimeC29, 5meC29; 14: 3meC29; 15: 3,xdimeC29, C30; 16: 14meC30, 12meC30; 17: 4meC30; 18: 3meC30; 19: C31:1; 20: C31; 21: 15meC31, 13meC31, 11meC31; 22: 11,15dimeC31; 23: 3meC31; 24: x, y-dimeC32; 25: 14meC32, 12meC32; 26: 15meC33, 13meC33, 11meC33; 27: 11,15dimeC33, 11,12dimeC33; 28: unID; 29: 17meC35, 15meC35, 13meC35, 11meC35; 30: 11,15dimeC35; 31: unID; 32: 11,xdimeC37 ('imp.' denotes impurity, 'ster.' denotes steroid).

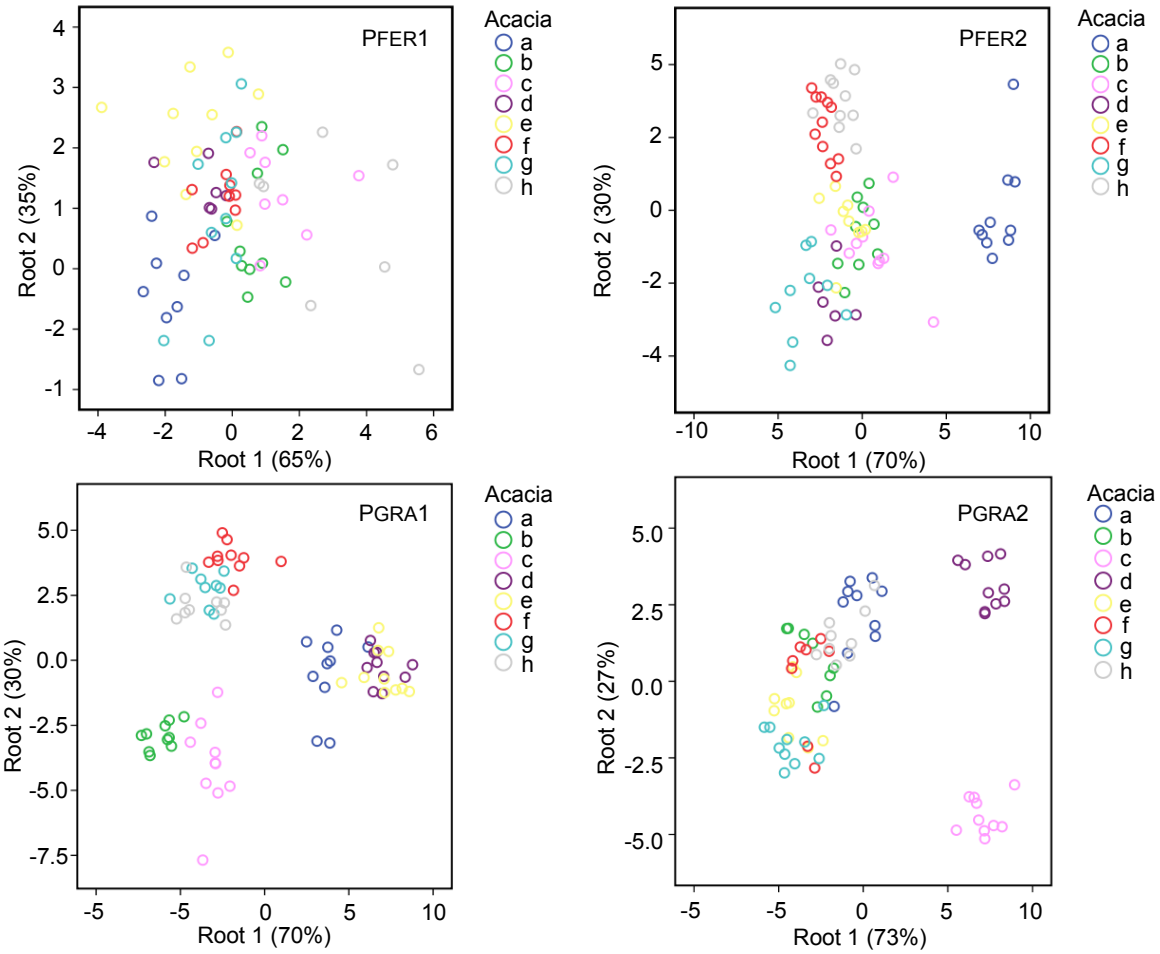


Fig. 3.3.2 Discriminant analysis based on factors extracted in a PCA.
Factors were extracted in a principal components analysis (PCA) based on the cuticular hydrocarbon profiles. Acacias (i.e., host trees) were used as grouping variable within each plot. Graphs were drawn from the first and second extracted root and are given with the variance each explains.

3.4 Colony composition of *Pseudomyrmex peperi*

The relative abundance of developmental stages and castes within supercolonies of *Pseudomyrmex peperi* differed significantly (univariate ANOVA PPEP103: $F_{caste\ (6,133)}$=36.196, P<0.001; PPEP132: $F_{caste\ (6,133)}$=28.309, P<0.001). In colony PPEP103, 1,532 female adults, 23 physogastric queens (66 females per queen; p.q.), 251 male adults, 740 female pupae (8 p.q.), 192 male pupae, 4,561 larvae (198 p.q.) and 6,536 eggs (284 p.q.) were found. In colony PPEP132, 61 physogastric queens,

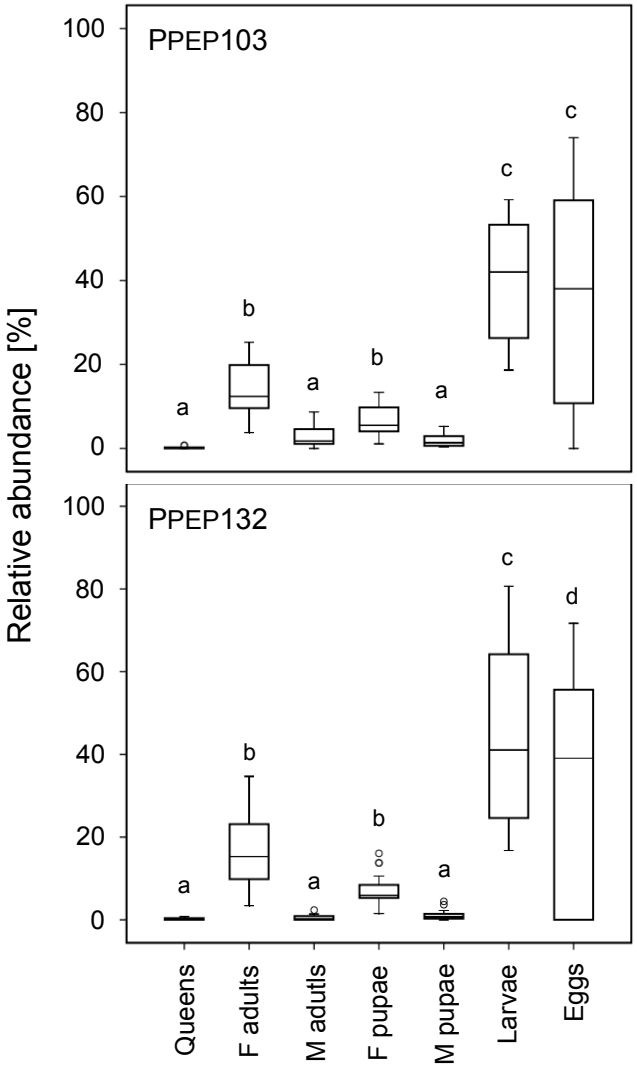


Fig. 3.4.1 Composition of supercolonies PPEP103 and PPEP132.
The colony composition of the two supercolonies PPEP103 and PPEP132 was estimated by relative abundance of castes based on collected thorns. Different letters indicate significant differences (P<0.05 according to LSD post hoc test after univariate ANOVA). Relative abundance was arcsine transformed prior statistical analysis due to non-independence of data. Upper and lower margins of boxes are 25% and 75% percentiles, whiskers represent 5% and 95% percentiles, lines in boxes are medians, circles are outliers.

2,384 female adults (39 p.q.), 93 male adults, 1,273 female pupae (20 p.q.), 159 male pupae, 7,055 larvae (115 p.q.) and 10,275 eggs (168 p.q.) were counted. Results are shown in Fig. 3.4.1. The number of queens per sub-sample (i.e., individual shoot in acacia cluster) was significantly correlated with number of eggs (Pearson's correlation PPEP103: r=0.717, P<0.001, PPEP132: r=0.880, P<0.001), but not with the other groups (P>0.05 in each case).

3.5 Molecular phylogeny

For this study, a total of 159 (126 new) sequences were used including 32 mtCOI, 32 28S rDNA, 31 *abd*-A, 32 LW *Rh* and 32 *wg* sequences (Table 3.5.1). A matrix with 3,329 unambiguously aligned nucleotide position characters was produced for analysis containing 1,014 positions in the mtCOI, 851 in the 28S rDNA, 600 in the *abd*-A, 456 in the LW *Rh* and 408 in the *wg* data sets. Unalignable portions of 28S and an intron in LW *Rh* had previously been excluded from the analysis. I attempted to exclude nuclear pseudogenes from the mtCOI data set by removing sequences with gaps in the alignment. However, numts (pseudogenes) in pseudomyrmecines are often of the same length as the true mtDNA fragment (Ward, unpubl.). A more important criterion is whether, for the same individual extract, one observes mismatched bases in overlapping fragments amplified with different primer pairs. Thus, two mtCOI fragments of each specimen were sequenced. One was amplified using the primer combination COI-LCO1490/Ben, while the overlapping fragment was amplified using the primer pair Jerry/Pat. In the cases of disagreeing sequences the specimen was then excluded from the alignment. In some cases, the primer pair Jerry/Pat did not yield a PCR-product and two shorter fragments using the primer pairs COI-LCO1490/COI-HCO2198 as well as Jerry/Ben were amplified. The ends of all sequences were trimmed to a segment covering the region amplified by COI-LCO1490/Ben. The combined alignment is available in TREEBASE (http.//www.treebase.org/treebase; accession no. SN4188). Maximum Likelihood analysis of the combined data set yielded a tree that did not contradict the Bayesian tree topology. The mean log likelihood of the Bayesian tree sampling was –14567.99. Detailed information on nucleotide composition of data partitions is given in Table 3.5.2. The base composition of the mtCOI locus in the study species was highly AT biased (0.756), as expected for mitochondrial DNA in general and particularly for

hymenopterans (Brady *et al.* 2000; Chenuil & McKey 1996; Whitfield & Cameron 1998). AT bias at third codon positions was more pronounced (0.821) than at first (0.650) or second (0.634) positions. Since the topologies of the ML and B/MCMC analyses did not show any strongly supported conflicts, only the 50% majority-rule consensus tree of Bayesian tree sampling is shown. Those nodes that received strong support (i.e., posterior probability (pp) ≥ 0.95 in B/MCMC analysis and ML bootstrap (BM) ≥ 75%) in both the Bayesian and ML analyses are in bold (Fig. 3.5.1).

In the majority-rule consensus tree of the combined data set shown in Fig. 3.5.1, two main clades within the genus *Pseudomyrmex* can be recognized. One is formed by species of the *gracilis* group, while the other comprises all other species. The *gracilis* group is strongly supported as monophyletic in both analyses (BM 100, pp 1.0) and within the clade the three specimens of *P. nigropilosus* are well separated from *P. gracilis*.

Within the second major clade, *P. salvini*, *P. haytianus*, *P.* spec. PSW-01 and *P.* spec. PSW-06 take a sister position to one clade that comprises all species of the *ferrugineus* group as well as the undescribed species *P.* spec. PSW-54. Thus, the obligate acacia-ants in the *ferrugineus* group do not form a monophyletic group. The undescribed species *P.* spec. PSW-54, which is a generalist inhabitant of dead twigs (P.S. Ward, unpubl. data), is sister to *P. nigrocinctus*. *P. ferrugineus*, *P. flavicornis* and *P. mixtecus* form one well-supported group (BM 99, pp 1.0) that is a sister to *P. peperi*.

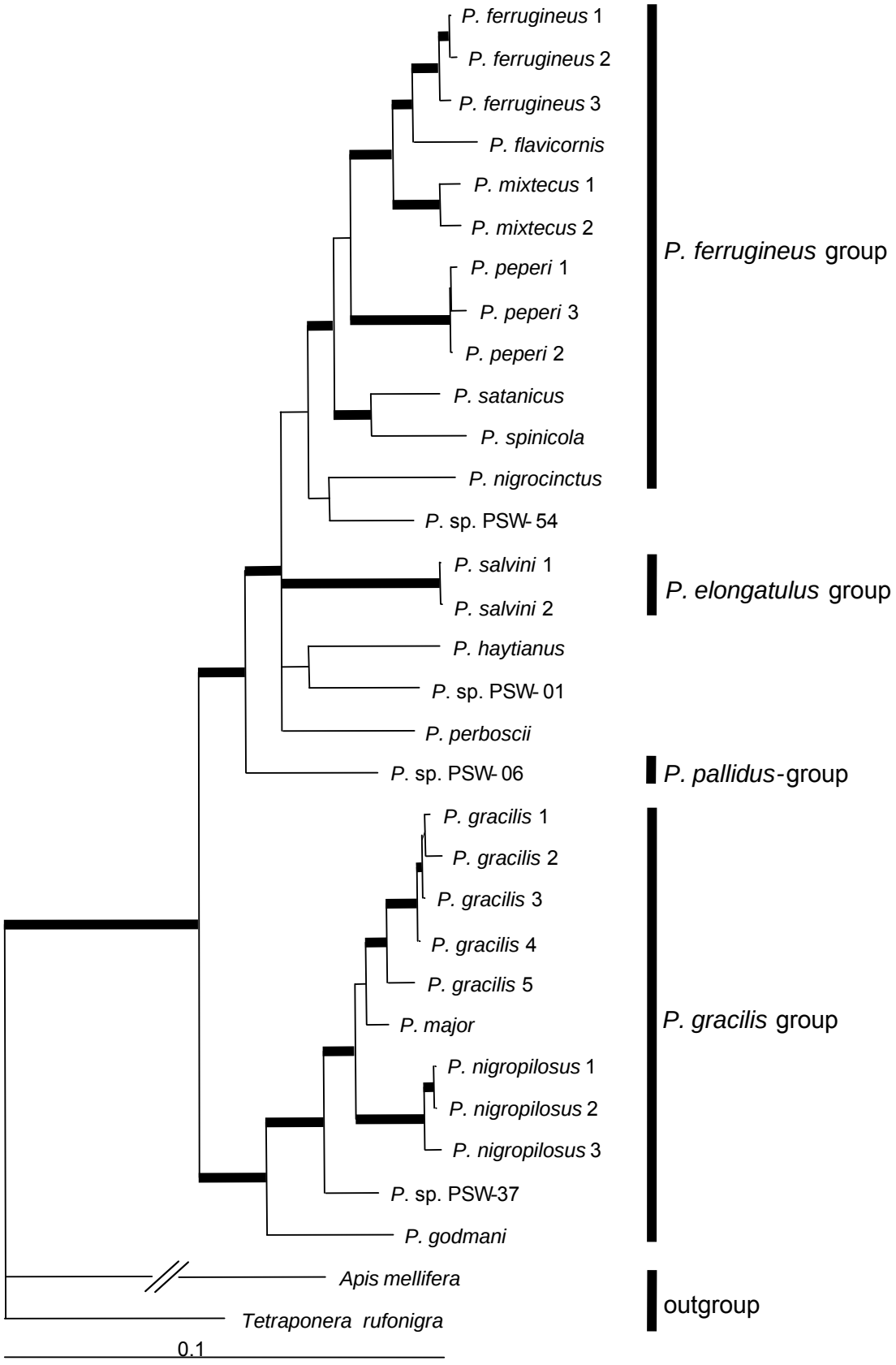


Fig. 3.5.1 Molecular phylogeny of *Pseudomyrmex* ants.
The phylogeny was inferred from a five gene fragments partition analysis totaling 3,224 bp. Displayed is a 50% majority rule consensus tree based on 74,000 trees from a B/MCMC tree sampling procedure. Branches with posterior probabilities equal or above 0.95 and ML bootstrap support values equal and above 75% are indicated in bold.

Table 3.5.1 Species and specimens included in the phylogenetic study.
Voucher Specimen and GENBANK Accession Nos. BL=Bolivia; CS=Costa Rica; DR=Dominican Republic; EC=Ecuador; GT=Guatemala; LT=San Andres Tuxtla; MR=Matias Romero; MX=Mexico; PM=Panama; PTO=Puerto Escondido; VZ=Venezuela; ZP=Zipolite. *A.*=*Acacia* ; *P.*=*Pseudomyrmex*. LWC=Lars W. Clement. n.det.=not determined.

Specimen	Location; year	Collection	host plant	association with myrmecophytes	Invertase activity in workers	GENBANK accession numbers				
						mtCOI	*wg*	LW *Rh*	*abd*-A	28S
P. ferrugineus 1	ZP, MX; 2005	Heil (Kautz#02-1)	*A. cornigera*	mutualistic	no	FJ436816	FJ436847	FJ436871	FJ436895	FJ436918
P. ferrugineus 2	PTO, MX; 2005	Heil (Kautz#05-1)	*A. collinsii*	mutualistic	no	FJ436817	FJ436848	FJ436872	FJ436896	FJ436919
P. ferrugineus 3	MR, MX; 2004	LWC (Kautz#49-1)	*A. chiapensis*	mutualistic	no	FJ436818	FJ436849	FJ436873	FJ436897	FJ436920
P. flavicornis	CS; 2000	Ward#14180		mutualistic	n.det.	FJ436819	AY703661	AY703795	AY703728	AY703594
P. godmani	BL; 1993	Ward#12235		generalist	n.det.	FJ436820	AY703662	AY703796	AY703729	AY703595
P. gracilis 1	MR, MX; 2005	Heil (Kautz#11-1)	*A. chiapensis*	parasitic	yes	FJ436821	FJ436850	FJ436874	FJ436898	FJ436921
P. gracilis 2	MR, MX; 2006	Kautz#205-1	*A. chiapensis*	parasitic	yes	FJ436822	FJ436851	FJ436875	FJ436899	FJ436922
P. gracilis 3	MR, MX; 2004	LWC (Kautz#53-1)	*A. chiapensis*	parasitic	yes	FJ436823	FJ436852	FJ436876	FJ436900	FJ436923
P. gracilis 4	LT, MX; 2005	Kautz#131-1		generalist	yes	FJ436824	FJ436853	FJ436877	FJ436901	FJ436924
P. gracilis 5	CS; 2000	Ward#14184		generalist	n.det.	FJ436825	AY703663	AY703797	AY703730	AY703596
P. haytianus	DR; 1992	Ward#11772		generalist	n.det.	FJ436826	AY703664	AY703798	AY703731	AY703597
P. major	MX; 2000	MacKay#19185		generalist	n.det.	FJ436827	FJ436854	FJ436878	FJ436902	FJ436925
P. mixtecus 1	PTO, MX; 2005	Heil (Kautz#04-1)	*A. collinsii*	mutualistic	no	FJ436828	FJ436855	FJ436879	FJ436903	FJ436926
P. mixtecus 2	PTO, MX; 2004	LWC (Kautz#44-1)	*A. collinsii*	mutualistic	no	FJ436829	FJ436856	FJ436880	FJ436904	FJ436927
P. nigrocinctus	CS; 2000	Ward#14179		mutualistic	n.det.	FJ436830	AY703668	AY703802	AY703735	AY703601
P. nigropilosus 1	PTO, MX; 2005	Kautz#142-1	*A. hindsii*	parasitic	yes	FJ436831	FJ436857	FJ436881	FJ436905	FJ436928
P. nigropilosus 2	MR, MX; 2004	LWC (Kautz#32-1)	*A. chiapensis*	parasitic	yes	FJ436832	FJ436858	FJ436882	FJ436906	FJ436929

P. nigropilosus 3	CS; 1989	Longino#2554		parasitic	yes	FJ436833	AY703669	AY703803	AY703736	AY703602
P. peperi 1	ZP, MX; 2005	Heil (Kautz#07-1)	*A. cornigera*	mutualistic	no	FJ436834	FJ436859	FJ436883	FJ436907	FJ436930
P. peperi 2	PTO, MX; 2005	Heil (Kautz#01-1)	*A. hindsii*	mutualistic	no	FJ436835	FJ436860	FJ436884	FJ436908	FJ436931
P. peperi 3	GT; 2004	MacKay#20784		mutualistic	n.det.	FJ436836	FJ436861	FJ436885	FJ436909	FJ436932
P. perboscii	BL; 1992	P. Bettella (3.iii.1992)		generalist	n.det.	FJ436837	FJ436862	FJ436886	—	FJ436933
P. salvini 1	LT, MX; 2005	Kautz#115-1		generalist	yes	FJ436838	FJ436863	FJ436887	FJ436910	FJ436934
P. salvini 2	LT, MX; 2005	Kautz#118-1		generalist	yes	FJ436839	FJ436864	FJ436888	FJ436911	FJ436935
P. satanicus	PM; 2004	A. Dejean (19.v.2004)		mutualistic	n.det.	FJ436840	FJ436865	FJ436889	FJ436912	FJ436936
P. spinicola	CS; 2000	Ward#14181		mutualistic	n.det.	FJ436841	FJ436866	FJ436890	FJ436913	FJ436937
P. spec. PSW-01	VZ; 1987	Ward#9027		generalist	n.det.	FJ436842	FJ436867	FJ436891	FJ436914	FJ436938
P. spec. PSW-06	PTO, MX; 2005	Kautz#151-1		generalist	yes	FJ436843	FJ436868	FJ436892	FJ436915	FJ436939
P. spec. PSW-37	EC; 1991	Ward#11387		generalist	n.det.	FJ436844	FJ436869	FJ436893	FJ436916	FJ436940
P. spec. PSW-54	GT; 2003	Ward#15038		generalist	n.det.	FJ436845	FJ436870	FJ436894	FJ436917	FJ436941
Tetraponera rufonigra	India, 1999	Ward#13844		generalist	n.det.	FJ436846	AY703649	AY703783	AY703716	AY703582
Apis mellifera				—	—	L06178	AY703618	AY703752	AY703685	AY703551

Table 3.5.2 Parameters of Bayesian tree sampling of data partitions.

Gene partition	Partition 1 mtCOI 1st position	Partition 2 mtCOI 2nd position	Partition 3 mtCOI 3rd position	Partition 4 *wg* 1st position	Partition 5 *wg* 2nd position	Partition 6 *wg* 3rd position	Partition 7 LW *Rh* 1st position	Partition 8 LW *Rh* 2nd position	Partition 9 LW *Rh* 3rd position	Partition 10 *abd*-A 1st position	Partition 11 *abd*-A 2nd position	Partition 12 *abd*-A 3rd position	Partition 13 28S
$\pi A_{mean/all}$	0.364	0.176	0.410	0.315	0.355	0.190	0.331	0.166	0.198	0.195	0.216	0.192	0.201
$\pi C_{mean/all}$	0.156	0.241	0.165	0.212	0.148	0.358	0.203	0.236	0.284	0.232	0.499	0.308	0.293
$\pi G_{mean/all}$	0.192	0.152	0.035	0.337	0.315	0.279	0.216	0.214	0..252	0.361	0.148	0.262	0.314
$\pi T_{mean/all}$	0.288	0.431	0.390	0.135	0.182	0.173	0.250	0.384	0.266	0.211	0.137	0.238	0.192
$r(AC)_{mean/all}$	0.033	0.095	0.036	N/A	N/A	N/A	N/A	N/A	N/A	0.023	0.004	0.110	0.143
$r(AG)_{mean/all}$	0.107	0.328	0.544	N/A	N/A	N/A	N/A	N/A	N/A	0.047	0.325	0.464	0.163
$r(AT)_{mean/all}$	0.071	0.067	0.058	N/A	N/A	N/A	N/A	N/A	N/A	0.027	0.257	0.037	0.096
$r(CG)_{mean/all}$	0.020	0.215	0.023	N/A	N/A	N/A	N/A	N/A	N/A	0.009	0.005	0.075	0.014
$r(CT)_{mean/all}$	0.690	0.279	0.310	N/A	N/A	N/A	N/A	N/A	N/A	0.857	0,006	0.269	0.503
$r(GT)_{mean/all}$	0.080	0.015	0.030	N/A	N/A	N/A	N/A	N/A	N/A	0.037	0.402	0.044	0.081
$\alpha_{mean/all}$	0.829	0.302	1.160	N/A	N/A	N/A	0.233	0.097	76.802	0.059	0.030	96.646	0.497
$P_{(invar)\ mean/all}$	0.610	0.674	0.896	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.350

3.5.1 Alternative hypothesis testing

Alternative topologies with the *P. ferrugineus* group forming a monophyletic group are rejected with the current data set (P<0.001 in both tests).

3.5.2 Ancestral states reconstruction 1

Among the 30 *Pseudomyrmex* specimens (19 species) included in this study, eleven were mutualists (seven species), six (two species) were parasites of the mutualism with acacia and for thirteen specimens (ten species) no interaction with this plant genus has been reported. For *P. gracilis*, specimens from two generalist colonies and three exploiting colonies were included. Ancestral character mapping of ant-plant associations on the phylogeny (Fig. 3.5.2) leads to the conclusion that the ancestors to pseudomyrmecines were generalists and that mutualism has arisen once or twice within the *ferrugineus* group. Whether the ancestor to the *ferrugineus* group, including *P*. spec. PSW-54, was generalist or mutualistic cannot be determined with the current data set. Both scenarios appear possible (Fig. 3.5.2). However, the ancestral state to the *P. gracilis* group was generalist and exploitation has arisen twice within the species group: once for *P. nigropilosus* and once within the species *P. gracilis*. Most importantly, the data presented here clearly show that parasitism of acacias by *P. gracilis* and *P. nigropilosus* has evolved independently of the mutualistic interaction. Invertase activity could only be quantified in seven of these ant species and the data suggest that inducible invertase activity in workers is the ancestral state (Fig. 3.5.2).

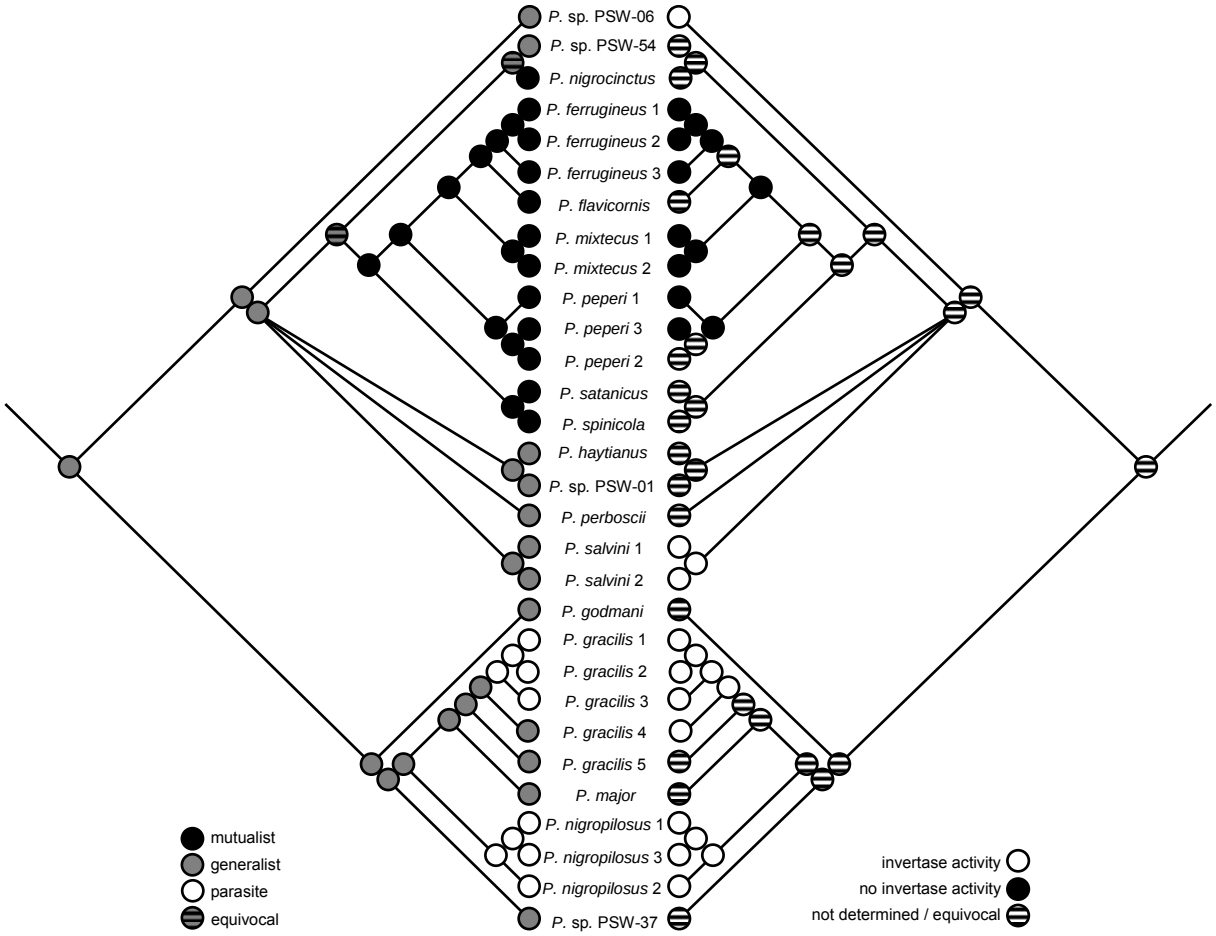


Fig. 3.5.2 Ancestral states reconstruction 1.
Associations of ants with myrmecophytic acacia plants (mutualistic, generalist and parasitic; left) and invertase activity in workers of *Pseudomyrmex* ants (invertase activity, no invertase activity and not determined; right) traced on the phylogeny of *Pseudomyrmex* ants, as inferred from a five gene fragments analysis.

3.6 Microsatellite primer development

Twelve primer pairs were flanking polymorphic loci that comprised two to ten alleles per population of *Pseudomyrmex ferrugineus* (Table 3.6.1), suggesting that they are sufficiently variable for population genetic analyses. All primers were published (Kautz *et al.* 2009b) and the sequences have been submitted to GENBANK (accession numbers: EU864160:EU864162, EU864172- EU864180). Observed and expected heterozygosities and exact Hardy-Weinberg probability test using the Markov chain method with default parameters are given in Tables 3.6.1 and 3.6.2. No significant deviation between expected and observed heterozygosity was detected. In tests for null alleles using MICROCHECKER (van Oosterhout *et al.* 2004) no evidence for null-alleles was detected. Linkage disequilibrium between the loci Psfe06, Psfe07 and Psfe13 in the Matias Romero population was detected based on Fisher's exact test as implemented in the online version of the GENEPOP software (Raymond & Rousset 1995; $P<0.05$). Thus, at least nine primer pairs for *P. ferrugineus* were developed that show no deviations from Hardy-Weinberg equilibrium or linkage disequilibrium and amplify reliable. PCR primers and characteristics for ten additional microsatellite loci can be found in Table 3.6.2 (accession numbers: EU864155-EU864159, EU864163-EU864164, EU919670-EU919671 and EU919681). These primers were tested on a different set of ant individuals due to a run out of DNA. For *P. mixtecus* and *P. peperi*, ten primers amplified successfully and showed variability (Table 3.6.3). Deviations from Hardy-Weinberg equilibrium at four loci in *P. mixtecus* and at seven loci in *P. peperi* might be due to the sampling strategy. Several individuals from the same colony were sampled, which do not represent independent samples, since workers are related.

Table 3.6.1 Primer sequences and characteristics of microsatellite loci.
Twelve loci developed for *Pseudomyrmex ferrugineus* are shown here. The repeat motif is given for the cloned allele. GB, GENBANK; Ta, annealing temperature; N, number of genotypes that amplified from eleven individuals of each population screened; A, observed number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity.

						Puerto Escondido				Matias Romero			
Locus	GB accession	Primer sequence	Repeat motif	T_a (°C)	Size range (bp)	N	A	H_E	H_O	N	A	H_E	H_O
Psfe06	EU864160	5' TET-ACGAAAAGGTTTTTAATAAGC	$(TC)_{20}$	50	76-103	11	3	0.31	0.32	11	7	0.43	0.41
		5' GCTGACAGATTAATAGTATGC											
Psfe07	EU864161	5' 6-FAM-AAGGCTTGAAATATCGTTCGTTGC	$(GA)_{11}$	55	108-123	11	3	0.30	0.27	11	6	0.42	0.50
		5' AAAGTAGATGTTTCAGTCCATCGC											
Psfe08	EU864162	5' 6-FAM-ATTAATGCTCGAAGGCAAA	$(CT)_{25}$	55	124-150	10	2	0.25	0.30	11	7	0.44	0.41
		5' ATCGAACACGTTGAATGATAC											
Psfe13	EU919672	5' HEX-TATTGAGAAGTCAGACGGTTTCGC	$(AG)_{20}AA(AG)_9$	55	161-205	11	4	0.35	0.41	11	10	0.46	0.50
		5' GAGTCATTACTATCTATTAACAGG											
Psfe14	EU919673	5' TET-AATAGTAATTACCGAGATAATAAC	$(CT)_{28}$	50	112-164	11	4	0.35	0.41	11	10	0.44	0.50
		5' ACAAGACAAGCTCGAGGATTTAAA											
Psfe15	EU919674	5' TET-TTGCGTTTCCGAGAAACAAC	$(CT)_{22}$	55	106-134	11	4	0.25	0.14	11	7	0.38	0.27
		5' CCGATGCGTTCATTAAAA											
Psfe16	EU919675	5' TET-TTCGCTAAAAGATTCTTCCGTATT	$(GAA)_9(AG)_3AA(AG)_{13}AAAGAA$	55	180-207	11	6	0.39	0.36	11	7	0.44	0.50
		5' AAACCTTTCATGTGCGTTACATCG	$(AG)_{14}AA(AG)AA(AG)_{17}$										
Psfe17	EU919676	5' HEX-AGTGCCAATTCTAAACATTATCGC	$(TC)_{26}$	55	111-154	11	5	0.39	0.28	11	9	0.43	0.41
		5' ATCGTGCTGTTAGAATGATGGACC											
Psfe18	EU919677	5' HEX-TTTTGATAATGACAGGTTTTGGTA	$(TC)_{19}$	55	128-150	11	4	0.08	0.09	11	8	0.46	0.50
		5' ATAATGCATTCCGATTGACTGTGC											
Psfe19	EU919678	5' TET-TCACAAAACGTCTTGAAACTTTCC	$(AG)_{32}$	55	113-174	10	8	0.41	0.35	11	5	0.29	0.32
		5' TAAAGCAAAAGAGATTCTACCCTA											
Psfe20	EU919679	5' HEX-ACTCTGAATTGTTGCATTGTTTGC	$(TC)_{10}GTTCTTTCCG(TCTG)_2$	55	159-169	11	3	0.08	0.00	11	5	0.34	0.19
		5' CCATTACGAATATTCAAATACGTG	$(TC)_3TT(TC)_3TT(TC)_{10}$										
Psfe21	EU919680	5' 6-FAM-TCGCCGGAGATAGGGAGGAAC	$(GA)_3AA(GA)_{20}$	55	100-132	11	5	0.37	0.45	11	3	0.24	0.32
		5' TAAGGAGCGTGGAAGTTAGC											

Table 3.6.2 Primer sequences and characteristics of additional microsatellite loci.
Ten additional primer pairs were developed for *P. ferrugineus* and tested on a different set of individuals derived from a total of four colonies. The repeat motif is given for the cloned allele. GB, GenBank; T_a, annealing temperature; N, number of genotypes that amplified from twelve individuals of each population screened / number of colonies screened; A, observed number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; * significant deviation (P<0.001) between expected and observed heterozygosities based on Hardy-Weinberg probability tests, † Hardy-Weinberg probability tests not possible.

						Puerto Escondido				Matias Romero			
Locus	GB accession	Primer sequence	Repeat motif	T_a (°C)	Size range (bp)	N	A	H_E	H_O	N	A	H_E	H_O
Psfe01	EU864155	5' TET-AACAAACGACCAGGTCGGC	$(CT)_2AT(CT)_{11}TT(CT)_2$	58	84-114	12/2	2	0.08	0.08†	12/2	4	0.68	0.60
		5' TGGTGCAAAACTGTAAACGACGAT											
Psfe02	EU864156	5' HEX-GTCGTTTACAGTTTTGCACCATGC	$(TC)_{12}TT(TC)_7TT(TC)_6$	58	92-138	12/2	3	0.45	0.25	12/2	6	0.81	0.40
		5' TCTCGTGTTTGGAATCAGTAAAGC	$TA(TC)_2TA(TC)_3$										
Psfe03	EU864157	5' 6-FAM-TGTACGGTTAACGTGATACGCTGC	$(CT)_{30}$	58	98-140	12/2	2	0.23	0.25	12/2	5	0.75	0.56
		5' AGTTCTACTTCCGGTATCACCTGC											
Psfe04	EU864158	5' HEX-GTACAATGGATCTCGTTGACG	$(GA)_3AA(GA)_9GC(GA)_{10}$	50	113-143	12/2	3	0.36	0.42	12/2	5	0.70	0.80
		5' TCAAACAGCTTTTCCAGTCTA											
Psfe05	EU864159	5' 6-FAM-TCTTTTATGAAATGGAAGGCC	$(GA)_{23}$	55	107-125	12/2	2	0.08	0.08†	12/2	5	0.80	0.89
		5' TATCGTTGTCAGGTGTATTCC											
Psfe09	EU864163	5' TET-AAAATATCGAAAAATACATAACACGGC	$(GA)_{45}$	55	143-185	12/2	4	0.72	0.75*	12/2	6	0.74	0.89
		5' TTGCGCGTTATCTGTTTCGCAAGC											
Psfe10	EU864164	5' TET-CGCGGATGTTTTCTCGATAATCTC	$(TC)_{30}TT(TC)_6$	55	130-151	12/2	7	0.70	0.75	12/2	4	0.65	0.70
		5' TATTCGGACGGAGCTGAACCCTGC											
Psfe11	EU919670	5' TET-TAGATGATGATATGAGCATGTTGG	$(GA)_{12}AA(GA)_5AA(GA)_{11}$	55	115-133	12/2	2	0.16	0.00	12/2	6	0.86	0.89
		5' GTATCTAAAATAACTGAGAAAGC											
Psfe12	EU919671	5' 6-FAM-ATTTGACGATACACCGC	$(CT)_{11}TT(CT)_5$	55	150-156	12/2	3	0.69	0.50	12/2	1	-	-
		5' TAACAACCGGTACGTTTCTCC	$TT(CT)_3GT(CT)_6$										
Psfe22	EU919681	5' HEX-TAGAGGAACATGGAGAACGGCG	$(AG)_{19}AA(AG)_3$	55	148-174	12/2	1	-	-	12/2	5	0.81	0.67
		5' TAAGGAGCGTGGAAGTTAGC											

Table 3.6.3 Cross-species testing of microsatellite primers.
Primers developed for *Pseudomyrmex ferrugineus* were tested on *P. mixtecus* and *P. peperi*. Shown are only those loci that amplified in at least one of the two species. N, number of genotypes screened/number of colonies screened; A, observed number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; * significant deviation (P<0.001) between expected and observed heterozygosities based on Hardy-Weinberg probability tests, † Hardy-Weinberg probability tests not possible.

	P. mixtecus					*P. peperi*				
Locus	Size range (bp)	N	A	H_E	H_O	Size range (bp)	N	A	H_E	H_O
Psfe06	93	20/3	1	-	-	85-93	24/5	4	0.59	0.54
Psfe13	164-166	20/3	2	0.33	0.30	179-187	24/5	2	0.51	0.17*
Psfe14	126-146	20/3	8	0.77	0.55*	136-154	24/5	7	0.84	0.75*
Psfe15	109-127	20/3	4	0.62	0.35*	105-143	24/5	7	0.78	0.58*
Psfe16	160-210	20/3	6	0.83	0.95*	152-170	24/5	6	0.84	0.54*
Psfe17	110-150	20/3	5	0.73	0.80	112-146	24/5	6	0.80	0.42*
Psfe18	126-130	20/3	4	0.63	0.50	132-154	24/5	5	0.56	0.46
Psfe19	120-166	20/3	7	0.64	0.55*	101-103	24/5	2	0.08	0.08†
Psfe20	163-171	20/3	6	0.60	0.70	163-191	24/5	7	0.81	0.54*
Psfe21	103-109	20/3	5	0.57	0.45	124-130	24/5	4	0.64	0.13*

3.7 Social structure of a mutualist and a parasite

3.7.1 Genetic diversity

All loci were polymorphic in each plot. Within each plot, two to eleven alleles were found per locus for *Pseudomyrmex ferrugineus* and two to 19 alleles per locus for *P. gracilis* (Tables 3.7.1 and 3.7.2). Genetic diversity among workers of each acacia was high. Heterozygosity ranged from 0.07 to 1.00 (mean 0.67) in *P. ferrugineus* and from 0.29 to 0.96 (mean 0.73) in *P. gracilis*. Tests for conformity of genotype proportions to Hardy-Weinberg expectations revealed that most loci showed significant deviation ($P<0.05$, Tables 3.7.1 and 3.7.2). These deviations can be explained, since workers inhabiting the same acacia are related and not independent samples. Among the twelve loci analyzed for *P. ferrugineus*, there was evidence for null alleles in plot PFER1 at two loci (Psfe17 and Psfe20) and in plot PFER2 at three loci (Psfe08, Psfe17 and Psfe20). For *P. gracilis*, evidence for null alleles was given at the loci Psgr03 in plot PGRA1 as well as Psgr05 and Psgr12 in plot PGRA2. According to MICROCHECKER, null alleles may be present at the respective loci due to homozygote excess based on Hardy-Weinberg equilibrium. The tests for null alleles were repeated using only one individual per sampled tree and consecutively no evidence for null alleles was given in any of the plots. Thus, no locus was discarded for further analyses.

Table 3.7.1 Genetic diversity measures for *P. ferrugineus.*
Two different plots (PFER1 and PFER2) were analyzed separately. N denotes the total number of female individuals for each population; A denotes observed number of alleles found at each locus from each population; H_E, expected heterozygosity; H_O, observed heterozygosity; * significant deviation according to Hardy-Weinberg probability test (P<0.05).

	PFER1 (N=44)			PFER2 (N=47)		
Locus	A	H_E	H_O	A	H_E	H_O
Psfe14	5	0.68	0.86*	11	0.88	0.96*
Psfe17	6	0.77	0.57*	10	0.87	0.85*
Psfe20	3	0.25	0.07*	5	0.70	0.47*
Psfe21	5	0.69	0.93*	3	0.46	0.62*
Psfe15	4	0.19	0.20	9	0.81	0.64*
Psfe16	6	0.76	0.73*	8	0.86	1.00*
Psfe18	3	0.19	0.21	9	0.87	1.00*
Psfe19	9	0.74	0.66*	6	0.62	0.64*
Psfe06	4	0.65	0.68*	10	0.86	0.83*
Psfe07	3	0.60	0.49*	8	0.84	1.00*
Psfe08	2	0.50	0.52	8	0.84	0.70*
Psfe13	3	0.65	0.61*	11	0.88	1.00*
Total	53			98		
Mean	4.4	0.56	0.54	8.1	0.79	0.81

Table 3.7.2 Genetic diversity measures for *P. gracilis.*
Two different plots (PGRA1 and PGRA2) were analyzed separately. N denotes the total number of female individuals for each population; N_A denotes observed number of alleles found at each locus from each population; H_E, expected heterozygosity; H_O, observed heterozygosity; * significant deviation according to Hardy-Weinberg probability test (P<0.05).

	PGRA1 (N=48)			PGRA2 (N=48)		
Locus	A	H_E	H_O	A	H_E	H_O
Psgr03	8	0.80	0.62*	8	0.82	0.83*
Psgr04	12	0.89	0.94*	11	0.86	0.91
Psgr05	7	0.61	0.54*	5	0.67	0.51*
Psgr06	2	0.43	0.63*	2	0.25	0.29
Psgr07	7	0.81	0.96*	7	0.71	0.80*
Psgr09	4	0.62	0.67*	3	0.66	0.52
Psgr10	13	0.89	0.88*	19	0.91	0.92*
Psgr11	8	0.75	0.83*	9	0.74	0.68
Psgr12	9	0.82	0.85*	13	0.81	0.67*
Total	80			64		
Mean	8.9	0.74	0.77	7.1	0.71	0.68

3.7.2 Population genetic structure

AMOVA and pairwise genetic distance (F_{ST}) results were similar between the four different plots. For the mutualist acacia-ant *Pseudomyrmex ferrugineus*, 37.48% variation was found between groups (group comprise all ants derived from one acacia) and 62.53% variation was found within groups according to AMOVA in plot PFER1. The portion of 89% of F_{ST} values showed significant difference between pairs of groups, i.e., 25 of 28 group pairs were genetically significantly different (supplementary Table 2, page 143). Significant group pairwise F_{ST} values averaged 0.40±0.08 (N=25, range 0.25-0.55). In plot PFER2, 30.19% variation was among groups and 69.80% variation was within groups according to AMOVA. Again, 89% of F_{ST} values were significantly different (supplementary Table 3, page 144). Significant group pairwise F_{ST} was lower as compared to plot PFER1 and averaged 0.33±0.05

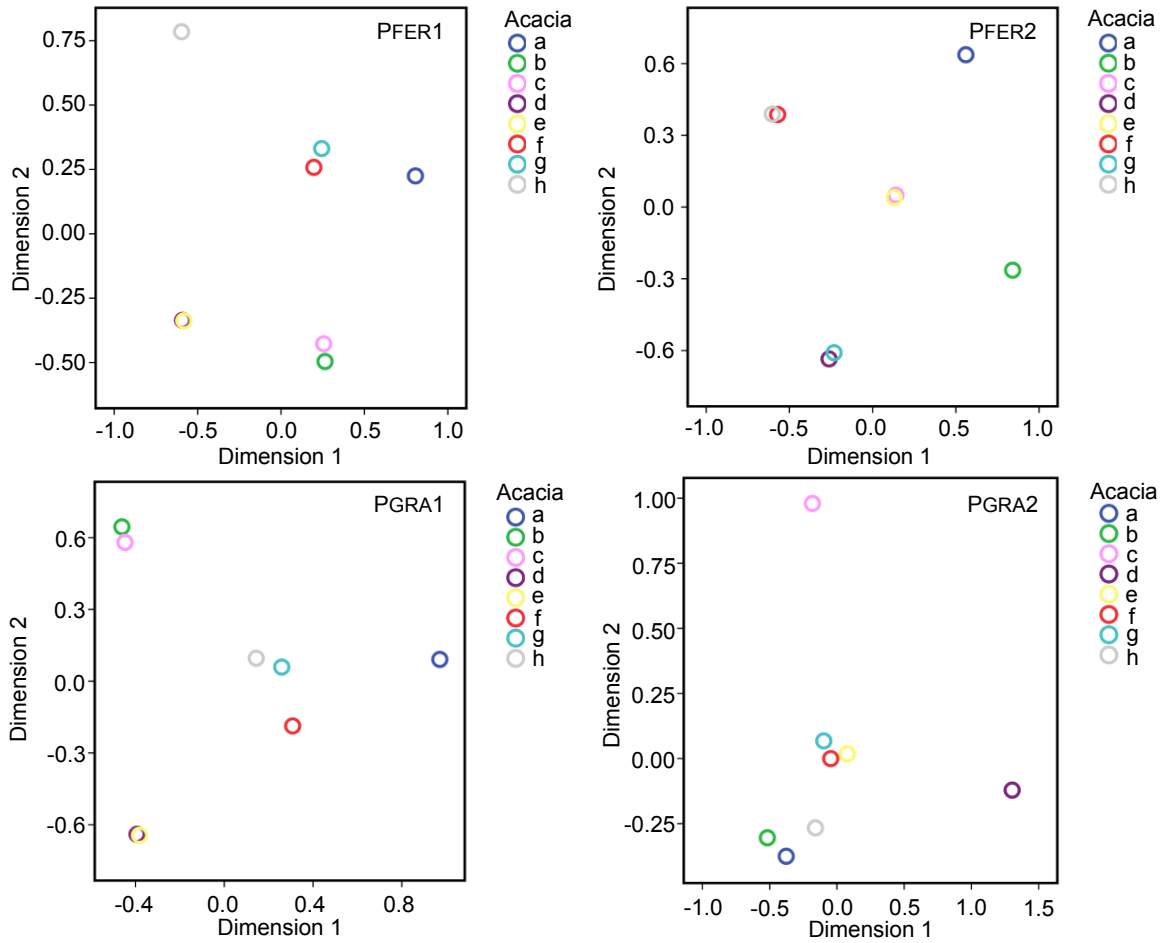


Fig. 3.7.1 Genetic distances between workers of eight acacias in each plot.
Distances were drawn using multidimensional scaling (MDS) to represent the genetic distance between the eight colonies in each plot. MDS is based on F_{ST} genetic distances. Pairwise genetic distances can be found in supplementary Tables 2-5.

(N=25, range 0.26-0.41). For *P. gracilis*, 23.33% variation was among and 76.67% variation was within groups in plot PGRA1. 89% of F_{ST} values were significant (supplementary Table 4) and these averaged 0.25±0.08 (N=25, range 0.07-0.39). In plot PGRA2, 15.59% variation was detected among and 84.41% within groups. With 75%, a lower portion of F_{ST} values were significant (supplementary Table 5) and significant pairwise F_{ST} averaged 0.19±0.10 (N=21, range 0.06-0.35). Pairwise differences of F_{ST} values of each plot were illustrated using multidimensional scaling (Fig. 3.7.1).

3.7.3 Genetic structure and relatedness

All sampled individuals from one plot were grouped into full-sib (monoandry) and half-sib families (polyandry) using COLONY. When assuming monoandry, COLONY detected six full-sib families in plot PFER1. Three family groups consisted of individuals that were collected from two acacias each: 1b and 1c; 1d and 1e, 1f and 1g. However, the individual 1f-1 from acacia 1f was isolated and was the single member of the fourth group. Two additional groups contained all workers collected from a single acacia: 1a and 1h. Multiple mating was not likely in plot PFER1. On average, one monogynous, monoandrous colony inhabited 1.77 acacias, or each acacia housed 0.56 monogynous, monoandrous ant colonies. Plot PFER2 was divided into six family-groups under the assumption of singly mated queens, of which two groups contained ants collected from two acacia trees: 2d and 2g as well as 2f and 2h. The individuals collected from acacias 2c and 2e fell into two groups, but the two groups did not correspond to the origin of the individuals. The individuals from acacia 2a and acacia 2b made up two distinct groups. One monogynous, monoandrous colony inhabited 1.33 trees, i.e., one acacia housed 0.75 monogynous, monoandrous colonies. When assuming multiple mating, the individuals from acacias 2c and 2e formed one half-sib family-group containing two full-sib family groups. This means that the two trees are either inhabited by the offspring of one queen that mated twice or the offspring of two closely related queens, each mated once, colonize two acacias and workers admix.

When looking at the family structure of *Pseudomyrmex gracilis*, 15 full-sib family groups were detected in plot PGRA1. Two of these groups were composed of ants derived from two acacias each: 1b+1c and 1d+1e. One group contained all ants collected from acacia 1a. The remaining twelve groups each contained one to three workers collected from acacias 1f, 1g and 1h. On average, one monogynous, monoandrous colony inhabited 0.53 acacias and one acacia housed an average of 1.88 monogynous, monoandrous *P. gracilis* colonies in plot PGRA1. When assuming multiple mating in this plot, seven half-sib family groups are formed from the 15 full-sib groups. The three largest groups (1b+1c; 1d+1e and 1a) remain unchanged. The members collected from the three acacias 1f, 1g and 1h are grouped into four half-sib families containing twelve half-sib families. In plot PGRA2, a total of 21 full-sib family groups were formed. All individuals sampled off acacia 2c and all individuals sampled off acacia 2d each formed one full-sib family group. One group was formed by five

individuals sampled from acacias 2a, 2b and 2g. Two groups contained four ant individuals each, of which one group comprised ants from acacias 2b and 2e and the other group comprised ants from acacia 2e and 2f. One group contained three ant workers sampled from acacia 2g. Four groups contained two members each and ten groups contained one member each. Thus, an average of 2.63 monoandrous, monogynous colonies of *P. gracilis* inhabited one tree and each monoandrous, monogynous colony inhabited 0.38 acacias. Under the assumption of multiple mating, eight half-sib family groups were formed containing a total of 21 full-sib family groups. Twelve of 18 ant individuals from acacias 2a, 2b and 2h were gathered in one of the half-sib family groups and nine of 18 workers collected from colonies 2e, 2f and 2g. Four members of acacia 2a fell into one half-sib family.

The overall relatedness was estimated among workers derived from one acacia (Table 3.7.3). For *P. ferrugineus* plot PFER1, relatedness was lowest among workers from acacia 1f with 0.49±0.31 (mean±SD) and highest among workers from acacia 1a with 0.89±0.08. According to T-test relatedness values were not significantly different from 0.75 (as among nestmates in monogynous singly mated colonies) among workers from acacias 1c (T=0.196, df=9, P=0.849), 1d (T=0.638, df=14, P=0.534) and 1g (T=-0.484, df=9, P=0.640). Results were similar in plot PFER2 with average relatedness ranging from 0.44±0.27 (acacia 2e) to 0.77±0.08 (acacia 2a) (Table 3.7.3). Values were not significantly different from 0.75 among workers from acacias 2a (T=1.154, df=14, P=0.268), 2b (T=-0.730, df=14, P=0.477), 2d (T=-1.118, df=9, P=0.293), 2f (T=-0.333, df=9, P=0.747) and 2h (T=0.566, df=14, P=0.580). Observed relatedness among workers of *P. gracilis* plot PGRA1 derived from one tree was moderate to high among workers from acacias 1a, 1b, 1c, 1d and 1e with average relatedness varying from 0.65±0.13 among workers of acacia 1e to 0.82±0.02 among workers of acacia 1a. However, in the colonies 1f, 1g and 1h, relatedness was lower ranging from 0.11±0.18 to 0.39±0.31. Regression relatedness was significantly different from 0.75 in all cases. Looking at plot PGRA2, values of relatedness among workers derived from one acacia individual were rather low. Average values were extremely low among workers from trees 2e, 2f, 2g and 2h ranging from 0.00±0.18 to 0.14±0.35. Moderate to high values were found in colonies 2a through 2d with the highest value of 0.74±0.12 (acacia 2d) (Table 3.7.3). Among workers from acacia 2d, values were not significantly different from 0.75 according to T-test (T=-0.173, df=14, P=0.865).

Table 3.7.3 Relatedness among the workers sampled from each acacia.
The regression relatedness (R-value) is given as mean±SD. (*) indicates significant deviation of relatedness among workers from 0.75 according to T-test. N denotes number of individuals included of each acacia.

Acacia	R-value	N
Plot PFER1		
1a	0.89±0.08*	6
1b	0.82±0.09*	6
1c	0.76±0.08	5
1d	0.76±0.07	6
1e	0.82±0.07*	5
1f	0.49±0.31*	5
1g	0.73±0.14	5
1h	0.83±0.06*	6
Plot PFER2		
2a	0.77±0.08	6
2b	0.73±0.11	6
2c	0.46±0.25*	6
2d	0.70±0.13	5
2e	0.44±0.27*	6
2f	0.74±0.14	6
2g	0.68±0.08*	6
2h	0.76±0.09	6
Plot PGRA1		
1a	0.82±0.09*	6
1b	0.66±0.14*	6
1c	0.67±0.15*	6
1d	0.67±0.13*	6
1e	0.65±0.13*	6
1f	0.39±0.31*	6
1g	0.28±0.23*	6
1h	0.11±0.18*	6
Plot PGRA2		
2a	0.36±0.16*	6
2b	0.40±0.22*	6
2c	0.62±0.26*	6
2d	0.74±0.12	6
2e	0.12±0.20*	6
2f	0.00±0.18*	6
2g	0.14±0.35*	6
2h	0.11±0.21*	6

3.7.4 Correlations of geography, behavior, chemistry and genetics

Partial correlation analysis showed that the genetic and chemical distances between the colonies were significantly associated in all four plots (Mantel test; plot PFER1: $r_{gen,chem}$=0.585, P=0.0015; plot PFER2: $r_{gen,chem}$=0.384, P=0.0070; plot PGRA1: $r_{gen,chem}$=0.613, P<0.001; plot PGRA2: $r_{gen,chem}$=0.729, P=0.0250). The geographic and genetic distances between the colonies in each plot were not significantly associated in three plots, but in plot PFER2. Also, the chemical distances between colonies were not significantly correlated with geographic or behavioral distances in all plots but in PFER2. The behavioral distance between workers derived from one acacia was significantly correlated with geographic, chemical and genetic distance in plots PFER2 and PGRA1, but not in plots PFER1 and PGRA2. For detailed Mantel test results see supplementary Figs. 1-4 (pages 161-164).

3.8 Polygyny in the acacia-mutualist *Pseudomyrmex peperi*

3.8.1 Genetic diversity between colonies

At each microsatellite locus, two to five alleles were found when combining both supercolonies (18 and 17 alleles in the supercolonies PPEP103 and PPEP132, respectively, Table 3.8.1). A total of 27 private alleles (77%; i.e., unique to a single supercolony) across all eight microsatellite loci was found. 43.92% variation was found among supercolonies (SS=192.02; df=1, P<0.001), 1.55% variation among sub-samples within supercolonies (SS=56.24, df=27, P=0.937), no variation among individuals within sub-samples (SS=243.90, df=163, P<0.001) and 57.21% variation among all individuals (SS=317.00, df=192, P<0.001) according to AMOVA (see Table 3.8.2 for summary of statistics and results of fixation indices). One sub-sample refers to all ants derived from the same individual acacia shoot.

Table 3.8.1 Genetic diversity measures of *P. peperi*.
Each supercolony was analyzed separately and only female genotypes were included. N denotes the total number of female individuals (queens, virgin queens, workers) for each supercolony; A denotes observed number of alleles found at each locus from each supercolony; H_E, expected heterozygosity; H_O, observed heterozygosity; No significant deviations according to Hardy-Weinberg probability test after Bonferroni-correction for multiple tests, P<0.05.

	712 (N=54)			732 (N=139)		
Locus	A	H_E	H_O	A	H_E	H_O
Pf14	3	0.64	0.64	2	0.39	0.39
Pf17	2	0.43	0.47	3	0.66	0.76
Pf20	3	0.66	0.69	2	0.46	0.46
Pf21	2	0.40	0.35	1	—	—
Pf15	1	—	—	3	0.66	0.71
Pf16	3	0.67	0.65	3	0.66	0.63
Pf18	3	0.65	0.54	1	—	—
Pf19	1	—	—	2	0.42	0.35
Total	18			17		
Mean	2.3	0.58	0.56	2.1	0.54	0.55

Table 3.8.2 Results of hierarchical AMOVA.
Results of hierarchical AMOVA comparing genetic variation at four levels: among supercolonies, among sub-samples (individuals derived from one acacia shoot) within supercolonies, among individuals within sub-samples (i.e., among individuals derived from one acacia shoot), among individuals within sub-samples (individuals derived from one acacia shoot) and among all individuals of *Pseudomyrmex peperi*. Significance was tested against a null distribution of 10,000 random permutations.

Source of variation	df	Sum of squares	Variance component	Percent variation	Fixation index	*P*-value
Among supercolonies	1	192.015	1.26767	43.92	F_{IS} = -0.04915	< 0.001
Among sub-samples within supercolonies	27	56.243	0.04881	1.55	F_{SC} = 0.02769	0.946
Among individuals within sub-samples	163	243.904	-0.07735	-2.68	F_{CT} = 0.43922	< 0.001
Among all individuals	192	317.000	1.65104	57.21	F_{IT} = 0.42795	< 0.001
Total	383	809.161	2.88617	100		

3.8.2 Genetic structure of supercolonies in *Pseudomyrmex peperi*

Six of the eight loci were polymorphic in each supercolony. Within each supercolony, one to three alleles were detected per locus (Table 3.8.1). Allele frequencies were balanced (supplementary Table 11, page 159) with frequencies ranging from 0.17 to 1.00 and were equally distributed among the different social castes: no rare alleles were detected. Despite the low number of alleles, genetic diversity within each colony was high. Heterozygosity ranged from 0.35–0.69 in supercolony PPEP103 and from 0.35–0.76 in supercolony PPEP132. Tests for conformity of genotype proportions to Hardy-Weinberg expectations revealed no significant deviation (P=0.05) after Bonferroni correction (Table 3.8.1). At loci, for which three alleles were present in one supercolony, expected heterozygosities ranged from 0.64 to 0.67. The original expected heterozygosity in the absence of genetic drift is 0.67 (see equations 2 and 3). When two alleles are present, the expected original heterozygosity is 0.44 and the observed values are 0.39 to 0.46 (see equations 2 and 4). These findings suggest almost no drift in each of the two supercolonies and hence a large contribution of the founding female and her daughters. Using MICROCHECKER, there was no evidence for

null alleles at any of the loci in both populations. More importantly, the amplification success in males allows making predictions about null alleles. At the loci Psfe15 and Psfe20, amplification failed at one out of 72 males (1.34%) and at loci Psfe14 and Psfe18 at two loci (2.78%). The two latter loci seemed to be sensitive to low DNA quality, since locus Psfe14 failed to amplify in 13 of 193 females (7.29%) and locus Psfe18 in 4 of 192 cases (2.08%). The loci Psfe15 and Psfe20 each failed to amplify in one case (0.52%). The four loci Psfe16, Psfe17, Psfe19 and Psfe21 amplified in all 264 cases. Thus, results obtained with loci Psfe14 and Psfe18 should be treated with care. However, rerunning analyses under the exclusion of both loci did not lead to different conclusions as given (data not shown). No signal was obtained at any of the eight microsatellite loci for four individuals (three males and one worker). DNA isolation was visualized on 1.5% TBE agarose gels strained with ethidium bromide for these four ant individuals and no positive signal could be detected suggesting failure of DNA isolation.

The expected Heterozygosity H_E is calculated from the equation:

$$H_E = 1 - \sum_{i=1}^{k} p_i^2 \quad \text{(Equation 2)}$$

where p_i is the frequency of the i^{th} of k alleles.

In absence of genetic drift, the frequency of each allele does not change. Given three alleles are present in one colony (two derived from the diploid mother and one derived from the haploid father), the frequency of each allele is 0.33. This equals an expected heterozygosity of 0.67:

$1 - [(0.\overline{3})^2 + (0.\overline{3})^2 + (0.\overline{3})^2] = 1 - 0.\overline{1} - 0.\overline{1} - 0.\overline{1} = 0.\overline{6}$ (Equation 3).

Given two alleles are present in one colony, the frequency of one allele is 0.66 and the frequency of the other is 0.33. This equals an expected heterozygosity of 0.44:

$1 - [(0.\overline{6})^2 + (0.\overline{3})^2] = 1 - 0.\overline{4} - 0.\overline{1} = 0.\overline{4}$ (Equation 4).

3.8.3 Ancestral states reconstruction 2

A molecular phylogeny of *Pseudomyrmex* species, of which the colony structure was known, was established. The mean log likelihood of the Bayesian tree sampling was -11270. In the majority-rule consensus tree shown in Fig. 3.8.1, two main clades within the genus *Pseudomyrmex* can be recognized. One is formed by species of the *gracilis* group, while the other comprises all other species. The *gracilis* group is strongly supported as monophyletic in both analyses (BM 100, pp 1.0). Within the second major clade, *P. salvini* and *P.* spec. PSW-06 take a basal position to one clade that comprises all species of the *ferrugineus* group. *Pseudomyrmex spinicola* and *P. satanicus* are sisters. *Pseudomyrmex nigrocinctus*. *P. ferrugineus*, *P. flavicornis* and *P. mixtecus* form one well-supported group (BM 99, pp 1.0) that is a sister to *P. peperi*.

Among the 21 *Pseudomyrmex* specimens (11 species) included in this study, seventeen (nine species) were monogynous while four (two species) were polygynous. Ancestral character mapping of colony structure on the phylogeny (Fig. 3.8.2) suggest that monogyny is the ancestral state to the *P. ferrugineus* group. Ancestors to the genus *Pseudomyrmex* also are monogynous. Among the taxa included, the analysis suggested that polygyny has evolved independently twice within the *ferrugineus* group, in *P. satanicus* and *P. peperi*.

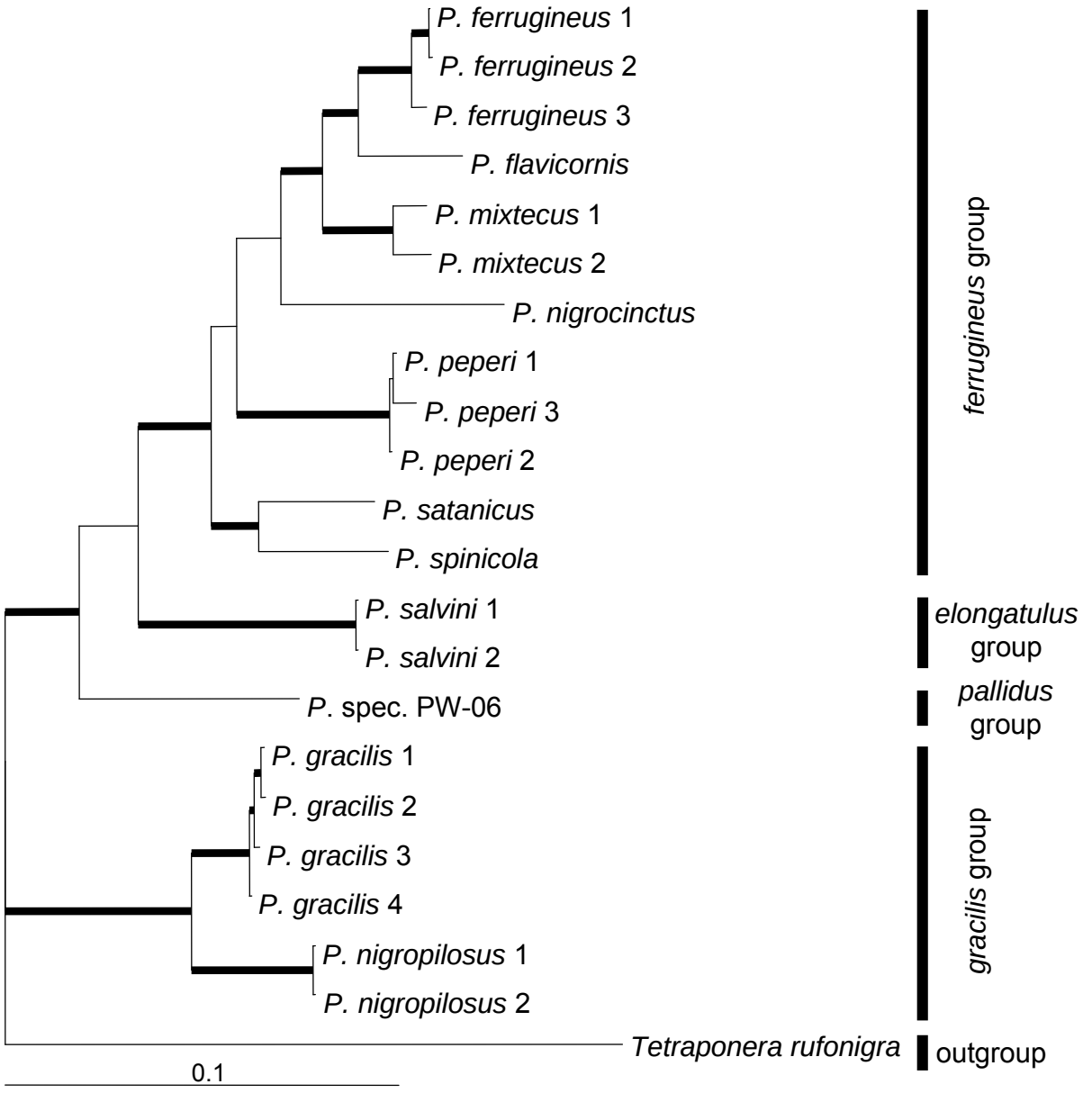


Fig. 3.8.1 Molecular phylogeny of *Pseudomyrmex* acacia-ants.
A Phylogeny of selected *Pseudomyrmex* ants as inferred from a five gene fragments partition analysis (3,313bp). This is a 50% majority rule consensus tree based on 74,000 trees from a B/MCMC tree sampling procedure. Branches with posterior probabilities equal or above 0.95 and ML bootstrap support values above 74% are indicated in bold.

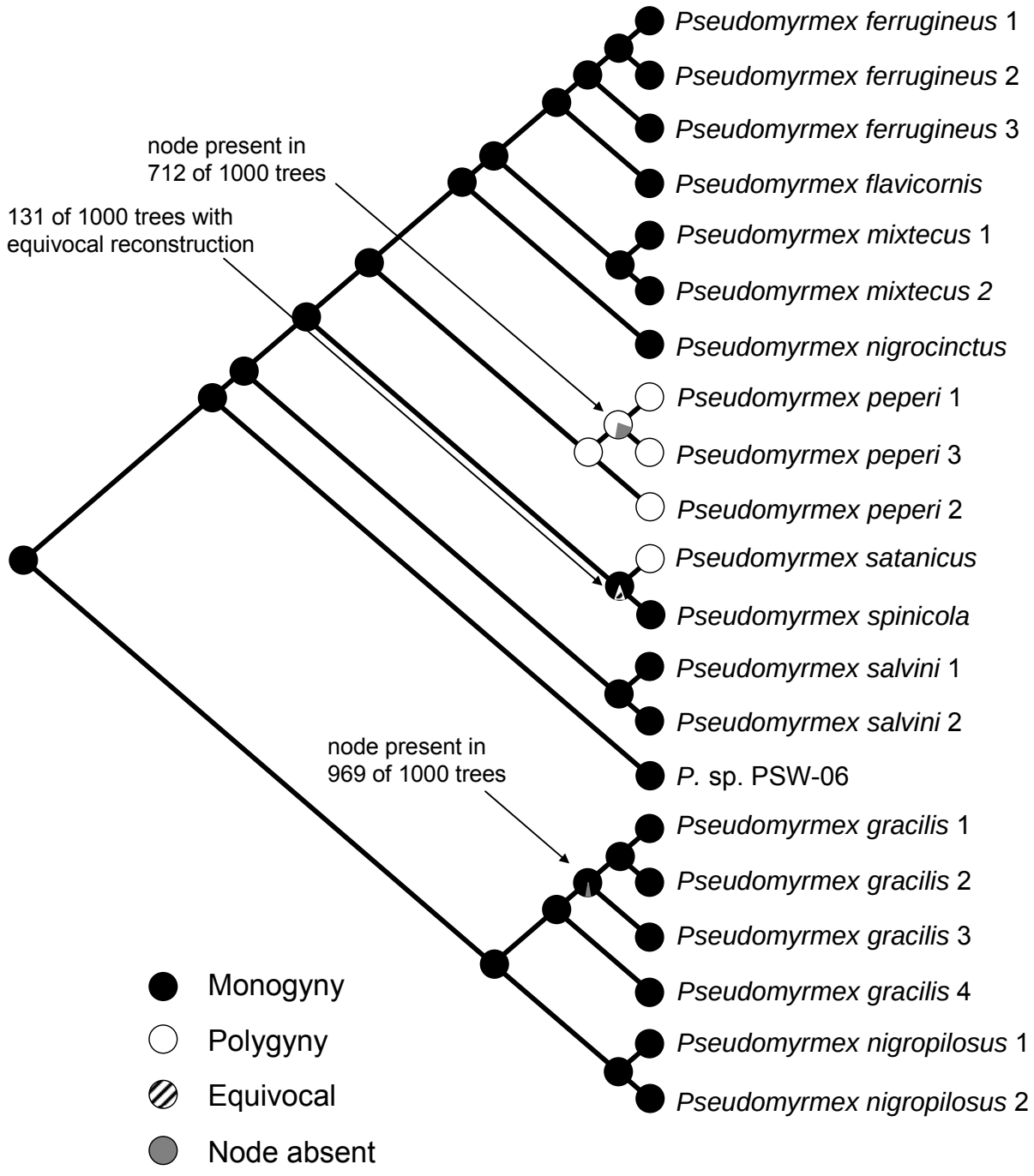


Fig. 3.8.2 Ancestral states reconstruction 2.
Colony structure (monogyny vs. polygyny) of *Pseudomyrmex* species traced on a 1,000 trees inferred from a five gene fragments analysis (shown in Fig. 3.8.1).

4 Discussion

The aim of the present study was to understand mechanisms that stabilize the specific acacia-*Pseudomyrmex* interaction with special emphasis on coevolutionary aspects. I investigated putative adaptations of acacia-ants towards their mutualistic life-style and compared them to parasitic and generalist ants.

4.1 Physiological adaptations of acacia-inhabiting *Pseudomyrmex* ants

First, I aimed at understanding the behavioral, physiological and phylogenetic basis of a filter supposedly stabilizing the mutualism of acacia myrmecophytes with their defending *Pseudomyrmex* ant inhabitants: acacias' secretion of sucrose-free extrafloral nectar (EFN) and the corresponding lack of the sucrose-cleaving enzyme, invertase, in the mutualist ant workers (Heil *et al.* 2005b). As several exploiting ant species have been described for this system, I also aimed at understanding the evolutionary origin of these exploiters. In this context, my goal was to determine whether the exploiters in the system have evolved from mutualists or from generalists.

A comparison of obligate acacia-mutualists with exploiters and generalist species revealed that the digestive capacities (Figs. 3.1.1, 3.1.2) of the various ant species predicted their feeding preferences (Heil *et al.* 2005b; Kautz *et al.* 2009a). Workers of all three mutualist species lacked invertase activity, which could not even be induced when they were feeding on a sucrose-containing diet (Fig. 3.1.2). These ant workers therefore appear dependent on the sucrose-free EFN that is provided by their plant hosts. To investigate the degree of the ants' dependency, long-term experiments would have to be conducted, in which the ants are only fed with sucrose. Sucrose-free EFN, on the other hand, was less attractive to generalist and parasitic ant species, which all possess substrate-inducible invertases (Heil *et al.* 2005b; Kautz *et al.* 2009a). Secretion of EFN without sucrose by obligate acacia ant-plants apparently reduces the competition of their mutualistic *Pseudomyrmex* inhabitants with generalist ants. These findings indicate that sucrose-free EFN functions as a partner choice mechanism allowing the plant to attract the desired ant partner.

However, this filter does not entirely exclude exploiters, since at least three parasitic ant species have been described for acacia myrmecophytes (Clement *et al.*

2008; Janzen 1975; Raine *et al.* 2004), two of which (*P. gracilis* and *P. nigropilosus*) even belong to the same genus as the mutualistic ants. What is the evolutionary history of these species and why does sucrose-free EFN not exclude them from exploitation?

Phylogenetic relationships

In a molecular phylogeny, the two exploiters investigated here clustered within the *gracilis* group, which was well separated from the *ferrugineus* group (Fig. 3.5.1, page 83) — a pattern that confirms earlier findings (Ward & Downie 2005). *Pseudomyrmex gracilis* and *P. nigropilosus* are not closely related to the mutualistic *ferrugineus* group, they consequently must have evolved their association to myrmecophytic acacia plants independently of the mutualists. Surprisingly, the mutualistic acacia-ants (*ferrugineus* group) formed a paraphyletic group, since the generalist *P*. spec. PSW-54 clustered within the same clade (Fig. 3.5.1, page 83). The male genitalia of this species resemble those of the species of the *ferrugineus* group (P.S. Ward, unpubl. data). This evidence supports the molecular data of the present study (Fig. 3.5.1, page 83), showing that this species is closely related to the *ferrugineus* group. Alternative topologies can therefore be rejected.

Ancestral character mapping revealed that *P. gracilis* and *P. nigropilosus* are most likely derived from a generalist ancestor (Fig. 3.5.2, page 88). Another exploiter of acacia myrmecophytes, *Camponotus planatus* (Raine *et al.* 2004) even belongs to a genus that has no known mutualism with acacia. All exploiters of Mesoamerican acacia myrmecophytes that have been described so far, thus, evolved from generalists and not from mutualists. On the other hand, a species with no current obligate association with acacia myrmecophytes (*P*. spec. PSW-54) appears to have evolved from a mutualistic ancestor. Both findings confirm recent observations by Sachs and Simms (2006), who found that mutualists rarely evolve into exploiters, while they reported several cases, in which species with no association to the respective host had a mutualistic ancestor.

Behavioral and physiological basis of the filter

Cafeteria experiments revealed that the ants distinguished among different sugar solutions and that this behavior has a physiological basis (Heil *et al.* 2005b; Kautz *et al.* 2009a). The digestive capacities of workers and larvae were determined by the food sources that are naturally used by the respective ontogenetic stage. First, invertase activity was found in larvae of all species investigated, most probably since larvae of mutualistic *Pseudomyrmex* species feed on food bodies (Janzen 1966) and these do contain sucrose (Heil *et al.* 2004a). Second, workers and larvae of parasites feed also on host-independent food sources (Clement *et al.* 2008), including sucrose-containing plant saps. Thus, all groups of ants turned out to have digestive capacities that are highly adjusted to their respective food source.

The two parasites studied here differ in their life histories, as *P. nigropilosus* represents an obligate — though not defending — inhabitant of acacia myrmecophytes (Janzen 1975), while *P. gracilis* only facultatively exploits acacias. Although all colonies used in the present study were living on *Acacia hindsii*, both parasite species possessed inducible invertase activity (Fig. 3.1.2) and discriminated against glucose and fructose (Heil *et al.* 2005b; Kautz *et al.* 2009a) — the main carbohydrates in the EFN of *A. hindsii* (Heil *et al.* 2005b). The physiology and behavior of the parasites in this respect resembled generalists rather than mutualists. A lack of invertase activity was found only in the *P. ferrugineus* group and ancient character mapping made a loss of invertase activity from workers within this clade likely (Fig. 3.5.2). Workers that lack invertase characterize this specific taxonomic group rather than obligate acacia-ants and represent, thus, a phylogenetic rather than an ecological phenomenon.

Coevolutionary stabilization

Due to the mutualist workers' lack of capacity to digest sucrose, these species would not be able to live on solely sucrose-based diets. Former mutualists that stopped providing the protective service would still rely on the host-derived food resources only. Ceasing the protective service harms the plant, since the acacia plants require ant-mediated protection from being overgrown by competing plants and lianas and

also from herbivores (Janzen 1966; Raine *et al.* 2002). As lack of protection from these threats and the resulting increase in, for example, dead shoot tips (see Clement *et al.* 2008) reduces the amount of host-derived food rewards, cheaters would be strongly counter-selected. Thus, the gain of invertase activity in the nectar of myrmecophytic acacia species and the corresponding loss of the enzyme in the specific *Pseudomyrmex* workers reinforce the cooperation between the two actors and apparently prevent the evolution of cheaters. Remarkably, this adaptation exclusively applies to workers. Since larvae are not able to move off the host plant, a factor that evolved for the exclusion of non-desired species is only required to act against workers, yet not against larvae. However, more information is needed to test this key aspect of the stabilization hypothesis presented here. Future experiments would need to include removal of ants in field studies and quantifying plant survival and fitness compared to plants inhabited by ants.

In contrast, the digestive physiology and behavior of both parasites resembled generalists. The ‘filter’, which apparently serves to discourage generalist ants, might, thus, also reduce the attractiveness of myrmecophyte EFN to more specialized exploiters. But why do parasites still exist in the system? First, the parasites make use of host-independent food resources and reproduce at smaller colony sizes than the mutualists (see also Clement *et al.* 2008). They are, therefore, much less dependent on the state of their acacia host plant than are the mutualists. Second, hollow structures that can be used as nesting space are a generally limiting resource for many tropical ant species (Fonseca 1993; Heil & McKey 2003; Philpott & Foster 2005) and some specific filters excluding non-mutualists from entering these thorns (Brouat *et al.* 2001) would therefore be required to completely protect a myrmecophyte from exploitation.

A coevolutionary scenario

How might the adaptive specialization of ants and plants have taken place in evolutionary terms? As soon as a specialized mutualism — such as the mutualism of acacia myrmecophytes and obligate acacia-ants — has been established, it should be in the host’s interest to exclude less desirable partners from the interaction (Davidson & McKey 1993b). Traces of invertase have also been detected in EFNs of

non-myrmecophytes (Heil *et al.* 2005b). Thus, the high invertase activity of acacia myrmecophytes EFNs represents a quantitative rather than a qualitative change. After establishing the mutualism, acacia myrmecophytes did not have the necessity to attract ants from the vicinity any longer and most probably it was only then that the invertase activity increased in their EFN, since there was no selection pressure on maintaining a highly attractive EFN. In general, highly specialized symbionts, such as endosymbionts, have reduced their metabolic capacities after establishing a symbiosis (Zientz *et al.* 2004). In correspondence, the mutualistic acacia-ants most probably have lost their invertase activity after the plants had increased theirs. Two different hypotheses have been formulated to explain the evolution of this trait in mutualist ants. The neutral mutation hypothesis (Kimura 1968; Wilkens 1988) suggests that loss of invertase activity is caused by random mutations in digestive genes, which gradually accumulate in the absence of selective pressure. In contrast, the adaptation hypothesis (Ayala 2007; Sket 1985) suggests that natural selection causes the loss of invertase activity due to advantages in losing this digestive capacity. The same hypotheses aim at explaining the loss of eyes in cave fish (Jeffrey *et al.* 2003).

Mutualistic *Pseudomyrmex* plant-ants were much more specialized on the host-derived food sources than the congeneric parasitic species. The filter as described here apparently did prevent the successful establishment of cheaters in the acacia-*Pseudomyrmex* association, as no exploiters with an evolutionary history as mutualists have ever been described for this system. Mutualisms are often regarded as being destabilized by cheaters (Axelrod & Hamilton 1981; Herre *et al.* 1999; Trivers 1971). However, parasite species that have no mutualistic ancestor appear to be the much more common problem. Distinguishing cheaters from parasites is important, since the latter can simply be seen as one further strategy by which organisms obtain a useful resource (Bronstein 2001) and since mechanisms for the stabilization of mutualisms against the evolution of cheaters are different from those that exclude parasites. In the system investigated here, the secretion of sucrose-free EFN by the hosts and the corresponding lack of invertase in mutualist workers could successfully prevent the establishment of cheaters. Further studies will have to investigate, however, how the mutualism is protected from the more common threat: the exploitation by parasites.

4.2 The social structure of a mutualist and a parasite

I combined behavioral, chemical and genetic data to compare the social structure of a mutualistic (*Pseudomyrmex ferrugineus*) and a parasitic (*P. gracilis*) acacia-ant. I analyzed the relative importance of these approaches at two different levels: among workers derived from the same host tree and between workers of different hosts.

On the level of individual acacias, for both species fighting of workers that inhabited the same host tree was never observed. This observation on peaceful coexistence of workers inhabiting one acacia was confirmed in experiments at natural sites. Ants of both species never showed aggressive behavior after being experimentally re-transferred onto their original host tree (Fig. 3.2.1, page 75).

Findings on ant behavior are in line with analyses of cuticular hydrocarbon profiles. For both *Pseudomyrmex* species investigated, comparative analyses of cuticular hydrocarbons revealed that chemical profiles of workers inhabiting the same tree were always very similar (Fig. 3.3.2, page 79). Cuticular compounds are considered crucial recognition cues that allow social insects for discrimination of non-nestmates (Howard & Blomquist 2005; Vander Meer & Morel 1998).

These compounds possess a genetic and an environmental component. Inhabiting the same host plants represents exactly the same environment to the respective ant workers and the hosts confer a specific cuticular blend to the inhabiting ants, regardless of their genetic origin (Debout *et al.* 2009). Debout and co-workers (2009) observed that after being experimentally placed onto a different tree, workers of the plant-ant *Cataulacus mckeyi* start to rub antennae on leaves for ‘odor capture’ and by this behavior try to avoid being attacked by resident ants. For *P. gracilis* no aggression and a common cuticular blend of workers inhabiting the same acacia regardless of the ants’ genetic identity was observed. Relatedness among these workers was low to moderate (R ranging from 0.00 to 0.40) in nine of 16 cases analyzed. Sibship reconstructions indicated that the offspring of an average of 2.23 queens shared the same host tree. This leads to the suggestion that their common environment conveys a similar blend of cuticular hydrocarbons, which is then responsible for the lack of aggressiveness among non-related neighbors. This strategy reduces fighting and increases ant number on hosts and saturates nesting space and potentially enables *P. gracilis* to persist against the mutualist with larger colony sizes (Clement *et al.* 2008).

The total number of individuals inhabiting one host tree is much lower in *P. gracilis* as compared to *P. ferrugineus* (Clement *et al.* 2008). In addition, my present study now demonstrates that the actual output per queen in the parasitic *P. gracilis* is even lower than previously assumed, since not all *P. gracilis* workers inhabiting the same tree were sisters. In the mutualist *P. ferrugineus*, relatedness among individuals that inhabited the same acacia was usually high (R=0.68-0.89) and the most likely scenario is that all workers inhabiting one host are the offspring of one singly mated queen. Moreover, the offspring of one queen of the mutualist *Pseudomyrmex ferrugineus* inhabited up to several acacias (average 1.56) as colonies of the mutualist tend to expand and can colonize up to five closely located host trees (Janzen 1973). Only in the case of workers inhabiting the acacias Pfer2c and Pfer2e, it seems possible that all workers are the offspring of one queen that was mated twice. Thus, in contrast to *P. gracilis*, observations on aggression and cuticular hydrocarbons are in line with genetic data for *P. ferrugineus*. The life style as plant-ant shapes the social structure of the resident ants and allows two competing species to each stabilize their ecological niche in evolutionary terms using different ways.

On the 'inter-host plant' level, overall aggression between ants of different acacias was high (71% of inter-host plant encounters were aggressive). Intruding non-nestmates were usually attacked and chased away or killed by the residents as known for other ant species (Bourke & Franks 1995). The discrimination of non-nestmates occurs following antennal contact and probably involved olfactory perception of cues residing in or on the cuticle (Howard & Blomquist 2005).

The chemical analyses showed that in both species ants inhabiting the same acacia displayed a distinct chemical signature (Fig. 3.3.2, page 79). In three plots (PFER2, PGRA1, PGRA2), all individuals collected from the same tree shared a characteristic cuticular profile and were well separated from most other groups (Fig. 3.3.2, page 79). In plot PFER1, colony boundaries based on cuticular hydrocarbons were somewhat arbitrary and a high portion of individuals were not correctly assigned to their original host tree (supplementary Table 6, page 147). This finding is in line with behavioral data, since many encounters were non-aggressive (Fig. 3.2.1, page 75) and might be due to the high chemical similarity and the short geographic distance of all colonies in this respective plot. Allelic richness and genetic diversity within this plot was low as compared to all other plots (4.4 alleles per locus as compared to 7.1 to 8.9 alleles per locus in the three other plots; mean H_O=0.54 as

compared to mean H_O ranging from 0.68-0.81 in the three other plots; Tables 3.7.1 and 3.7.2, pages 94 and 95) indicating that some colonies might be headed by related foundresses.

The observed chemical patterns between workers of different acacias could be predicted by their genetic distance (P<0.001 according to Mantel-test). Even in *P. gracilis*, where relatedness among individuals inhabiting the same acacia was often low but cuticular profiles were similar, genetic distances between workers of one host (as F_{ST} values) were significantly correlated with chemical distances (P<0.001 in plot PGRA1 and P=0.025 in plot PGRA2 according to Mantel-test; see additional Figs. 3 and 4, see pages 164 f.). Mantel tests do not directly allow testing for the proportions of variance explained by the different predictor variables (Ugelvig *et al.* 2008), but the partial correlation coefficients suggest that genetic distance was the best predictor of chemical distance (see additional Figs. 1-4, pages 162 ff.). This is in line with other studies showing that chemical recognition cues are encoded in the ants' hydrocarbon profiles (Lahav *et al.* 1999; Tsutsui *et al.* 2001) and suggests that these hydrocarbon profiles have a significant genetic component (Dronnet *et al.* 2006; Stuart 1988; Suarez *et al.* 1997). However, considerable amounts of behavior could not be explained by chemical and genetic markers. Other factors, such as colony age and size might influence the aggression potential of a respective colony (Howard & Blomquist 2005).

My multidisciplinary study revealed that there are many ways for ants to meet the requirements in an ant-plant mutualism. High numbers of workers are required when ants colonize myrmecophytes. Acacia plants provide numerous domatia to their resident ants and constantly grow, either as individual trees or via subterraneous stolons as clusters. Consequently, the inhabiting ant colonies are polydomous and a constant growth is necessary to allow for effective plant inhabitation and defense of the host plant against coexisting species. The mutualist *P. ferrugineus* and the parasite *P. gracilis* express different strategies to meet these requirements. *Pseudomyrmex gracilis* is not able to build up large colonies. To make up for this, several colonies share hosts to reduce the risk of displacement by the mutualist and to meet the demands posed by the host tree. This has tremendous effects on recognition cues and ant behavior towards unrelated conspecifics. *P. ferrugineus* queens have a much higher output of offspring and colonies can reach large sizes. The social structure of the mutualist allows for efficient colonization of host plants.

Evaluation of the complementary methods

In this study, I simultaneously compared a mutualistic and a parasitic plant-ant using behavioral, chemical and genetic data. As in other studies, the three methods gave consistent but also complementary results (Ugelvig *et al.* 2008). Thus, both genetic and chemical variation can be used as powerful tool for the analysis of social structures. Microsatellites seem to be more informative to analyze colony boundaries, while behavioral observations of aggression were highly suitable for finally understanding colony boundaries in the ecological sense. Other studies showed that aggression levels between populations depend on the chemical profiles, which themselves are genetically based (Abbott *et al.* 2007; Ugelvig *et al.* 2008).

4.3 Polygyny in the acacia-mutualist *Pseudomyrmex peperi*

Polygyny has been described as an adaptation of invasive ant species to their highly competitive environment (e.g., Ugelvig *et al.* 2008) and has also been found in some mutualistic plant-ant systems (Dalecky *et al.* 2005; Feldhaar *et al.* 2005; Janzen 1973). The question arose whether polygyny has evolved in plant-ant species that are inferior competitors compared to rivaling congeners. Polygyny promotes colony survival and maintenance of a long-term association with an individual host plant or group of host plants, even when the original founding queen dies (Feldhaar *et al.* 2005). This colony structure should be particularly adaptive when there is a high pressure on the rapid colonization of new nesting sites, which then must be inhabited for long time spans and should be even more important for species that are competitively inferior colony founders. High competition exists among different *Pseudomyrmex* species in South Mexico (Kautz *et al.* submitted). However, individual myrmecophytic plants are rarely occupied by more than one ant colony (Davidson *et al.* 1989; Rico-Gray & Thien 1989; Vasconcelos 1993; Yu & Davidson 1997). *Pseudomyrmex peperi* is weaker in establishing new colonies as compared to its congeners: many founding queens of five *Pseudomyrmex* species were observed in parallel on acacia saplings and *P. peperi* foundresses (or young colonies) had completely disappeared after a six-month period (Kautz *et al.* submitted). Queens of this species were also least present in settling ant-free hosts. *Pseudomyrmex peperi* is not a successful colony founder, a limitation that poses an even higher pressure on

its capacities to maintain stable colonies once they have been successfully established.

In Africa, four ant species coexist on the ant-plant *Acacia drepanolobium*, of which the acacia-ant *Tetraponera penzigi* is superior in the early colony establishment, but disappears on mature hosts (Stanton *et al.* 2002, 2005). The authors came to the conclusion that the inferior colony establishers *Crematogaster sjostedti* and *C. mimosae* would dominate in the absence of disturbance in the system (Stanton *et al.* 2005). No 'hard' data exists from my study system in the absence of disturbance, since hackings of host trees by ranchers are regularly observed. However, five acacias that are six to eight years old are known (M. Heil, pers. comm.). All these individual plants are in fact inhabited by *P. peperi* and have never been observed to be inhabited by another acacia-ant. Over the years, both host and the inhabiting ant colony have reached large sizes. It is unlikely that such large colonies were formed recently. In contrast to these observations, *P. peperi* is the least abundant mutualistic inhabitant of mature acacias in the entire study area (pers. obs.). These findings lead to the conclusion that *P. peperi* is a weak colony establisher but strong in forming large colonies on large hosts once the ant was successful at founding a colony.

The relative numbers of larvae and of eggs in supercolonies of *Pseudomyrmex peperi* was much higher as compared to the monogynous species *P. gracilis* and *P. ferrugineus* (Clement 2005; Clement *et al.* 2008). In *P. peperi*, the relative abundance of workers was approximately 14%, while the brood (larvae, pupae and eggs) amounted to ca. 84% (Fig. 3.4.1, page 80; 42% larvae, 9% pupae and 33% eggs, respectively). In contrast, monogynous colonies of species of the same genus contained around 40% workers and 60% brood (Clement 2005). The higher proportion of brood found in *P. peperi* demonstrates a higher growth rate for this polygynous species (Passera *et al.* 1991). Thus, multiple-queen colonies possess an advantage in productivity at the colony-level, which facilitates the evolution of polygyny (Pamilo 1999).

Pseudomyrmex peperi exhibited fewer workers per queen and an intermediate number of brood per queen than the two congeneric species investigated by Clement and co-workers (2008), indicating that the reproductive output per queen is lower as compared to monogynous species (Bourke & Franks 1995; Komene *et al.* 1999; Ross & Keller 1995). For example, Janzen (1973) found 34 to 79 workers per queen

in mature colonies of *Pseudomyrmex veneficus*, values being similar to the findings for *P. peperi*. The plant-ant *Petalomyrmex phylax* shows a ratio of one queen for every 200 workers (McKey 1984) and number of queens is correlated with colony size (Dalecky *et al.* 2005), a pattern that corresponds to the findings of the study presented here. In contrast, queen numbers of the *Macaranga*-mutualist *Crematogaster* morphospecies 2 does not increase with colony growth but remains around seven per colony (Feldhaar *et al.* 2005). As both nesting space and food resources can limit colony growth of obligate plant-ants (Fonseca 1993; Heil *et al.* 2001), the increase in *P. peperi* queen numbers can be explained by the constant growth of the host plant and the increase of nesting space and food sources.

How are these supercolonies of *P. peperi* established? For *P. veneficus*, Janzen (1973) suggested that daughter queens may be re-adopted into the colony at small colony stages. However, genetic tools were missing at Janzen's times and the detailed strategy by which plant-ants can reach these colony sizes remained unexplored. Within each supercolony, no more than three alleles per locus were found (total of 264 individual ants derived from two unrelated supercolonies with eight polymorphic microsatellite loci analyzed). This leads to the conclusion that the *P. peperi* supercolonies investigated here had been founded by one singly mated queen and that polygyny results in intranidal mating, as proposed for other ant species (e.g., Schrempf *et al.* 2005). Given the high number of closely related queens present in the samples, it can be ruled out that unrelated queens are adopted into the colony as described for *Crematogaster* morphospecies 2 (Feldhaar *et al.* 2005). It can also be ruled out that queens mated with unrelated males and returned to nest, as described for other mutualistic ant-plant systems (Dalecky *et al.* 2005; Fonseca 1993; McKey 1984). If one of these strategies applied to *P. peperi*, many more alleles per supercolony would have been found. Due to the extremely large size of colonies, the high polydomy and the apparent migration of individuals between swollen thorns, sib-matings are rare and matings between remote relatives are the rule. The assumption of intranidal mating in *P. peperi* is supported by the fact that both types of alates were found within the same nest: winged males and winged non-physogastric queens (Figs. 2.5.3 and 3.4.1, pages 44 and 80) and that these individuals carried the same alleles as queens and workers. Such conditions may lead to the monopolization of large clusters of hosts by extended family groups composed of multiple generations (Chapuisat & Keller 1999).

If mating indeed takes place mainly or exclusively among alates of the same colony, how does *P. peperi* disperse to other plants? Swarming appears essential for the founding process and the colonization of distant plants, since single queens of *P. peperi* were found in individual thorns (Kautz *et al.* submitted). However, constant colony growth might enable colonies to spread over clusters of host plants and the ant colony could easily follow the vegetative growth of its host plant via budding (acacias largely reproduce vegetatively via subterraneous stolons).

A high genetic differentiation among the two supercolonies was found (77% private alleles). Thus, these two colonies were founded by individuals derived from different gene pools. Even unicolonial invasive species such as the Argentine ant (*Linepithema humile*) that spread over 2,500 kilometers can have colony borders within 30 meters due to the introduction history of the supercolonies (Giraud *et al.* 2002). Moreover, the ratio of 77% private alleles as observed in *P. peperi* is remarkable and suggests that the colonies are derived from different founding colonies. In a study on fire ants (*Solenopsis invicta*) from two supercolonies in Taiwan, the authors found a ratio of 24% private alleles (total of seven private alleles) and concluded that the two supercolonies investigated were derived from two different introductions (Yang *et al.* 2008). Behavioral assays also showed that *P. peperi* is not unicolonial (Kautz *et al.* submitted), as it has been described for invasive ants (e.g., Cremer *et al.* 2008). Thus, the term ‘supercolony’ is employed to describe the colonies of *P. peperi*, since the species forms large aggregations of nests that are non-aggressive to each other, although in this species aggression between nests can occur.

Ancestral states of the colony structure were reconstructed and found polygyny to be the derived state within obligate acacia-ants. In most other taxa, polygyny is also the derived state and has evolved from monogynous ancestors (but see Schrempf & Heinze 2007). Among the taxa included in the present thesis, polygyny evolved twice. This finding is consistent with the predictions made by Helanterä and co-workers (2009), that unicoloniality is an evolutionary dead-end and the case of unicoloniality arising from a unicolonial ancestor is unlikely.

In conclusion, polygyny is interpreted as further evolution towards an extreme specialization as an obligate mutualist. The obligate acacia-ant *Pseudomyrmex peperi* has found a way to be competitive in the long run. I hypothesize that this exceptional life history might be the consequence of a directed coevolutionary

process. In this situation, the growing pattern of the ant colony matches exactly the growth of its host plant and, thus, appears adapted to establish large and constantly growing colonies on a host that also constantly grows. The type of colony structure and breeding system as it was found in the obligate acacia-ant *Pseudomyrmex peperi* may play an important role for species coexistence in a dynamic and competitive habitat of ecologically successful plant-ants.

4.4 Conclusion

I was able to identify physiological, genetic and behavioral adaptations of mutualistic acacia-ants to their specific life style. These obligate acacia-ants are physiologically highly adapted to their myrmecophytic hosts. The ants physiologically depend on the diet provided by their hosts and thus, they are 'tied' to their hosts. In evolutionary terms, this physiological adaptation prevents the mutualists from evolving into parasites. Furthermore, large colonies with high numbers of individuals, which allow for efficient host defense, seem to be a key adaptation of mutualistic acacia-ants. Constant colony growth is possible especially in the polygynous acacia-ant *Pseudomyrmex peperi* and seems to be a higher adaptation towards the life style as acacia-mutualist. High relatedness among individuals colonizing the same host reduces conflicts and increases inclusive fitness of the individuals. The genes and consequently the behavior of the mutualist workers are then passed on to the next generation and allow the mutualism to persist in evolutionary terms. In contrast, ant species that parasitize the mutualism of acacias were not adapted to their hosts. They do not depend on the host plant in physiological terms and decrease plant fitness. These parasites can only persist in evolutionary terms because they coexist with mutualistic ant species that assure the maintenance of myrmecophytic traits in host plant populations.

5 Deutschsprachige Zusammenfassung

Mutualismen sind Interaktionen verschiedener Arten zu gegenseitigem Nutzen. Diese Wechselbeziehungen können Anpassungen der Partner einschließen. Als eine solche gegenseitige Adaptation sezernieren Ameisenakazien Saccharose-freien extrafloralen Nektar (EFN) und die auf den Pflanzen lebenden mutualistischen Ameisenarten haben in Anpassung an ihre Wirtspflanze die Fähigkeit verloren, das Enzym Invertase zur Spaltung von Saccharose zu expremieren. Zudem konnte experimentell gezeigt werden, dass die Aufnahme von Saccharose die Invertase-Aktivität von parasitischen (250%) und generalistischen (300%) Ameisenarten erhöht, nicht aber die von Mutualisten. Im Gegensatz zu adulten Tieren zeigten Larven aller drei untersuchten Ameisengruppen eine induzierbare Invertase-Aktivität (170-310%). Diese während ihrer Ontogenese reduzierte Verdauungskapazität bindet die mutualistischen Ameisenarten physiologisch an ihren Wirt. Andererseits verhindert jedoch der Verlust von Saccharose im EFN nicht die Ausbeutung des Mutualismus durch parasitische Ameisenarten. Anhand einer molekularen Phylogenie basierend auf DNA-Sequenzen wurde nachgewiesen, dass die Parasiten aus generalistischen Arten evolviert sind und nicht aus Mutualisten. Die physiologische Anpassung und die damit einhergehende Abhängigkeit der mutualistischen Ameisen von ihrer Wirtspflanze scheint die Evolution zu einem Parasiten zu verhindern, nicht aber die Ausbeutung des Systems durch Parasiten, welche aus Generalisten evolviert sind.

Die Koloniestruktur mutualistischer und parasitischer Ameisenarten wurde in der vorliegenden Arbeit mit Hilfe von Verhaltensbeobachtungen, chemischen Analysen kutikulärer Kohlenwasserstoffe und genetischen Mikrosatelliten-Daten vergleichend untersucht. Verwandtschaftsanalysen belegten, dass eine Kolonie des Mutualisten *P. ferrugineus* bis zu zwei (ø 1.56) Akazien-Pflanzen besiedelte, wohingegen sich mehrere Kolonien des Parasiten *P. gracilis* (ø 2.23) einzelne Wirtspflanzen teilten. Bei beiden Arten besaßen die Individuen, welche dieselbe Wirtspflanze bewohnten, charakteristische Profile kutikulärer Kohlenwasserstoffe. In Verhaltensexperimenten im Freiland zeigten die Bewohner einer Wirtspflanze keine Aggressivität untereinander, was durch die Profile der kutikulären Kohlenwasserstoffe erklärt werden konnte. Genetik, Chemie und Verhalten von *P. ferrugineus* wiesen somit ähnliche Muster auf, während bei *P. gracilis* die genetische Heterogenität und der z.T. geringe Verwandtschaftsgrad (R_{min}=0.00±0.18) im

Widerspruch zu Chemie und Verhalten standen. Durch die friedliche Koexistenz verschiedener Kolonien des Parasiten *P. gracilis* kann der limitierte Lebensraum „Akazie“ vor Besiedelung durch artfremde, konkurrierende Ameisen geschützt werden. Die Interaktion zwischen Ameisen und Pflanzen hat also Einfluss auf die genetische Identität von Ameisenkolonien, ihre chemischen Profile und ihr Verhalten gegenüber koloniefremden Artgenossen.

In einem kompetitiven Habitat sind große und schnell wachsende Kolonien von Vorteil, weil diese eine besonders effiziente Futtersuche und Nestverteidigung ermöglichen. In der vorliegenden Arbeit wurde untersucht, mit welchen Strategien die mutualistische Ameisenart *Pseudomyrmex peperi* Kolonien etabliert. Es konnte gezeigt werden, dass die Art extrem polygyne Kolonien bildet und so große Gruppen von Wirtspflanzen besiedelt. Mikrosatelliten-Daten zeigten, dass — trotz der großen Anzahl von Königinnen — Kolonien von einer einmal verpaarten Königin gegründet werden und durch Tochterköniginnen, welche mit Männchen aus derselben Kolonie verpaart sind, zu Superkolonien heranwachsen. Basierend auf DNA-Sequenzdaten wurde gezeigt, dass Polygynie innerhalb der mutualistischen Akazien-Ameisen das abgeleitete Merkmal darstellt. Die Polygynie von *P. peperi* ermöglicht es der Art, eine langjährige Beziehung mit ihrer Wirtspflanze einzugehen. Diese Sozialstruktur scheint eine weiterführende Anpassung der Ameisenart an ihre Wirtspflanze zu sein.

In der vorliegenden Arbeit konnten physiologische, genetische sowie verhaltensbiologische Anpassungen mutualistischer Akazien-Ameisen an ihre Lebensweise identifiziert werden. Extreme Koloniegrößen mit hohen Individuenzahlen, welche ihre Wirtspflanze effektiv verteidigen, scheinen eine wichtige Anpassung von Akazien-Ameisen zu sein. Besonders polygyne Arten sind zu einem kontinuierlichen Koloniewachstum fähig. Durch einen hohen Verwandtschaftsgrad unter den Ameisen einer Akazie werden Konflikte reduziert und die Gesamtfitness der Individuen erhöht. Die Gene und daher das Verhalten der mutualistischen Ameisen wird somit an die nächste Generation weiter gegeben und ermöglicht ein Bestehen des Mutualismus in evolutionären Zeiträumen. Ameisen hingegen, die den Mutualismus parasitieren, sind nicht an die Wirtspflanze angepasst. Sie sind nicht von ihrer Wirtspflanze abhängig und reduzieren durch ihr Verhalten die Fitness der Pflanzen. Diese Parasiten können evolutionär nur bestehen, da sie mit den mutualistischen Arten coexistieren und letztere den Erhalt myrmekophytischer Eigenschaften der Wirtspflanzen gewährleisten.

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Appendix

Supplementary Table 1 GPS data of collection sites.
GPS data of collection sites are given for each of eight colonies of the plots PFER1, PFER2, PGRA1, PGRA2.

Acacia	GPS N	GPS W	Elevation (ft)
PFER1			
1a	15°55.601	97°09.083	49
1b	15°55.607	97°09.082	39
1c	15°55.601	97°09.088	44
1d	15°55.595	97°09.091	43
1e	15°55.597	97°09.093	55
1f	15°55.598	97°09.091	55
1g	15°55.599	97°09.090	50
1h	15°55.598	97°09.095	48
PFER2			
2a	17°06.010	94°55.835	474
2b	17°06.022	94°55.855	438
2c	17°06.022	94°55.865	430
2d	17°06.021	94°55.876	403
2e	17°06.024	94°55.864	447
2f	17°06.024	94°55.876	416
2g	17°06.021	94°55.873	447
2h	17°06.022	94°55.875	429
PGRA1			
1a	17°06.013	094°55.855	440
1b	17°06.017	094°55.850	462
1c	17°06.020	094°55.850	473
1d	17°06.016	094°55.842	477
1e	17°06.016	094°55.840	465
1f	17°06.034	094°55.823	496
1g	17°06.038	094°55.831	491
1h	17°06.033	094°55.831	506
PGRA2			
2a	17°06.038	94°55.828	464
2b	17°06.041	94°55.825	467
2c	17°06.047	94°55.827	460
2d	17°06.045	94°55.831	459
2e	17°06.041	94°55.834	460
2f	17°06.037	94°55.842	457
2g	17°06.034	94°55.838	461
2h	17°06.034	94°55.831	465

Supplementary Table 2 Colony differentiation in plot PFER1.
Colony differentiation is based on genetic, chemical, behavioral and geographic pairwise distances of *P. ferrugineus* in plot PFER1.

a) Pairwise genetic distances (F_{ST}) between sampled colonies of *P. ferrugineus* in plot PFER1, using twelve microsatellite loci (Table 3.7.1, Fig. 3.7.1, pages 94 and 96). Bold numbers indicate significance after multiple comparisons, P<0.05.

1a	—							
1b	**0.417**	—						
1c	**0.364**	0.000	—					
1d	**0.495**	**0.390**	**0.368**	—				
1e	**0.493**	**0.390**	**0.362**	0.000	—			
1f	**0.255**	**0.312**	**0.269**	**0.352**	**0.344**	—		
1g	**0.297**	**0.350**	**0.308**	**0.408**	**0.406**	0.000	—	
1h	**0.551**	**0.467**	**0.468**	**0.481**	**0.490**	**0.423**	**0.471**	—
	1a	1b	1c	1d	1e	1f	1g	1h

b) Pairwise chemical (Mahalanobis) distances based on discriminant analysis of the cuticular hydrocarbon profiles between the sampled *P. ferrugineus* colonies of plot PFER1 (Table 3.3.1, Fig. 3.3.2, pages 77 and 79). Bold numbers indicate significance.

1a	—							
1b	**8.32**	—						
1c	**14.82**	1.05	—					
1d	**4.80**	**4.31**	**6.68**	—				
1e	**8.29**	**7.05**	**8.94**	1.62	—			
1f	**6.69**	**2.36**	**4.73**	**4.19**	**3.78**	—		
1g	**4.96**	1.82	**4.46**	2.79	**3.24**	0.21	—	
1h	**25.97**	**7.85**	**5.63**	**17.04**	**20.80**	**15.07**	**14.27**	—
	1a	1b	1c	1d	1e	1f	1g	1h

c) Pairwise behavioral distance (proportions of aggressive encounters) between the sampled *P. ferrugineus* colonies in plot PFER1 (Fig. 3.2.1, page 75).

1a	—							
1b	1.0	—						
1c	0.9	1.0	—					
1d	0.3	1.0	0.5	—				
1e	0.0	1.0	0.2	0.0	—			
1f	0.7	0.5	1.0	1.0	0.6	—		
1g	0.5	0.7	0.5	1.0	1.0	0.0	—	
1h	1.0	1.0	1.0	0.9	0.9	0.5	0.3	—
	1a	1b	1c	1d	1e	1f	1g	1h

d) Pairwise geographic distances (in m) between the study colonies of *P. ferrugineus* in plot PFER1 (Fig. 2.4.1, page 41).

1a	—							
1b	11	—						
1c	8	15	—					
1d	18	27	12	—				
1e	19	26	11	5	—			
1f	15	23	7	5	4	—		
1g	13	20	5	7	6	2	—	
1h	22	28	13	9	4	7	9	—
	1a	1b	1c	1d	1e	1f	1g	1h

Supplementary Table 3 Colony differentiation in plot PFER2.
Colony differentiation is based on genetic, chemical, behavioral and geographic pairwise distances of *P. ferrugineus* in plot PFER2.

a) Pairwise genetic distances (F_{ST}) between sampled colonies of *P. ferrugineus* in plot PFER2, using twelve microsatellite loci (Table 3.7.1, Fig. 3.7.1, pages 94 and 96). Bold numbers indicate significance after multiple comparisons, P<0.05.

2a	—							
2b	**0.328**	—						
2c	**0.302**	**0.306**	—					
2d	**0.392**	**0.399**	**0.285**	—				
2e	**0.300**	**0.300**	0.000	**0.284**	—			
2f	**0.382**	**0.403**	**0.275**	**0.351**	**0.272**	—		
2g	**0.373**	**0.383**	**0.266**	0.000	**0.263**	**0.346**	—	
2h	**0.391**	**0.413**	**0.297**	**0.348**	**0.295**	0.000	**0.348**	—
	2a	2a	2a	2a	2a	2a	2a	2a

b) Pairwise chemical (Mahalanobis) distances based on discriminant analysis of the cuticular hydrocarbon profiles between the sampled *P. ferrugineus* colonies of plot PFER2 (Table 3.3.1, Fig. 3.3.2, pages 77 and 79). Bold numbers indicate significance.

2a	—							
2b	**73.17**	—						
2c	**59.64**	**5.94**	—					
2d	**105.04**	**9.67**	**11.06**	—				
2e	**84.63**	**9.17**	**11.65**	**8.19**	—			
2f	**115.69**	**19.43**	**21.05**	**25.65**	**13.07**	—		
2g	**138.23**	**20.40**	**24.03**	**7.29**	**13.38**	**28.04**	—	
2h	**97.68**	**17.93**	**24.32**	**32.79**	**12.71**	**4.33**	**34.12**	—
	2a	2a	2a	2a	2a	2a	2a	2a

c) Pairwise behavioral distance (proportions of aggressive encounters) between the sampled *P. ferrugineus* colonies in plot PFER2 (Fig. 3.2.1, page 75).

2a	—							
2b	0.70	—						
2c	1.00	1.00	—					
2d	1.00	1.00	1.00	—				
2e	1.00	0.60	0.00	1.00	—			
2f	1.00	0.80	1.00	1.00	0.90	—		
2g	1.00	1.00	0.50	0.00	1.00	1.00	—	
2h	0.40	1.00	1.00	1.00	1.00	0.00	1.00	—
	2a	2a	2a	2a	2a	2a	2a	2a

d) Pairwise geographic distances (in m) between the study colonies of *P. ferrugineus* in plot PFER2 (Fig. 2.4.1, page 41).

2a	—							
2b	41	—						
2c	57	17	—					
2d	75	37	19	—				
2e	57	16	4	21	—			
2f	77	37	19	5	21	—		
2g	70	31	14	5	16	7	—	
2h	74	35	17	2	19	4	3	—
	2a	2a	2a	2a	2a	2a	2a	2a

Supplementary Table 4 Colony differentiation in plot PGRA1.
Colony differentiation is based on genetic, chemical, behavioral and geographic pairwise distances of *P. gracilis* in plot PGRA1.

a) Pairwise genetic distances (F_{ST}) between sampled colonies of *P. gracilis* in plot PGRA1, using nine microsatellite loci (Table 3.7.2, Fig. 3.7.1, pages 95 and 96). Bold numbers indicate significance after multiple comparisons, P<0.05.

	1a	1b	1c	1d	1e	1f	1g	1h
1a	—							
1b	**0.389**	—						
1c	**0.375**	0.000	—					
1d	**0.376**	**0.327**	**0.297**	—				
1e	**0.371**	**0.332**	**0.300**	0.000	—			
1f	**0.233**	**0.291**	**0.271**	**0.239**	**0.240**	—		
1g	**0.197**	**0.265**	**0.250**	**0.249**	**0.239**	**0.084**	—	
1h	**0.168**	**0.170**	**0.163**	**0.210**	**0.212**	**0.075**	0.000	—

b) Pairwise chemical (Mahalanobis) distances based on discriminant analysis of the cuticular hydrocarbon profiles between the sampled *P. gracilis* colonies of plot PGRA1 (Table 3.3.1, Fig. 3.3.2, pages 77 and 79). Bold numbers indicate significance.

	1a	1b	1c	1d	1e	1f	1g	1h
1a	—							
1b	**125.65**	—						
1c	**75.66**	**10.72**	—					
1d	**42.14**	**179.93**	**120.71**	—				
1e	**43.50**	**180.06**	**120.35**	1.66	—			
1f	**64.01**	**67.18**	**66.29**	**103.59**	**106.78**	—		
1g	**82.14**	**39.29**	**46.80**	**120.72**	**123.51**	6.64	—	
1h	**93.83**	**32.99**	**42.52**	**123.53**	**123.87**	**15.22**	**2.61**	—

c) Pairwise behavioral distance (proportions of aggressive encounters) between the sampled *P. gracilis* colonies in plot PGRA1 (Fig. 3.2.1, page 75).

	1a	1b	1c	1d	1e	1f	1g	1h
1a	—							
1b	0.8	—						
1c	1.0	0.0	—					
1d	1.0	1.0	1.0	—				
1e	0.8	1.0	0.9	0.0	—			
1f	0.8	0.9	1.0	0.9	0.9	—		
1g	1.0	1.0	0.9	1.0	1.0	0.8	—	
1h	1.0	0.9	1.0	1.0	1.0	0.7	0.0	—

d) Pairwise geographic distances (in m) between the study colonies of *P. gracilis* in plot PGRA1 (Fig. 2.4.1, page 41).

	1a	1b	1c	1d	1e	1f	1g	1h
1a	—							
1b	11	—						
1c	15	5	—					
1d	23	14	15	—				
1e	27	17	19	3	—			
1f	68	57	54	47	44	—		
1g	62	51	47	45	43	15	—	
1h	56	44	41	31	35	14	9	—

Supplementary Table 5 Colony differentiation in plot PGRA2.
Colony differentiation is based on genetic, chemical, behavioral and geographic pairwise distances of *P. gracilis* in plot PGRA2.

a) Pairwise genetic distances (F_{ST}) between sampled colonies of *P. gracilis* in plot PGRA2, using nine microsatellite loci (Table 3.7.2, Fig. 3.7.1, pages 95 and 96). Bold numbers indicate significance after multiple comparisons, P<0.05.

2a	—							
2b	0.041	—						
2c	**0.267**	**0.245**	—					
2d	**0.342**	**0.347**	**0.348**	—				
2e	**0.129**	**0.126**	**0.192**	**0.189**	—			
2f	**0.061**	**0.103**	**0.187**	**0.246**	0.000	—		
2g	**0.077**	**0.090**	**0.141**	**0.266**	0.006	0.000	—	
2h	0.036	0.050	**0.208**	**0.242**	**0.060**	**0.068**	0.072	—
	2a	2a	2a	2a	2a	2a	2a	2a

b) Pairwise chemical (Mahalanobis) distances based on discriminant analysis of the cuticular hydrocarbon profiles between the sampled *P. gracilis* colonies of plot PGRA2 (Table 3.3.1, Fig. 3.3.2, pages 77 and 79). Bold numbers indicate significance.

2a	—							
2b	**17.46**	—						
2c	**99.04**	**131.38**	—					
2d	**74.64**	**119.89**	**63.27**	—				
2e	**34.27**	**6.32**	**142.19**	**151.00**	—			
2f	**19.95**	**8.63**	**129.94**	**125.07**	**5.83**	—		
2g	**45.76**	**17.78**	**139.79**	**162.21**	**4.75**	**6.61**	—	
2h	**5.28**	**12.87**	**105.23**	**84.09**	**19.60**	**8.66**	**27.52**	—
	2a	2a	2a	2a	2a	2a	2a	2a

c) Pairwise behavioral distance (proportions of aggressive encounters) between the sampled *P. gracilis* colonies in plot PGRA2 (Fig. 3.2.1, page 75).

2a	—							
2b	1.0	—						
2c	1.0	1.0	—					
2d	1.0	1.0	1.0	—				
2e	1.0	1.0	1.0	1.0	—			
2f	1.0	1.0	1.0	1.0	0.9	—		
2g	1.0	1.0	0.0	1.0	1.0	1.0	—	
2h	0.0	0.7	1.0	1.0	1.0	1.0	1.0	—
	2a	2a	2a	2a	2a	2a	2a	2a

d) Pairwise geographic distances (in m) between the study colonies of *P. gracilis* in plot PGRA2 (Fig. 2.4.1, page 41).

2a	—							
2b	7	—						
2c	16	11	—					
2d	13	12	7	—				
2e	11	15	16	9	—			
2f	24	31	32	24	15	—		
2g	19	26	30	23	14	8	—	
2h	9	16	25	20	13	20	12	—
	2a	2a	2a	2a	2a	2a	2a	2a

Supplementary Table 6 Assignment of workers in plot PFER1.
Correct and incorrect posterior assignment of individual workers to their colony based on cuticular hydorcarbon profiles (plot PFER1). Posterior probabilities of each individual worker to be assigned to its original host tree, of which it was sampled, based on the chemical hydrocarbon profile, Cases, in which posterior probabilities were higher for another acacia (bold), this posterior probability, as well as the identification for the other acacia are given.

		Posterior probability for		
Original acacia	Individual	Original acacia	Alternative acacia	Alternative acacia
1a	1a-01	0.83		
1a	1a-02	**0.31**	**0.34**	**1g**
1a	1a-03	**0.02**	**0.47**	**1f**
1a	1a-04	0.88		
1a	1a-05	0.77		
1a	1a-06	0.88		
1a	1a-08	0.97		
1a	1a-09	0.96		
1a	1a-10	0.45		
1b	1b-01	0.48		
1b	1b-02	0.39		
1b	1b-03	**0.26**	**0.58**	**1c**
1b	1b-04	0.53		
1b	1b-05	**0.20**	**0.31**	**1c**
1b	1b-06	0.56		
1b	1b-07	0.30		
1b	1b-08	**0.25**	**0.26**	**1g**
1b	1b-09	0.54		
1b	1b-10	0.36		
1c	1c-01	0.48		
1c	1c-02	0.90		
1c	1c-03	**0.20**	**0.23**	**1b**
1c	1c-04	0.53		
1c	1c-05	**0.35**	**0.41**	**1b**
1c	1c-07	0.51		
1c	1c-08	0.30		
1c	1c-08	**0.12**	**0.43**	**1b**
1c	1c-10	**0.18**	**0.40**	**1f**
1d	1d-01	0.48		
1d	1d-03	**0.15**	**0.28**	**1g**

1d	1d-04	0.24		
1d	1d-08	0.42		
1d	1d-09	0.50		
1d	1d-10	**0.14**	**0.32**	**1g**
1e	1e-01	0.85		
1e	1e-02	0.47		
1e	1e-03	0.56		
1e	1e-04	0.56		
1e	1e-05	0.30		
1e	1e-06	0.82		
1e	1e-07	0.75		
1e	1e-08	**0.26**	**0.30**	**1c**
1e	1e-09	**0.03**	**0.30**	**1g**
1e	1e-10	0.75		
1f	1f-01	0.49		
1f	1f-02	0.49		
1f	1f-03	0.39		
1f	1f-04	**0.04**	**0.44**	**1d**
1f	1f-05	0.44		
1f	1f-06	0.39		
1f	1f-08	**0.30**	**0.33**	**1g**
1f	1f-09	**0.24**	**0.25**	**1e**
1f	1f-10	0.37		
1g	1g-01	**0.05**	**0.91**	**1a**
1g	1g-02	0.40		
1g	1g-03	0.34		
1g	1g-04	**0.33**	**0.55**	**1f**
1g	1g-05	**0.23**	**0.26**	**1e**
1g	1g-07	**0.19**	**0.42**	**1e**
1g	1g-08	**0.13**	**0.49**	**1e**
1g	1g-09	**0.26**	**0.42**	**1a**
1g	1g-10	0.31		
1h	1h-02	0.72		
1h	1h-03	**0.01**	**0.35**	**1b**
1h	1h-04	1.00		
1h	1h-07	**0.23**	**0.41**	**1b**
1h	1h-08	**0.01**	**0.31**	**1b**
1h	1h-09	1.00		

Supplementary Table 7 Assignment of workers in plot PFER2.
Correct and incorrect posterior assignment of individual workers to their colony based on cuticular hydrocarbon profiles (plot PFER2). Posterior probabilities of each individual worker to be assigned to its original host tree, of which it was sampled, based on the chemical hydrocarbon profile. Cases, in which posterior probabilities were higher for another acacia (bold), this posterior probability, as well as the identification for the other acacia are given.

Original acacia	Individual	Posterior probability for		
		Original acacia	Alternative acacia	Alternative acacia
2a	2a-01	1.00		
2a	2a-02	1.00		
2a	2a-03	1.00		
2a	2a-04	1.00		
2a	2a-05	1.00		
2a	2a-06	1.00		
2a	2a-07	1.00		
2a	2a-08	1.00		
2a	2a-09	1.00		
2a	2a-10	1.00		
2b	2b-01	0.99		
2b	2b-02	0.91		
2b	2b-03	0.62		
2b	2b-04	**0.46**	**0.54**	**2c**
2b	2b-05	**0.21**	**0.63**	**2c**
2b	2b-06	0.92		
2b	2b-07	0.94		
2b	2b-08	**0.46**	**0.51**	**2e**
2b	2b-09	0.86		
2c	2c-01	0.57		
2c	2c-02	0.56		
2c	2c-03	**0.27**	**0.50**	**2b**
2c	2c-04	0.97		
2c	2c-05	0.98		
2c	2c-06	0.89		
2c	2c-07	0.98		
2c	2c-08	0.99		
2c	2c-09	0.96		
2c	2c-10	0.90		
2d	2d-01	0.97		

2d	2d-04	0.75		
2d	2d-05	0.93		
2d	2d-06	**0.10**	**0.57**	**2c**
2d	2d-08	0.59		
2d	2d-09	0.43		
2e	2e-01	0.48		
2e	2e-02	**0.14**	**0.74**	**2f**
2e	2e-03	0.99		
2e	2e-04	1.00		
2e	2e-05	0.99		
2e	2e-06	0.98		
2e	2e-07	0.53		
2e	2e-08	0.99		
2e	2e-09	**0.22**	**0.61**	**2b**
2e	2e-10	0.93		
2f	2f-01	0.71		
2f	2f-02	0.77		
2f	2f-03	0.86		
2f	2f-04	0.95		
2f	2f-05	0.81		
2f	2f-06	0.82		
2f	2f-07	0.84		
2f	2f-08	0.77		
2f	2f-09	0.91		
2f	2f-10	0.96		
2g	2g-01	1.00		
2g	2g-02	0.97		
2g	2g-03	1.00		
2g	2g-04	1.00		
2g	2g-06	**0.23**	**0.64**	**2d**
2g	2g-07	**0.33**	**0.39**	**2e**
2g	2g-08	**0.20**	**0.79**	**2d**
2g	2g-09	0.48		
2g	2g-10	0.72		
2h	2h-01	0.89		
2h	2h-02	0.95		
2h	2h-03	0.89		
2h	2h-04	**0.48**	**0.51**	**2f**
2h	2h-05	0.99		

2h	2h-07	0.50
2h	2h-08	0.85
2h	2h-09	0.77
2h	2h-10	0.94

Supplementary Table 8 Assignment of workers in plot PGRA1.
Correct and incorrect posterior assignment of individual workers to their colony based on cuticular hydrocarbon profiles (plot PGRA1). Posterior probabilities of each individual worker to be assigned to its original host tree, of which it was sampled, based on the chemical hydrocarbon profile. Cases in which posterior probabilities were higher for another acacia (bold), this posterior probability as well as the identification for the other acacia are given.

		Posterior probability for		
Original acacia	Individual	Original acacia	Alternative acacia	Alternative acacia
1a	1a-01	1.00		
1a	1a-02	1.00		
1a	1a-03	1.00		
1a	1a-04	1.00		
1a	1a-05	1.00		
1a	1a-06	1.00		
1a	1a-07	1.00		
1a	1a-08	1.00		
1a	1a-09	1.00		
1a	1a-10	1.00		
1b	1b-01	0.97		
1b	1b-02	1.00		
1b	1b-03	0.96		
1b	1b-04	1.00		
1b	1b-05	1.00		
1b	1b-06	0.99		
1b	1b-07	0.98		
1b	1b-08	0.99		
1b	1b-09	1.00		
1b	1b-10	0.84		
1c	1c-01	1.00		
1c	1c-02	1.00		
1c	1c-03	1.00		
1c	1c-04	1.00		
1c	1c-05	0.65		
1c	1c-06	0.59		
1c	1c-07	1.00		
1c	1c-08	1.00		
1c	1c-09	1.00		
1c	1c-10	0.70		

1d	1d-01	1.00		
1d	1d-02	0.73		
1d	1d-03	**0.43**	**0.57**	**1e**
1d	1d-04	**0.48**	**0.52**	**1e**
1d	1d-05	0.89		
1d	1d-06	0.66		
1d	1d-07	0.58		
1d	1d-08	**0.28**	**0.72**	**1e**
1d	1d-09	0.51		
1d	1d-10	0.18	0.82	1e
1e	1e-01	0.76		
1e	1e-02	**0.41**	**0.59**	**1d**
1e	1e-03	0.55		
1e	1e-04	0.78		
1e	1e-05	0.61		
1e	1e-06	0.81		
1e	1e-07	0.79		
1e	1e-08	0.81		
1e	1e-09	0.65		
1e	1e-10	0.68		
1f	1f-01	1.00		
1f	1f-02	0.78		
1f	1f-03	0.98		
1f	1f-04	0.99		
1f	1f-05	0.77		
1f	1f-06	0.97		
1f	1f-07	0.81		
1f	1f-08	0.97		
1f	1f-09	**0.25**	**0.52**	**1g**
1f	1f-10	1.00		
1g	1g-01	0.66		
1g	1g-02	0.76		
1g	1g-03	0.76		
1g	1g-04	0.86		
1g	1g-05	0.85		
1g	1g-07	0.62		
1g	1g-08	**0.44**	**0.56**	**1h**
1g	1g-09	0.67		
1g	1g-10	0.80		

1h	1h-01	**0.42**	**0.57**	**1g**
1h	1h-02	**0.45**	**0.54**	**1g**
1h	1h-03	**0.28**	**0.72**	**1g**
1h	1h-04	1.00		
1h	1h-05	0.67		
1h	1h-07	0.86		
1h	1h-08	0.90		
1h	1h-09	0.78		
1h	1h-10	0.71		

Supplementary Table 9 Assignment of workers in plot PGRA2.
Correct and incorrect posterior assignment of individual workers to their colony based on cuticular hydorcarbon profiles (plot PGRA2). Posterior probabilities of each individual worker to be assigned to its original host tree, of which it was sampled, based on the chemical hydrocarbon profile. Cases, in which posterior probabilities were higher for another acacia (bold), this posterior probability, as well as the identification for the other acacia are given.

		Posterior probability for		
Original acacia	Individual	Original acacia	Alternative acacia	Alternative acacia
2a	2a-01	0.50		
2a	2a-02	**0.00**	**0.88**	**2b**
2a	2a-03	0.99		
2a	2a-04	1.00		
2a	2a-05	0.94		
2a	2a-06	0.96		
2a	2a-07	0.98		
2a	2a-08	**0.03**	**0.88**	**2b**
2a	2a-09	1.00		
2a	2a-10	0.98		
2b	2b-03	0.94		
2b	2b-04	0.62		
2b	2b-05	0.99		
2b	2b-06	0.86		
2b	2b-07	0.98		
2b	2b-08	0.50		
2b	2b-09	1.00		
2b	2b-10	0.85		
2c	2c-01	1.00		
2c	2c-02	1.00		
2c	2c-03	1.00		
2c	2c-04	1.00		
2c	2c-05	1.00		
2c	2c-06	1.00		
2c	2c-07	1.00		
2c	2c-08	1.00		
2c	2c-09	1.00		
2c	2c-10	1.00		
2d	2d-01	1.00		
2d	2d-02	1.00		

2d	2d-03	1.00		
2d	2d-04	1.00		
2d	2d-05	1.00		
2d	2d-06	1.00		
2d	2d-07	1.00		
2d	2d-08	1.00		
2d	2d-09	1.00		
2d	2d-10	1.00		
2e	2e-01	0.80		
2e	2e-02	0.92		
2e	2e-03	**0.38**	**0.60**	**2g**
2e	2e-04	**0.08**	**0.87**	**2b**
2e	2e-05	0.96		
2e	2e-06	0.76		
2e	2e-07	**0.37**	**0.61**	**2g**
2e	2e-08	**0.08**	**0.91**	**2b**
2e	2e-09	0.96		
2f	2f-02	0.76		
2f	2f-03	0.69		
2f	2f-04	**0.27**	**0.69**	**2g**
2f	2f-05	0.95		
2f	2f-06	0.62		
2f	2f-07	0.54		
2f	2f-08	**0.12**	**0.83**	**2g**
2f	2f-10	0.90		
2g	2g-01	0.99		
2g	2g-02	0.67		
2g	2g-03	0.88		
2g	2g-04	0.96		
2g	2g-05	0.98		
2g	2g-06	**0.29**	**0.71**	**2e**
2g	2g-07	0.95		
2g	2g-08	**0.48**	**0.51**	**2e**
2g	2g-09	0.68		
2g	2g-10	**0.18**	**0.73**	**2f**
2h	2h-01	0.84		
2h	2h-02	0.55		
2h	2h-03	0.62		
2h	2h-04	**0.18**	**0.82**	**2a**

2h	2h-05	0.90		
2h	2h-06	0.91		
2h	2h-07	**0.35**	**0.62**	**2f**
2h	2h-08	0.98		
2h	2h-09	0.92		

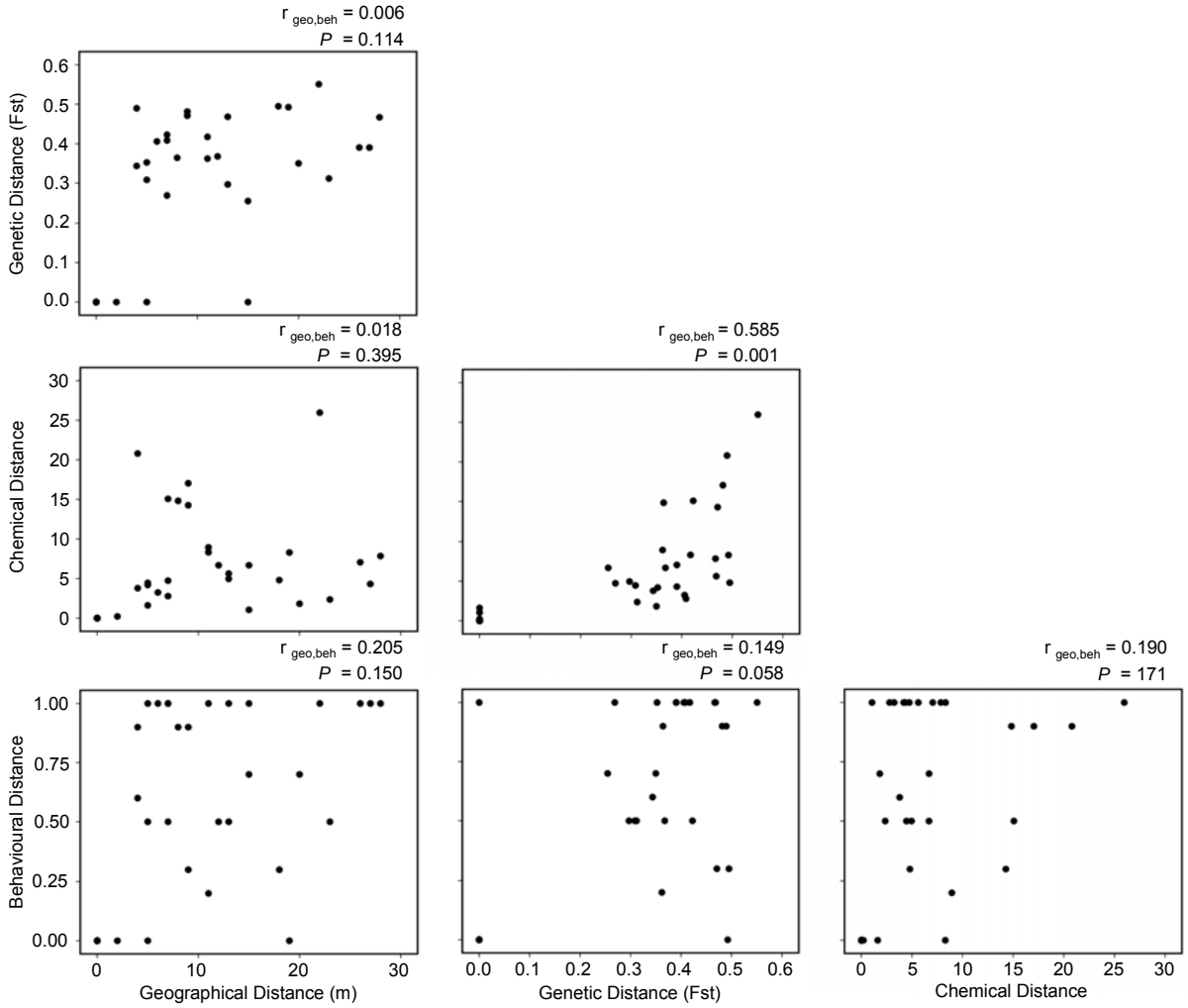
Supplementary Table 10 GPS data of sampled *P. peperi* supercolonies.
GPS data is given for each sub-sample of each of the two *P. peperi* supercolonies.

Acacia-shoot	GPS N	GPS W	Elevation (ft)
PPEP103			
a	15°57.518	97°20.666	110
b	15°57.517	97°20.667	105
c	15°57.522	97°20.664	174
d	15°57.520	97°20.664	178
e	15°57.523	97°20.661	173
f	15°57.522	97°20.661	166
g	15°57.524	97°20.661	154
h	15°57.526	97°20.661	158
i	15°57.527	97°20.659	296
j	15°57.526	97°20.659	169
k	15°57.527	97°20.660	175
l	15°57.528	97°20.658	175
m	15°57.530	97°20.657	163
n	15°57.531	97°20.658	159
0	15°57.533	97°20.658	160
p	15°57.535	97°20.658	158
q	15°57.534	97°20.657	154
r	15°57.536	97°20.656	154
s	15°57.537	97°20.655	162
t	15°57.538	97°20.654	165
PPEP132			
a	15°55.809	97°09.258	13
b	15°55.809	97°09.260	9
c	15°55.811	97°09.259	19
d	15°55.810	97°09.261	15
e	15°55.810	97°09.260	15
f	15°55.808	97°09.261	19
g	15°55.810	97°09.267	11
h	15°55.809	97°09.264	28
i	15°55.809	97°09.265	22
j	15°55.814	97°09.267	11
k	15°55.816	97°09.265	22
l	15°55.814	97°09.267	19
m	15°55.815	97°09.266	20
n	15°55.808	97°09.266	19
0	15°55.809	97°09.264	15
p	15°55.809	97°09.263	11
q	15°55.816	97°09.262	23
r	15°55.816	97°09.263	8
s	15°55.817	97°09.262	20
t	15°55.817	97°09.262	46

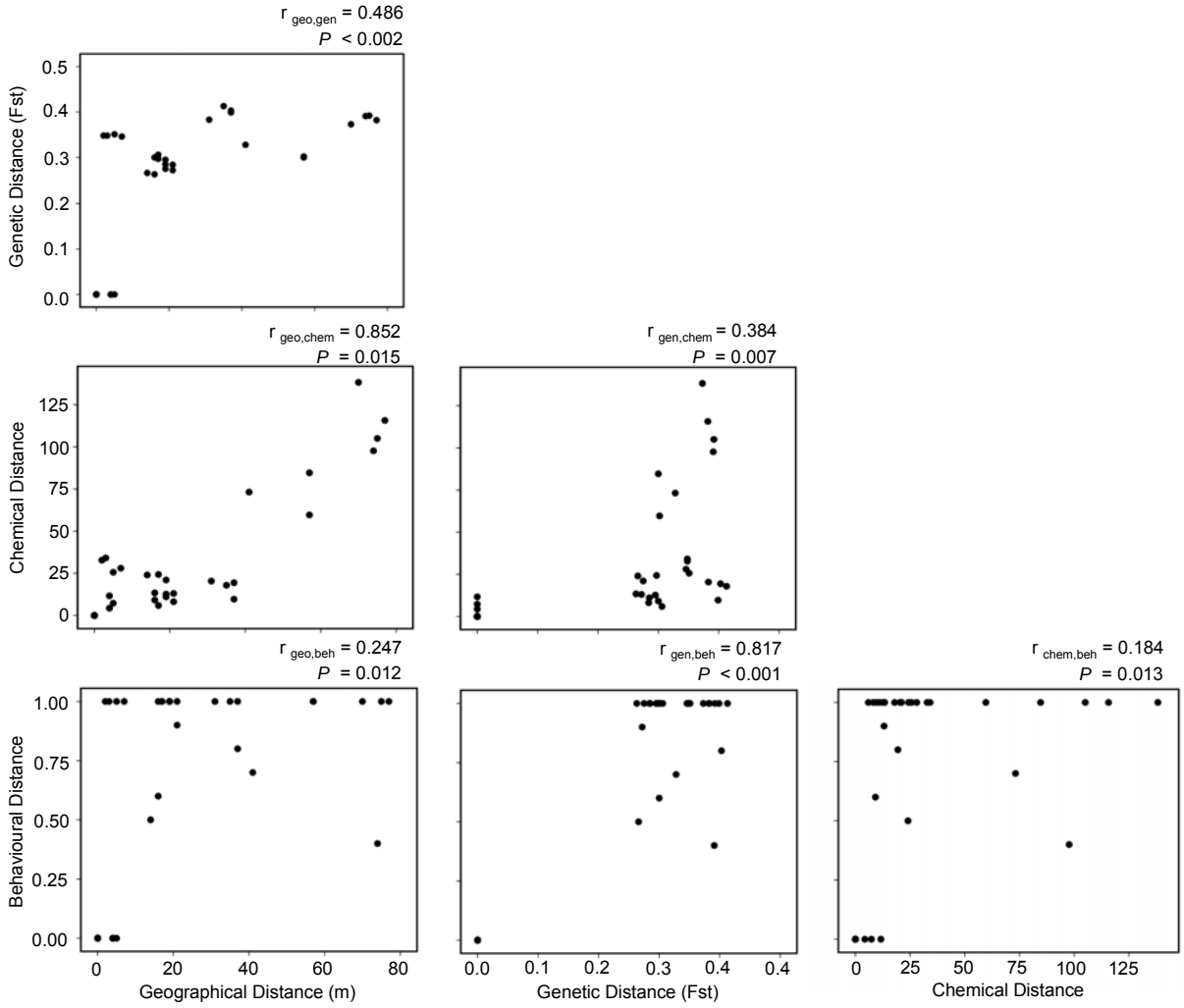
Supplementary Table 11 Allele frequencies in *P. peperi* supercolonies.
Allele frequencies are given within each colony of *Pseudomyrmex peperi*. Allele frequencies are given separately for females (queens and female progeny), queens, female progeny (*f* progeny) and male progeny (*m* progeny). N=number of alleles obtained from each group.

		PPEP103				PPEP132			
	Allele ID	1	2	3	N	1	2	3	N
Pf14	all	0.172	0.507	0.321	134	0.735	0.265		294
	females	0.167	0.480	0.353	102	0.734	0.266		256
	queens	0.174	0.457	0.370	46	0.717	0.283		106
	f progeny	0.161	0.500	0.339	56	0.747	0.253		150
	m progeny	0.188	0.594	0.219	32	0.737	0.263		38
Pf15	all	1.000			134	0.304	0.304	0.392	319
	females	1.000			102	0.296	0.318	0.386	280
	queens	1.000			46	0.315	0.315	0.370	108
	f progeny	1.000			56	0.285	0.320	0.395	172
	m progeny	1.000			32	0.359	0.205	0.436	39
Pf16	all	0.373	0.276	0.351	134	0.410	0.314	0.276	322
	females	0.392	0.284	0.343	102	0.397	0.316	0.287	282
	queens	0.370	0.304	0.326	46	0.389	0.306	0.306	108
	f progeny	0.411	0.268	0.321	56	0.402	0.322	0.276	174
	m progeny	0.312	0.250	0.438	32	0.500	0.300	0.200	40
Pf17	all	0.694	0.306		134	0.230	0.401	0.370	322
	females	0.686	0.314		102	0.248	0.411	0.340	282
	queens	0.739	0.261		46	0.231	0.398	0.370	108
	f progeny	0.643	0.357		56	0.259	0.420	0.322	174
	m progeny	0.719	0.281		32	0.100	0.325	0.575	40
	m progeny	0.719	0.281		32	0.100	0.325	0.575	40
Pf18	all	0.295	0.450	0.256	129	1.000			317
	females	0.280	0.460	0.260	100	1.000			276
	queens	0.326	0.370	0.304	46	1.000			104
	f progeny	0.241	0.537	0.222	54	1.000			172
	m progeny	0.345	0.414	0.241	29	1.000			40
Pf19	all	1.000			134	0.298	0.702		322
	females	1.000			102	0.298	0.702		282
	queens	1.000			46	0.315	0.685		108
	f progeny	1.000			56	0.287	0.713		174
	m progeny	1.000			32	0.300	0.700		40

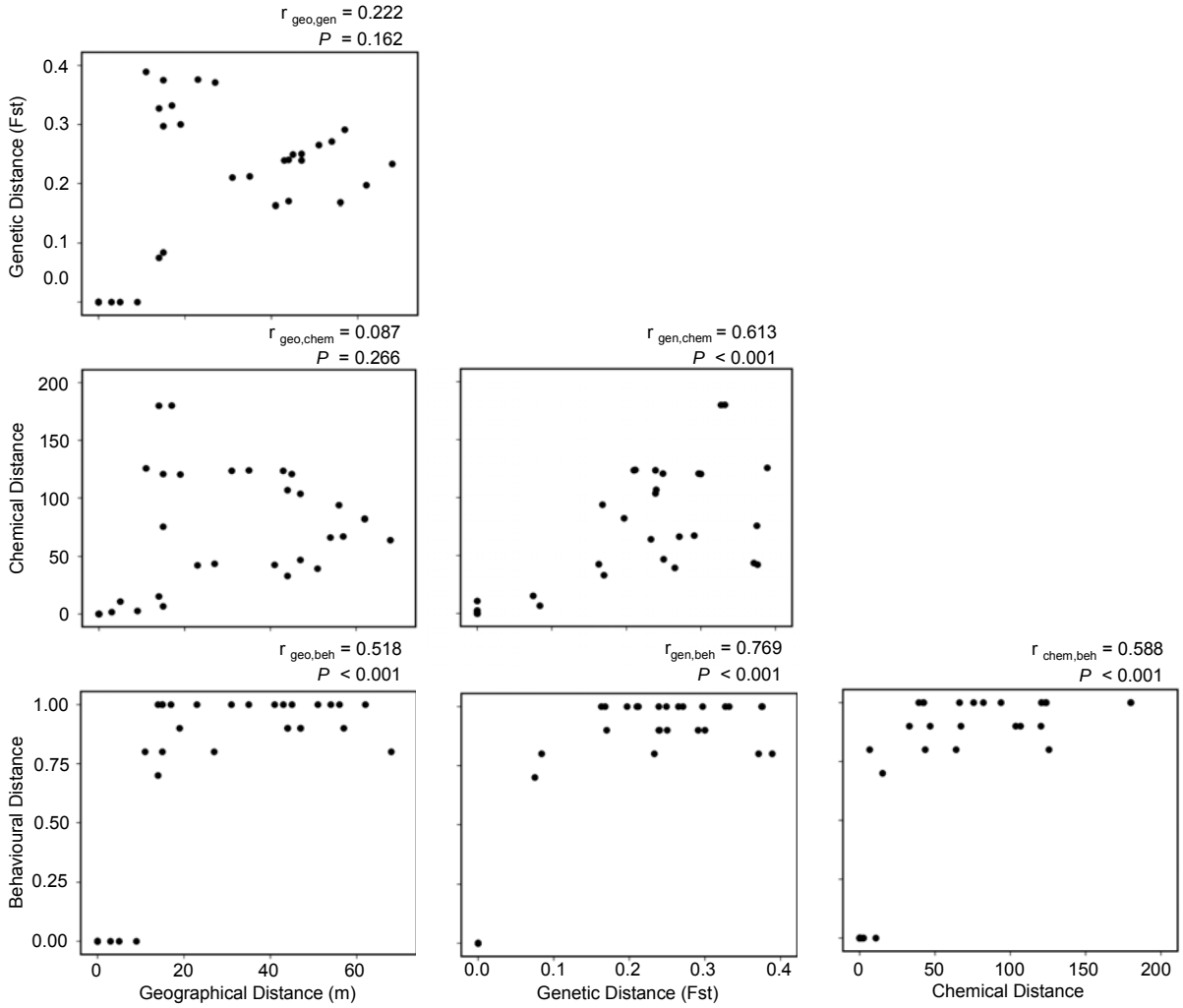
Pf20	all	0.216	0.396	0.388	134	0.639	0.361	319
	females	0.225	0.382	0.392	102	0.646	0.354	280
	queens	0.217	0.391	0.391	46	0.623	0.377	106
	f progeny	0.232	0.375	0.393	56	0.661	0.339	174
	m progeny	0.188	0.438	0.375	32	0.590	0.410	39
Pf21	all	0.739	0.261		134	1.000		322
	females	0.725	0.275		102	1.000		282
	queens	0.717	0.283		46	1.000		108
	f progeny	0.732	0.268		56	1.000		174
	m progeny	0.781	0.219		32	1.000		40



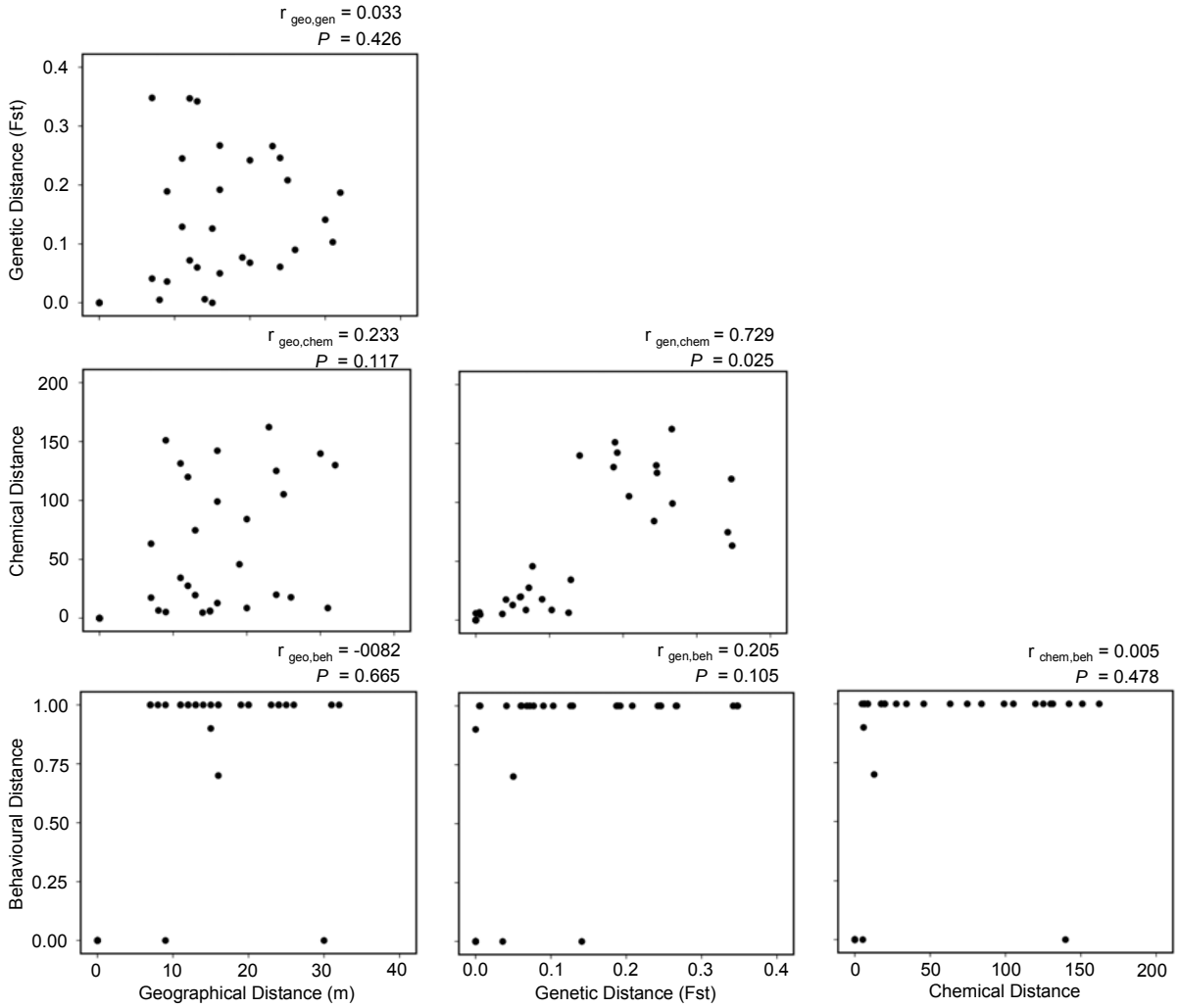
Supplementary Fig. 1 Correlations in plot PFER1.
Partial correlations between genetic, chemical, behavioral and geographic distance between the colonies of *Pseudomyrmex ferrugineus* in plot PFER1. Correlation coefficients ($r_{x,y}$) are given for each plot. Mantel tests showed that correlation coefficients were only significant for chemical vs. genetic distance.



Supplementary Fig. 2 Correlations in plot PFER2.
Partial correlations between genetic, chemical, behavioral and geographic distance between the colonies of *Pseudomyrmex ferrugineus* in plot PFER2. Correlation coefficients ($r_{x,y}$) are given for each plot. Mantel tests showed that all correlation coefficients were significant.



Supplementary Fig. 3 Correlations in plot PGRA1.
Partial correlations between genetic, chemical, behavioral and geographic distance between the colonies of *Pseudomyrmex gracilis* in plot PGRA1. Correlation coefficients ($r_{x,y}$) are given for each plot. Mantel tests showed that correlation coefficients were only significant for chemical vs. genetic distance and behavioral vs. geographic, genetic and chemical distance.



Supplementary Fig. 4 Correlations in plot PGRA2.
Partial correlations between genetic, chemical, behavioral and geographic distance between the colonies of *Pseudomyrmex gracilis* in plot PGRA2. Correlation coefficients ($r_{x,y}$) are given for each plot. Mantel tests showed that correlation coefficients were only significant for chemical vs. genetic distance.

Lebenslauf

Personalien

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Essen, den 15. April 2009

Stefanie Kautz

Erklärungen

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den _____ _____

Unterschrift des/r Doktoranden/in

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitgebiet, dem das Thema „Acacia-inhabiting *Pseudomyrmex* ants — integrating physiological, behavioral, chemical and genetic data to understand the maintenance of ant-plant mutualisms“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Stefanie Kautz befürworte.

Essen, den _____ _____

Unterschrift eines Mitglieds der Universität Duisburg-Essen

Erklärung:
Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner andern Fakultät abgelehnt worden ist.

Essen, den _____ _____

Unterschrift des Doktoranden