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Polygynous supercolonies of the acacia-ant *Pseudomyrmex peperi*, an inferior colony founder

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Abstract

In ant–plant protection mutualisms, plants provide nesting space and nutrition to defending ants. Several plant–ants are polygynous. Possessing more than one queen per colony can reduce nestmate relatedness and consequently the inclusive fitness of workers. Here, we investigated the colony structure of the obligate acacia-ant *Pseudomyrmex peperi*, which competes for nesting space with several congeneric and sympatric species. *Pseudomyrmex peperi* had a lower colony founding success than its congeners and thus, appears to be competitively inferior during the early stages of colony development. Aggression assays showed that *P. peperi* establishes distinct, but highly polygynous supercolonies, which can inhabit large clusters of host trees. Analysing queens, workers, males and virgin queens from two supercolonies with eight polymorphic microsatellite markers revealed a maximum of three alleles per locus within a colony and, thus, high relatedness among nestmates. Colonies had probably been founded by one singly mated queen and supercolonies resulted from intranidal mating among colony-derived males and daughter queens. This strategy allows colonies to grow by budding and to occupy individual plant clusters for time spans that are longer than an individual queen's life. Ancestral states reconstruction indicated that polygyny represents the derived state within obligate acacia-ants. We suggest that the extreme polygyny of *Pseudomyrmex peperi*, which is achieved by intranidal mating and thereby maintains high nestmate relatedness, might play an important role for species coexistence in a dynamic and competitive habitat.

Keywords: ancestral states reconstruction, ant–plant mutualism, behaviour, intranidal mating, microsatellites, polydomy

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Introduction

Ants are the world's major arthropod mutualists (Moreau *et al.* 2006). Over 40 genera of ants and 100 genera of angiosperms are involved in mutualisms, in which plants (so-called myrmecophytes) provide nesting space and/or food rewards to defending ants (Davidson & McKey 1993). The groups involved usually show a high taxonomic and functional diversity (Heil & McKey 2003; Rico-Gray & Oliveira 2007), with several closely related species participating in the mutualism on the side of both plants and ants (Davidson & McKey 1993). Different mechanisms have been proposed that enable species coexistence in ant–plant mutualisms. For example, competition–colonization trade-offs apply when some species are more successful in founding new colonies, while others succeed at occupying empty nesting space by expanding mature colonies (Stanton *et al.* 2002). Because individual host plants are usually occupied by a single ant colony (Davidson *et al.* 1989; Yu & Davidson 1997), co-occurring plant–ant species compete intensely for hosts (Janzen 1975; Davidson & McKey 1993; Clement *et al.* 2008). Moreover, many myrmeco-

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phytes are pioneer trees that continuously establish new populations at different and unpredictable sites, representing a highly dynamic environment for their ant-inhabitants (e.g. Fiala *et al.* 1989; Dejean *et al.* 2008). In such environments, fast growing and large colonies are generally selected for, which could lead to transitions in mating system and colony structure, for example from monogyny and monoandry to polygyny or polyandry (Rissing *et al.* 1989; Herbers 1993; Seppä *et al.* 1995; Pedersen & Boomsma 1999).

Several mutualistic plant–ants are characterized by exceptional colony structures. For example, colonies of the Mesoamerican obligate acacia-ant *Pseudomyrmex veneficus* are extremely polygynous (i.e. contain more than one queen): these colonies may comprise hundreds of thousands of queens and millions of workers, can colonize clusters of several hundreds of acacias (Janzen 1975), and are among the largest of all social insect societies (Ward 1993). Polygyny is also reported for the Asian *Macaranga triloba–Crematogaster* plant–ant association (Feldhaar *et al.* 2000, 2005), while facultative polygyny is known from the African myrmecophyte *Leonardoxa africana–Petalomyrmex phylax* mutualism (Dalecky *et al.* 2005). Polygynous colonies are often also polydomous (i.e. possess multiple nests) (Debout *et al.* 2007).

Besides plant–ants, polygyny and polydomy is found among many other ant species. One example is the highly polygynous and polydomous species *Formica yessensis* on Hokkaido, where 45 000 nests were formed by one large colony which contained 1 080 000 queens and 306 million workers (Higashi & Yamauchi 1979). Similarly, invasive ants often show extremely high queen numbers. They form supercolonies (large aggregations of nonaggressive nests) and may completely lose colony boundaries in the introduced range, often resulting in unicoloniality, with an entire population consisting of one supercolony (Heinze *et al.* 2006; Suarez *et al.* 2008). Unicoloniality seems to be associated with reduced heterozygosity and genetic uniformity resulting in reduced intraspecific aggression over large spatial scales (Tsutsui *et al.* 2003). This colony structure contributes significantly to the ecological dominance of invasive ants because it allows establishing very large colonies and high nest densities, thus greatly increasing foraging efficiency (Bourke & Franks 1995). While extreme polygyny and polydomy may be important during the invasion process of invasive ants (Ugelvig *et al.* 2008), for other ant species (e.g. *Formica* and *Pseudomyrmex veneficus*) this remarkable colony structure is not associated with invasiveness but instead may be related to the monopolization and inheritance of large, long-lived, resources such as nesting sites (e.g. thatch mounds or domatia-bearing trees) (Bourke & Franks 1995).

Many ant species are polygynous or polyandrous (i.e. queens mate with multiple males), thereby increasing the productivity of the individual colony (Bourke & Franks 1995; Crozier & Pamilo 1996). However, kin selection (Hamilton 1964) should favour ant colonies that are headed by one singly mated queen and, thus, characterised by high relatedness among nestmates, over polygynous or polyandrous colonies. Altruistic behaviour that does not increase the genetic contribution of an individual to the next generation may be maladaptive and evolutionarily unstable (Helanterä *et al.* 2009). However, low levels of relatedness can be favoured if more queens results in increased growth rates and colony sizes that allow the monopolization of more resources, or if increased genetic diversity benefits disease resistance (Queller & Strassmann 1998; Strassmann & Queller 2007).

In short, ecological requirements and kin selection theory place seemingly contrasting pressures on the colony structure of ants living in short-lived, dynamic and highly competitive habitats. To understand how ants solve this conflict, we studied the polygynous and polydomous plant–ant *Pseudomyrmex peperi* and sympatric species. We used this system to ask: (i) whether competition exists between *P. peperi* and its congeners, and if *P. peperi* is an inferior competitor; (ii) how *P. peperi* establishes polygynous colonies and how these colonies are genetically and behaviourally structured. To address these questions, we investigated competition for nesting sites among *P. peperi* and its congeners and used microsatellites and aggression assays to investigate the colony structure. Finally, we constructed a phylogenetic history of polygyny in obligate acacia-ants to test our 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 whether and how polygyny represents an adaptation of certain obligate plant–ants to strong competition.

Materials and methods

Study species

We examined competition among sympatrically occurring *Pseudomyrmex* acacia inhabiting ants. Furthermore, we investigated the colony structure of the polygynous and polydomous plant–ant *Pseudomyrmex peperi*, which is a member of the *Pseudomyrmex ferrugineus* group of obligate acacia inhabitants (Ward 1993; Kautz *et al.* 2009a). All members of the *P. ferrugineus* group only nest in hollow swollen thorns of Mesoamerican acacia myrmecophytes and exclusively feed on directly plant-derived food sources, i.e. extrafloral nectar and food

bodies (Janzen 1974; Heil *et al.* 2004; Clement *et al.* 2008). Congeneric ant species of the *P. ferrugineus* group inhabit the same plant hosts. Polygynous colonies have been described for *P. peperi* as well as for three other species of the *ferrugineus* group, i.e. *Pseudomyrmex janzeni*, *Pseudomyrmex satanicus* and *Pseudomyrmex veneficus*, while monogyny has been described for five species (*P. ferrugineus*, *Pseudomyrmex flavicornis*, *Pseudomyrmex mixtecus*, *Pseudomyrmex nigrocinctus* and *Pseudomyrmex spinicola*) (Ward 1993).

Study sites

The present study was conducted at two study sites (site 1 and site 2) in the state of Oaxaca in South Mexico (Fig. 1). Site 1 (N 15°55.809–15°55.817; W 97°09.258–97°09.267; elevation ~60 m) was about 30 km west of Puerto Escondido, site 2 (N 15°57.517–15°57.563; W 97°20.653–97°20.667; elevation ~15 m) was about 10 km west of the same city. At site 1, *P. ferrugineus*, *P. mixtecus* and *P. peperi* occurred sympatrically as inhabitants of *Acacia collinsii*, which represented the only host species at this site, and a large supercolony of *P. peperi* occupied an extensive *A. collinsii* cluster (referred to as col 1). At site 2, a supercolony (referred to as col 2) inhabited a large cluster of *Acacia hindsii*, which was surrounded by individuals of the same species and of *Acacia cornigera*. The latter were inhabited by *P. ferrugineus*, *P. mixtecus* and some colonies of the parasitic, i.e. nondefending species *Pseudomyrmex gracilis* and *Pseudomyrmex nigropilosus*. For genetic analyses, we sampled *P. peperi* about every metre along a transect at the edge of each cluster of host plants (Fig. 2).

Competition for nest sites

Two studies were conducted to estimate the intensity of competition among *Pseudomyrmex* ant queens for

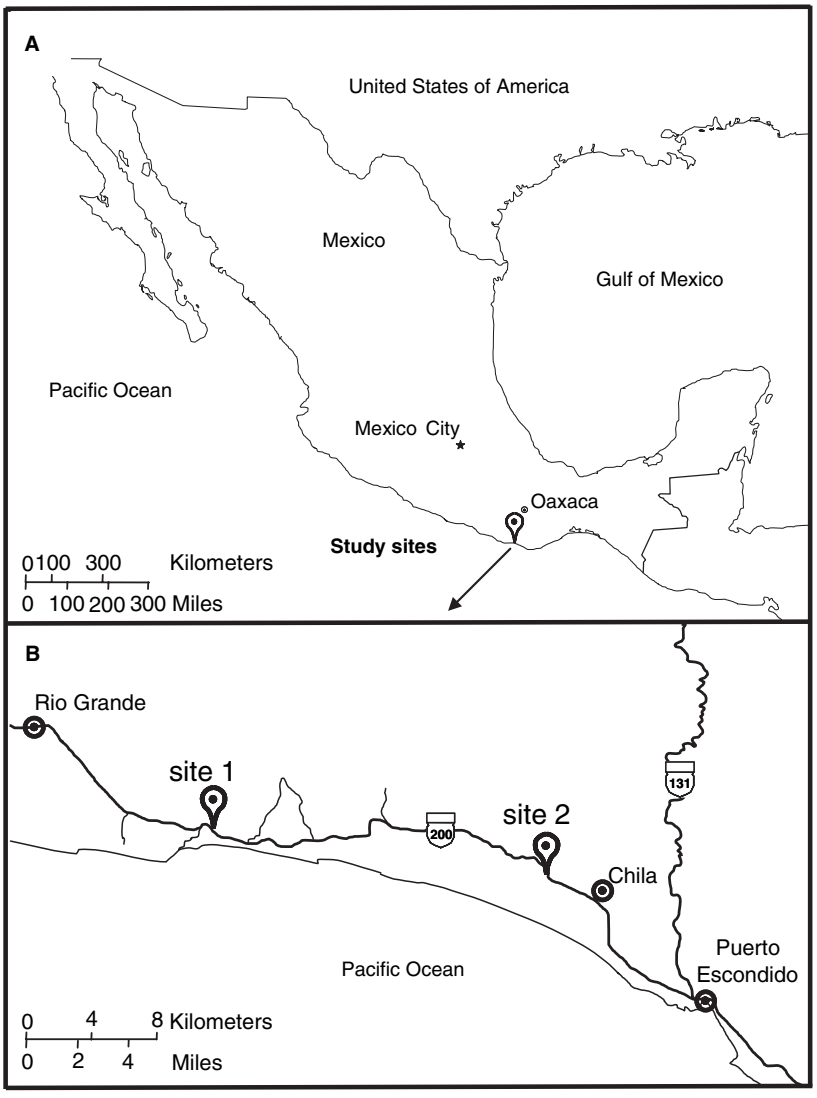

Fig. 1 Geographical location of the studied supercolonies of *Pseudomyrmex peperi*. (A) Overview of location in Mexico and (B) illustration of the sites' vicinity.

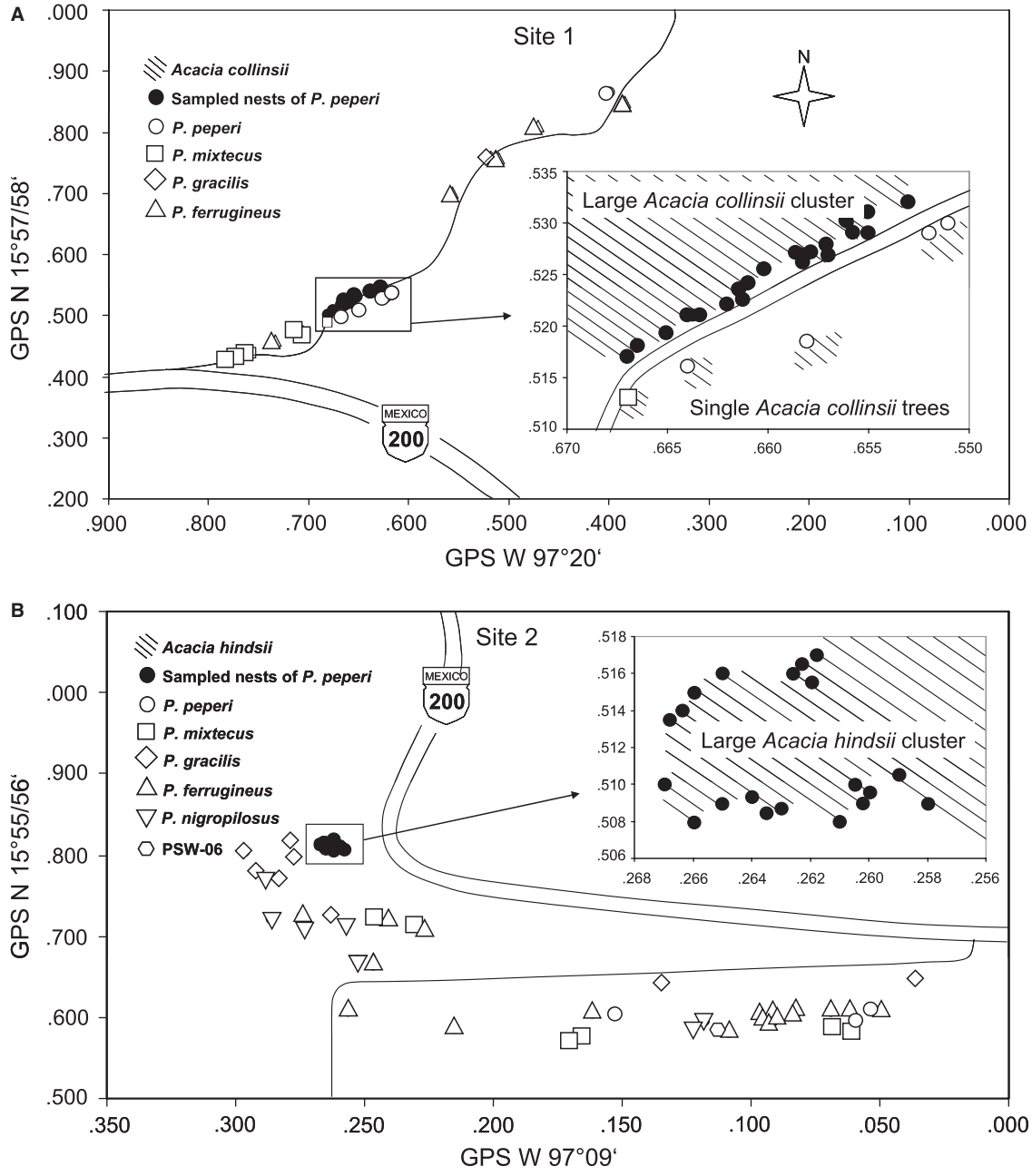

Fig. 2 Detailed illustrations of the collection sites of the two supercolonies investigated. At site 1, ants from a large colony (col 1) occupied an extensive *Acacia collinsii* cluster growing along a field path. At site 2, ants (col 2) inhabited a large cluster of *A. hindsii*.

nesting sites and the relative success of foundresses. We observed the colonisation frequencies of different acacia-ants on site 2, where acacias had recently been cropped by farmers so that new shoots emerged from the ground. Colonisation patterns on this pasture were recorded twice, during September 2006 and February 2007. For the first census, we selected 29 saplings (0.3–1.0 m high) of *A. cornigera* and 44 individuals of *A. hindsii* [species determined following Janzen (1974) and Seigler & Ebinger (1995)]. Every plant was simultaneously observed by two researchers from two opposite sides for 10 min to census all individual ants (workers and colony-founding queens) visible on the plant surface. Ants were determined according to Ward (1993). The presence of workers and foundresses of every species was rated on an ordinal scale following Clement *et al*. (2008): 0 = no ant of the respective species present, 1 = a single ant present, 2 = few (2–10) ants present

on <25% of leaves, 3 = workers on 25–50% of leaves, 4 = workers on 50–75% of leaves, 5 = entire plant occupied/patrolled. The site was revisited in February 2007 and plants were re-examined to obtain quantitative data on the development of young colonies on acacia host saplings.

To more directly asses the intensity of competition among foundresses for available nest space and the relative abundance of founding queens from each acacia-ant species, we excluded ants from the main branches of each of 15 *A. cornigera* and 20 *A. hindsii* plants (~1–3 m high) at site 2. Depriving parts of inhabited plants from their resident ants was necessary because entirely ant-free acacia myrmecophytes do virtually not exist in nature. Ants were removed by cutting off inhabited thorns and shoot tips were protected from remaining resident ants by applying a ring of Tangletrap® (Tanglefoot Co.). After 6 weeks, newly produced thorns were counted and the new branch parts were examined for founding queens, both moving on the plant surface and inside hollow thorns. These founding queens would have flown in and were thus not stopped by the Tangletrap®. We expect to find fewer queens of *P. peperi* as of congeneric species, if *P. peperi* is inferior in founding new colonies.

Aggression assays

Individual ants from two *P. peperi* supercolonies (col 1 and col 2, Figs 1 and 2) were experimentally confronted with other individuals to study colony boundaries at the behavioural level. From each supercolony, 40 workers were individually transferred as follows: (i) 10 ants were returned to the same single branch to test whether ants react aggressively to any other experimentally transferred ant (to serve as control); (ii) 10 further individuals were placed on a branch of an anatomically independent acacia shoot, which was inhabited by the same supercolony (identified by observing workers moving freely among the different plants); (iii) 10 further individuals were placed on a branch of a tree located at the same site but ~500 m away, being clearly separated by nonacacia vegetation from the tree of origin; (iv) The last 10 individuals were transferred to the supercolony at the other study site (hence, ~20 km away). Because several additional *P. peperi* colonies were available at site 2, we also transferred 10 workers of col 2 to a tree ~100 m away (v) and 10 to a tree ~1 km away (vi). Finally, two queens each were transferred to the trees at ~100 m, ~500 m and ~1 km distance. In every test, each ant was transferred individually. All ants obtained from one acacia shoot were kept together in a 250 ml plastic cup sealed with fabric (antiaphid net) until transferred to the plant. Each single ant was observed until it encountered an individual on the tree it was placed on. In all cases, the resulting behaviour could be clearly classified as aggressive (i.e. the transferred ant was attacked, which resulted in chasing, pairwise reciprocal stinging and eventually caused at least one of the opponents to fall off the tree) or as neutral (i.e. no attack took place over at least three minutes of observation time).

Colony composition

From each of the two supercolonies (col 1 and col 2, see Figs 1 and 2), we sampled ants from 20 different acacia shoots (Fig. 2) by collecting 3–7 swollen thorns per shoot and pooling them in Ziploc® bags (Toppits). The bags were stored at −20 °C before thorns were opened and all individuals from the same shoot were transferred to one tube using a funnel and stored in 96% ethanol until needed. The numbers of individuals in every sample were counted using a binocular microscope. We differentiated among reproductive queens (bigger in size than workers and strongly physogastric, i.e. gasters distended with eggs), virgin queens (possessing wings, not physogastric), workers (smaller in size with small gasters), males (winged and showing a characteristic morphology with long, slender bodies and long antennae) (Fig. 3). Virgin queens and workers were pooled as adults and pupae, because we were interested in the relative fecundity of queens rather than the production of sexuals.

Microsatellite analyses

DNA for microsatellite analysis was extracted from 76 workers, 75 males, 37 virgin queens and 80 queens derived from the two supercolonies following Kautz *et al.* (2009b). Gasters of queens were discarded before DNA extraction. For the other castes, entire individuals were used for DNA extraction. PCR was carried out in 10 μL reaction volume consisting of 1.0 μL 10× PCR buffer (Roche), 0.6 μL dNTPs (Epicentre), 2.0 μL 10× bovine serum albumin (New England BioLabs), 0.1 μL *Taq* Polymerase (Roche), 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 s, 55 °C for 30 s, 72 °C for 45 s and a final elongation for 10 min at 72 °C, holding temperature was set to 4 °C. The forward primer was 5′-labelled with either 6-FAM (blue), Vic (green), Ned (yellow) or Pet (red). Samples were analysed 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 four differently labelled PCR products. Loci were scored using the

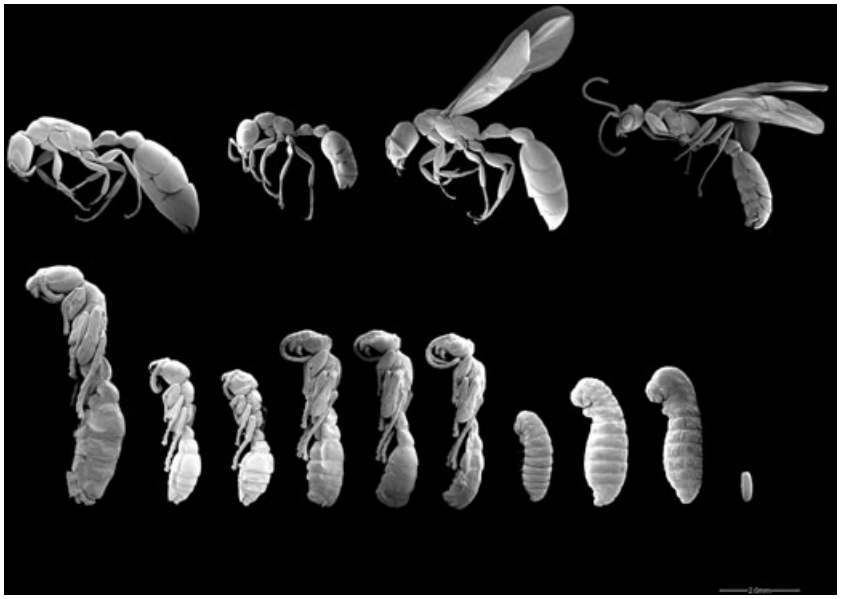

Fig. 3 REM photographs of different castes of *Pseudomyrmex peperi* ants. First row: physogastric queen, worker, virgin queen, male; second row: pupa queen, two pupae worker, three pupae males, three larvae and one egg. Queens were physogastric and showed relicts of wings, they were larger in size than workers. Virgin queens possessed wings and were not physogastric, larger than workers. Workers were of small size and had no signs of wings. Males had large antennae, small heads, wings and a characteristic habitus. Female pupae have shorter antennae than male pupae. Queen pupae had wing pads. Male pupae were characterized by long antennae and wing pads and were of larger size than workers.

GeneMapper 3.7 software (Applied Biosystems). We genotyped all specimens for loci Psfe14, Psfe15, Psfe16, Psfe17, Psfe18, Psfe19, Psfe20 and Psfe21 (Kautz *et al.* 2009b).

Genetic analyses

As inbreeding, which might occur in this study's supercolonies, would lead to an excess of homozygotes and violate Hardy–Weinberg equilibrium, we used results obtained from a males only data set to test for null alleles (i.e. nonamplified alleles that, when segregating with another allele, result in an apparent homozygote). If there were null alleles present, they would not show any amplification product in males, because male ants are haploid. The number of alleles, allele frequencies, observed heterozygosity and expected heterozygosity at each microsatellite locus for both supercolonies were calculated using the online version of the GENEPOP software (Raymond & Rousset 1995) based on female genotypes. Genotype proportions in the two supercolonies were tested for conformity to Hardy–Weinberg expectations using exact tests as implemented in GENEPOP. Input files were converted using Convert (Glaubitz 2004). We estimated pairwise relatedness of workers collected from one acacia tree using the program KINSPHIP 1.1.2 (Goodnight & Queller 1999).

Analysis of molecular variance (AMOVA) as implemented in ARLEQUIN 3.0 (Excoffier *et al.* 2005) was used to describe the genetic structure of the two supercolonies. This test partitions the total genetic variance and calculates fixation indices for each level of variance. We combined all female genotypes of each supercolony, i.e. queens and female progeny and examined the distribution of genetic variation at four hierarchical levels: among supercolonies, among subsamples within supercolonies (individuals derived from different acacia shoots but the same supercolony), among individuals within subsamples (that is among individuals derived from one acacia shoot), and among all female individuals.

Ancestral states reconstruction

The ancestral state of colony structure (monogyny vs. polygyny) was reconstructed based on a five gene phylogeny (mtCOI, *wg*, LW *Rh*, *abd*-A, 28S rDNA) with a total of 3313 base pairs. For phylogenetic analysis, we conducted a Bayesian analysis (B/MCMC) using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001) and a maximum likelihood (ML) analysis using GARLI version 0.951 (Zwickl 2006). We examined all taxa from the *P. ferrugineus* group for which sequence data were available from earlier studies (Ward & Downie 2005, Kautz *et al.* 2009a). GenBank accession nos are given in Table S1, Supporting information. Details of the analyses are given in Kautz *et al.* (2009a). The combined alignment is available in TreeBASE (http://www.treebase.org/treebase). We considered clades with a posterior probability of at least 0.95 in the B/MCMC and bootstrap support equal to or above 75% in the ML analysis well supported. Congruence among the data sets was assessed by comparing bootstrap support of clades above 70% for each locus (Lutzoni *et al.* 2004).

To infer whether polygyny in *P. peperi* is ancestral or derived within the *P. ferrugineus* group, we used ances-

tral character reconstruction (Pagel 1999). Information on the colony structure of all *P. ferrugineus* group taxa was obtained from Ward (1993). Of the 10 species that belong to this group, seven species were included. We further included four species for which we knew the colony structure from own field work. We examined nine to 20 colonies of the species *P. gracilis*, *P. nigropilosus* (both *gracilis* group), *P. salvini* and the undescribed *P.* spec. PSW-06 with 20 to 239 individuals per colony without ever finding more than one queen per nest (S. Kautz, D. J. Ballhorn & M. Heil, unpublished). These species are thus considered monogynous.

Our phylogenetic analyses revealed that polygyny evolved twice within the *P. ferrugineus* group. We tested whether our data are sufficient to reject the hypothesis that polygyny evolved twice employing hypothesis testing exactly as described in Kautz *et al.* (2009a).

Results

Competition for nest sites

Pseudomyrmex peperi is a poor colony founder compared with its sympatric congeners. On host plant saplings, workers and queens of *Pseudomyrmex ferrugineus*, *Pseudomyrmex mixtecus*, *P. peperi*, *Pseudomyrmex gracilis* and *Pseudomyrmex nigropilosus* were observed on the surfaces of the examined acacia individuals with *P. ferrugineus* being the dominant species. On 29 of the 73 examined plants, workers and/or queens of more than one ant species were active. After 6 months, we resampled 53 of the 73 plants. During this second census, *P. peperi* was found only on those plants that it had originally dominated (workers being active on more than 75% of the leaves), but not on plants where it before had been present but not dominant (Fig. 4A), including seven plants on which *P. peperi* queens had been recorded during the first census. Several factors indicate intense competition among foundresses and young colonies, and that *P. peperi* is a poor competitor in this context: Foundresses of different species co-occurred on several plants in 2006, whereas workers of only a single species per plant were found on the same plants in 2007, indicating successful colonisation by only one species in all cases. Far fewer queens of all species were observed on the surfaces during the second census as compared with the first census and no queens of *P. peperi* were observed during the second census. Thus, both the number of queens visible on the plant surfaces and the number of colonies of *P. peperi* decreased from the first to the second census.

The results of the ant-exclusion experiment confirmed this pattern. The observed 35 acacia plants had produced a total of 350 new thorns, which were inhabited by 255 queens, 81 additional queens were observed on the surface of branches. Thus, a total of 336 queens tried to colonise 350 thorns (0.96 queens per thorn) and, consequently, almost every newly produced thorn was occupied by a foundress. Among all mutualistic *Pseudomyrmex* species observed, *P. peperi* was the least abundant (Fig. 4B), although the study site was located close to a *P. peperi* supercolony (Fig. 2B).

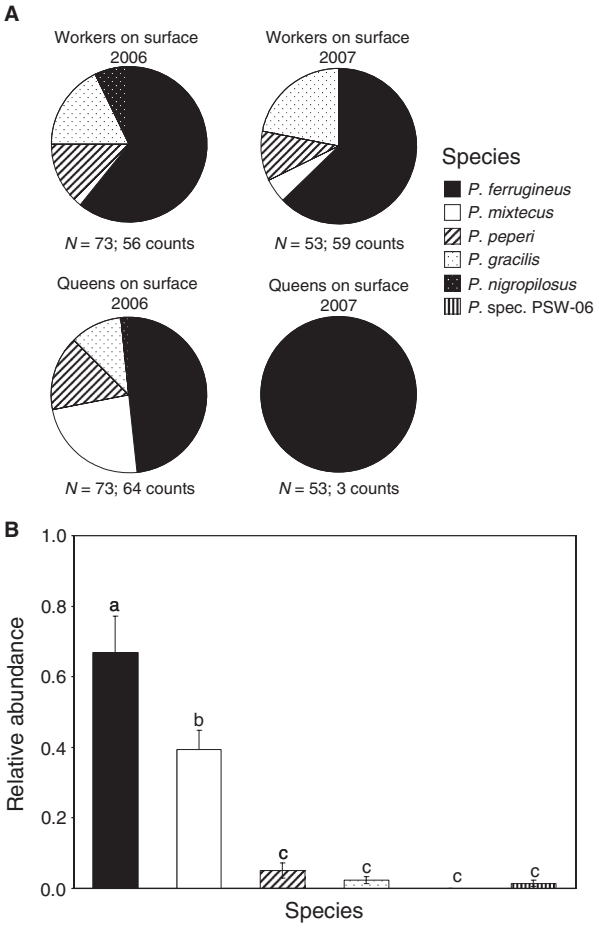

Fig. 4 Competition for nest sites. (A) *Pseudomyrmex* workers and queens observed on young acacia individuals in fall 2006 and spring 2007. *N* signifies the number of plants censused and counts denote the number of ant species on all plant individuals censused in the respective year. (B) The numbers of queens per thorn were determined for each species in plant parts experimentally deprived of resident ants. Bars are mean ± SD; different letters on top of bars indicate significant differences ($P < 0.05$) among numbers of queens for each species according to LSD test after univariate ANOVA.

Aggression assays

When workers from one acacia shoot were confronted with workers transferred from the same shoot or a dif-

ferent shoot belonging to the same acacia cluster (i.e. supercolony) they exhibited neutral behaviour in both supercolonies. For col 2, an additional experiment with a tree ~100 m away was conducted and resulted in nine neutral and one aggressive encounters, whereas six of 10 encounters with workers on a tree at ~500 m distance were neutral and four encounters were aggressive. All confrontations with workers of a tree at ~1 km distance and at ~20 km distance were of aggressive nature. Additionally, all queens that were placed on trees at ~100 m, ~500 m and ~1 km distance were violently attacked and chased off the tree. When using col 1 as donor colony, two trials (ants placed on same tree and another tree from the same cluster) were of neutral nature in all 10 cases and the two other (~500 m and ~20 km distance) resulted in fighting in all 10 cases (Fig. 5).

Colony composition

The relative abundances of developmental stages and castes within colonies differed significantly (univariate ANOVA col 1: $F_{\text{caste }(6,133)} = 36.196$, $P < 0.001$; univariate ANOVA col 2: $F_{\text{caste }(6,133)} = 28.309$, $P < 0.001$). In the samples of col 1, we counted a total of 23 physogastric queens as well as 1532 female workers and virgin queens (66 per queen; p.q.), 251 male adults, 740 female pupae (8 p.q.), 192 male pupae, 4561 larvae (198 p.q.) and 6536 eggs (284 p.q.). In col 2, we found 61 physogastric queens as well as 2384 workers and virgin queens (39 p.q.), 93 male adults, 1273 female pupae (20 p.q.), 159 male pupae, 7055 larvae (115 p.q.) and 10275 eggs (168 p.q.). Results are compiled in Fig. 6. The number of queens in each subsample (that is, ants collected from an individual shoot) was significantly correlated with the number of eggs (Pearson's correlation col 1: $R = 0.717$, $P < 0.001$, col 2: $r = 0.880$, $P < 0.001$), but not with the other groups ($P > 0.05$ in each case).

Colony structure and relatedness

Six of the eight microsatellite loci were polymorphic within each supercolony and we found no more than three alleles per locus (Table 1). Allele frequencies ranged from 0.172 to 1.000 and were equally distributed among the different castes. No rare alleles were detected (Table S2, Supporting information). Despite the low number of alleles, genetic diversity within each supercolony was high. Heterozygosity ranged from 0.35 to 0.69 in col 1 and from 0.35 to 0.76 in col 2. Tests for conformity of genotype proportions to Hardy–Weinberg expectations revealed no significant deviation after Bonferroni correction ($P = 0.05$; Table 1). At loci for which three alleles were present within a single supercolony, expected heterozygosities ranged from 0.64 to 0.67. In monogynous monoandrous colonies, the expected heterozygosity H_E in the absence of genetic drift is 0.67 as it is calculated from the equation:

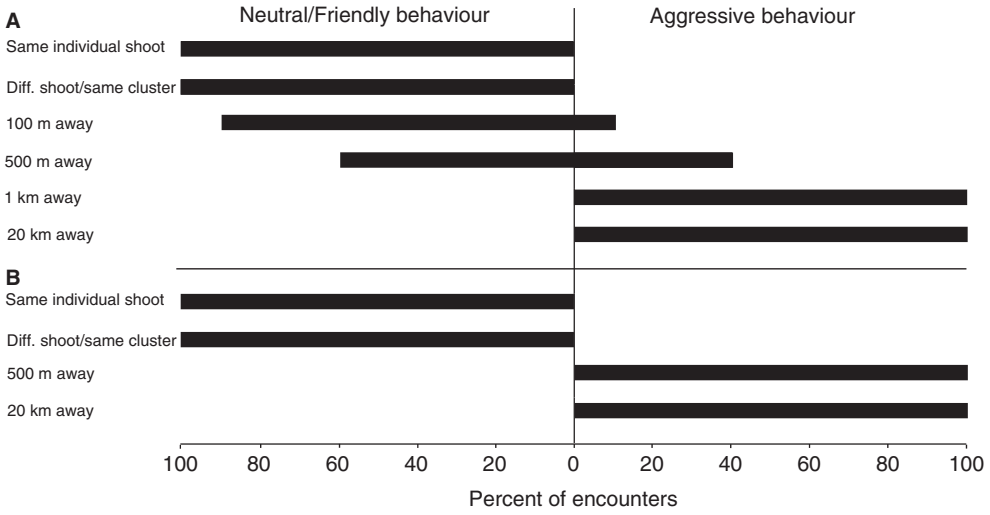

Fig. 5 Behavioural trials with *Pseudomyrmex peperi* workers. Proportion of reactions of workers of supercolony col 2 (A) and col 1 (B) that were placed onto an acacia shoot inhabited by *P. peperi* is indicated as neutral/friendly (left) or as aggressive (right). The shoot the ants were placed on was on either the same plant, another shoot inhabited by the same supercolony, another shoot located at the same site but at ~500 m away and clearly separated by nonacacia vegetation from the tree of origin, an acacia shoot at the other study site (~20 km away). Additionally, each 10 workers of col 2 were placed on a shoot 100 m away and on a shoot 1 km away.

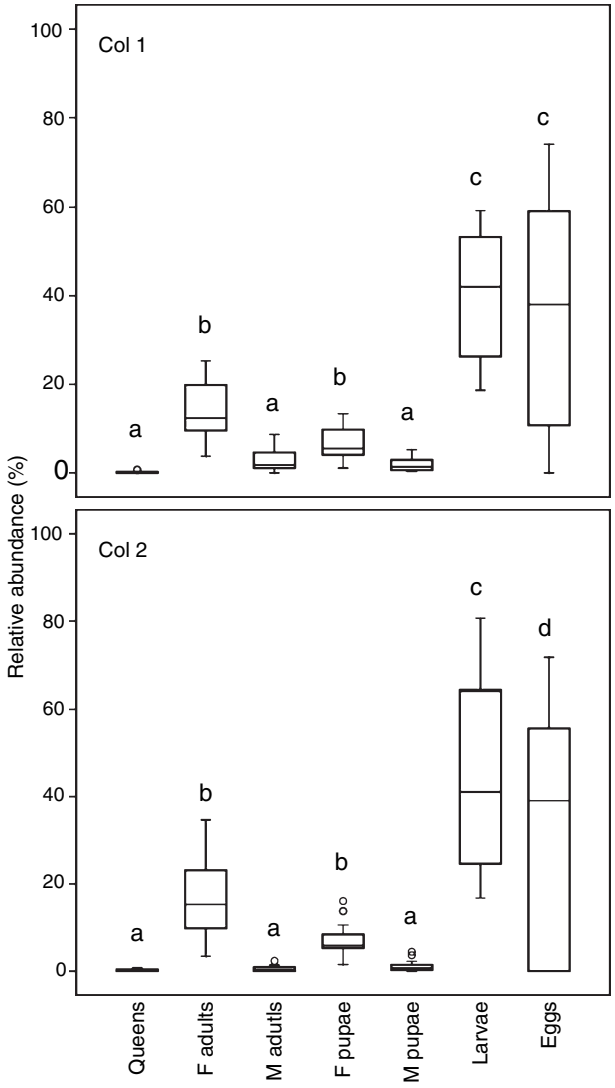

Fig. 6 Colony composition of the two supercolonies col 1 and col 2 estimated by relative abundance of castes within collected thorns. Different letters indicate significant differences ($P < 0.05$ according to LSD *post hoc* test after univariate ANOVA). Relative abundance was arcsine transformed prior to statistical analysis. Upper and lower margins of boxes represent 25% and 75% percentiles, whiskers represent 5% and 95% percentiles, lines in boxes are medians, circles are outliers.

$$H_E = 1 - \sum_{i=1}^{k} p_i^2$$

where p_i is the frequency of the ith of k alleles (Nei 1973). When two alleles are present in monogynous, monoandrous colonies, the expected heterozygosity is 0.44 and the observed values varied from 0.39 to 0.46. We used the amplification success in males to make predictions about null alleles. At the loci Psfe15 and Psfe20, amplification failed in one of 72 males (1.34%), and at loci Psfe14 and Psfe18 in two males (2.78%). The two latter loci seemed to be sensitive to low DNA quality, because locus Psfe14 failed to amplify in 13 of 192 females (6.77%) and locus Psfe18 failed in four of 192 cases (2.08%). The loci Psfe15 and Psfe20 each failed to amplify in one female (0.52%). The four loci Psfe16, Psfe17, Psfe19 and Psfe21 amplified in 264 cases. However, rerunning analyses under the exclusion of both loci did not lead to different conclusions as given. We did not obtain any signal for four individuals (three males and one worker) resulting from failure of DNA extraction (visualized on 1.5% TBE agarose gels stained with ethidium bromide).

Table 1 Genetic diversity measures within each study supercolony of *Pseudomyrmex peperi* in South Mexico as obtained from female genotypes

	Col 1 (N = 51)			Col 2 (N = 141)		
Locus	N_A	H_E	H_O	N_A	H_E	H_O
Pf14	3	0.64	0.64	2	0.39	0.39
Pf15	1	—	—	3	0.66	0.71
Pf16	3	0.67	0.65	3	0.66	0.63
Pf17	2	0.43	0.47	3	0.66	0.76
Pf18	3	0.65	0.54	1	—	—
Pf19	1	—	—	2	0.42	0.35
Pf20	3	0.66	0.69	2	0.46	0.46
Pf21	2	0.40	0.35	1	—	—

We found no significant deviation according to HW probability test after Bonferroni correction ($P = 0.05$).
N, total number of female individuals (queens, virgin queens, workers) for each supercolony; N_A, observed number of alleles found at each locus from each supercolony; H_E, expected heterozygosity; H_O, observed heterozygosity.

The overall relatedness among nestmate workers was R_{ww}(col 1) = 0.72 ± 0.11 (range 0.39–0.94) and R_{ww}(col 2) = 0.75 ± 0.10 (range 0.34–1.00), among nestmate queens R_{qq}(col 1) = 0.73 ± 0.11 (range 0.34–0.95) and R_{qq}(col 2) = 0.75 ± 0.10 (range 0.38–0.96).

Genetic diversity between the two supercolonies

We found 2–5 alleles per microsatellite locus when combining both supercolonies (18 and 17 alleles in col 1 and col 2, respectively, Table 1). A total of 27 private alleles (77%; i.e. unique to a single colony) across all eight microsatellite loci were found. AMOVA revealed 43.92% variation between supercolonies (SS = 192.02; d.f. = 1, $P < 0.001$), 1.55% variation among subsamples within supercolonies (SS = 56.24, d.f. = 27, $P = 0.937$), no variation among individuals within subsamples (SS = 243.90, d.f. = 163, $P < 0.001$) and 57.21% variation

Table 2 Results of hierarchical AMOVA comparing genetic variation at four levels

Source of variation	d.f.	Sum of squares	Variance component	Per cent variation	Fixation index	*P*-value
Among supercolonies	1	192.015	1.26767	43.92	F_{IS} = −0.04915	<0.001
Among subsamples within supercolonies	27	56.243	0.04881	1.55	F_{SC} = 0.02769	0.946
Among individuals within subsamples	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		

among all individuals (SS = 317.00, d.f. = 192, $P < 0.001$) (see Table 2 for summary of statistics and results of fixation indices).

Ancestral states reconstruction

The mean log likelihood of the Bayesian tree sampling was −11 270. In the majority-rule consensus tree shown in Fig. 7A, two main clades within the sample species of 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 (BM 100, pp 1.0). Within the second major clade, *Pseudomyrmex salvini*, and *Pseudomyrmex* spec. PSW-06 take a basal position to one clade that comprises all species of the *ferrugineus* group.

The 21 *Pseudomyrmex* specimens included in this study represented nine monogynous and two polygynous species. Ancestral character mapping (Fig. 7B) suggests that monogyny is the ancestral state to the *P. ferrugineus* group. Ancestors to the genus *Pseudomyrmex* were also identified as monogynous. For the included taxa, the analysis suggested that polygyny has evolved independently twice within the *ferrugineus* group, in *Pseudomyrmex satanicus* and *P. peperi*. Alternative topologies with the polygyny evolving once were rejected with the current data set ($P < 0.05$ in both tests).

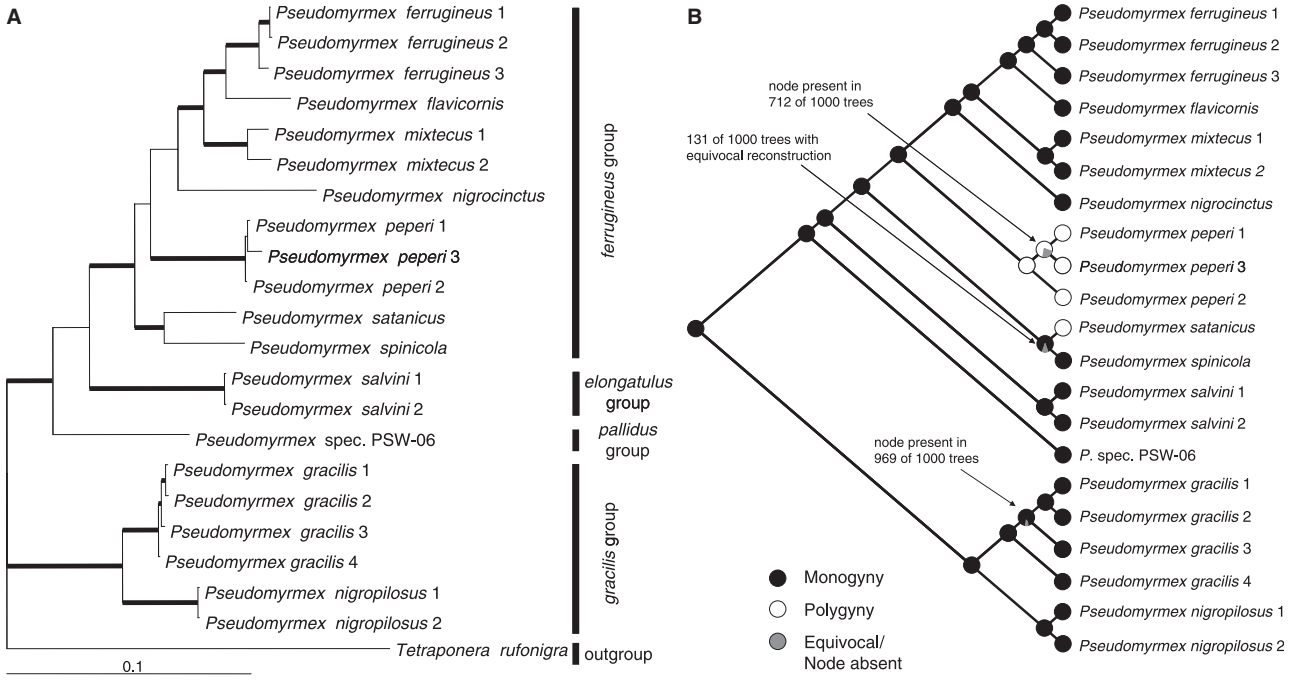

Fig. 7 Ancestral states reconstruction of colony structure in acacia-ants. (A) Phylogeny of selected *Pseudomyrmex* ants as inferred from a five gene-partition analysis (3313bp). This is a 50% majority rule consensus tree based on 74 000 trees from a B/MCMC tree sampling procedure. Branches with posterior probabilities equal or above 0.95 and ML bootstrap support values above 74% are indicated in bold. (B) Colony structure (monogyny vs. polygyny) of *Pseudomyrmex* species traced on 1000 trees inferred from the five gene fragments analysis.

Discussion

Extreme polygyny and polydomy is characteristic for invasive ants (Holway *et al.* 2002), but has also been found in other ant species (e.g. Higashi & Yamauchi 1979), including plant–ants (Janzen 1973). We examined whether polygyny has evolved in plant–ant species that are inferior competitors compared with rivalling congeners. Polygyny promotes colony survival and the 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). There is probably a trade-off between colony foundation efficiency and prolonged colony survival, and therefore we may expect to see a negative correlation between these two traits as documented for *Petalomyrmex phylax* (Léotard *et al.* 2009).

We found strong competition among different *Pseudomyrmex* species; a total of 336 queens attempted to colonise 35 acacia shoots, although mature individual myrmecophytic plants are usually occupied by a single ant colony (Davidson *et al.* 1989; Yu & Davidson 1997). We confirmed that *Pseudomyrmex peperi* is weaker in establishing new colonies than its congeners: many founding queens of five *Pseudomyrmex* species were observed together on acacia saplings, but *P. peperi* foundresses – or young colonies – were replaced by other species after a 6-month period. Queens of this species were also least represented among the queens that arrived on ant-free hosts. In conclusion, *P. peperi* appears to be a poor colony founder, a trait which the species might trade-off with prolonged colony survival.

On the African myrmecophytic plant species *Acacia drepanolobium* four ant species coexist, of which *Tetraponera penzigi* was found to be the superior competitor in early colony establishment, but then disappeared from mature hosts (Stanton *et al.* 2002, 2005). The authors concluded that the inferior colony establishers *Crematogaster sjostedti* and *Crematogaster mimosae* would dominate in the absence of disturbance in the system (Stanton *et al.* 2005). No data from undisturbed sites are available for the Mesoamerican case studied here. However, five *P. peperi* colonies were observed for at least 5 years and their host plants were never found to be inhabited by another ant species (M. Heil, personal observation). Because both host plant and ant inhabitants reached large sizes over these 5 years, it is unlikely that the colonies have repeatedly been re-established. In summary, we conclude that *P. peperi* is a weak colony founder, but has a high chance of maintaining colonies once they have reached a minimum size.

Our data on the colony composition of *P. peperi* reveal that the brood in this polygynous species makes up higher proportions as compared with the monogynous species *Pseudomyrmex gracilis* and *Pseudomyrmex ferrugineus* (Clement 2005; Clement *et al.* 2008). In *P. peperi*, brood (larvae, pupae and eggs) amounted to ~84% (Fig. 6), while monogynous colonies of species of the same genus contained only ~60% brood (Clement 2005). The higher proportion of brood found in *P. peperi* indicates a higher growth rate for this polygynous species (Keller & Vargo 1993). Thus, multiple-queen colonies possess an advantage in productivity at the colony level, which facilitates the evolution of polygyny (Pamilo 1999). However, *P. peperi* exhibited fewer workers per queen and an intermediate number of brood per queen than the two congeneric species investigated by Clement *et al.* (2008), indicating that the total reproductive output per queen is lower as compared with monogynous species (Bourke & Franks 1995; Ross & Keller 1995; Komene *et al.* 1999). For *Pseudomyrmex veneficus,* Janzen (1973) found values similar to those we observed. Colonies of the plant–ant *Petalomyrmex phylax* had 200 workers per queen (McKey 1984) and queen numbers were correlated with colony size (Dalecky *et al.* 2005), a pattern that corresponds to our findings. In contrast, queen numbers of the *Macaranga*-mutualist *Crematogaster* morphospecies 2 did not increase with colony growth but remained 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 corresponding increase of nesting space and food resources. In this case, colonies can occupy neighbouring plants via budding. This situation does not apply to *Macaranga*, where single colonies usually are restricted to one host tree (M. Heil, personal observation). It does, however, apply to *Leonardoxa africana* inhabited by *Petalomyrmex phylax* (Dalecky *et al.* 2005), although the latter species does not exhibit similarly high numbers of queens observed in *P. peperi*.

How are the supercolonies of *P. peperi* established? For *P. veneficus*, Janzen (1973) suggested that daughter queens may be readopted into the colony at small colony stages. However, genetic tools were missing at the time of Janzen's study and the detailed strategy by which plant–ants can reach these colony sizes remained unexplored. In our study, we found no more than three alleles per locus within each supercolony and conclude that the supercolonies investigated here had been founded by one singly mated queen. The loci used in the present study were sufficiently polymorphic to detect multiple mating (Kautz *et al.* 2009b). The high genetic differentiation between the two supercolonies also suggests that the marker system is sufficiently variable. Most probably, polygyny results from intranidal

mating, as proposed for other ant species (e.g. Schrempf *et al.* 2005). Alternatives, which have been described for other mutualistic ant–plant systems, such as adoption of unrelated queens into the colony or queens mating with unrelated males and returning to the nest (McKey 1984; Fonseca 1993; Feldhaar *et al.* 2005) seem unlikely, because both scenarios would have caused the presence of many more alleles per supercolony, or at least some rare alleles (Table S2). In *Petalomyrmex phylax* intranidal mating occurs and unrelated females that attempt to be accepted into a foreign colony are killed (Dalecky *et al.* 2005).

In each of the two supercolonies, we found no evidence for drift as observed heterozygosities were close to 0.67 when three alleles were present at one locus and close to 0.44 when two alleles were present (Table 1). This suggests a large contribution of the founding female and her daughters, which is also in line with the observation of all loci were in the Hardy–Weinberg equilibrium – an assumption that usually contradicts the idea of intranidal mating and inbreeding as brother sister matings should quickly lead to a reduction of heterozygote genotypes. The assumption of intranidal mating in *P. peperi* is also consistent with the occurrence of winged males and winged nonphysogastric queens within the same nest (Figs 3 and 6) and with the observation that these individuals carried the same alleles as queens and workers. Relatedness among nestmate egg-laying queens and among workers in *P. peperi* was extremely high, which is in line with the assumption of intranidal mating among colony members. Such conditions may lead to the monopolization of large clusters of hosts by extended family groups (Chapuisat & Keller 1999).

It might be that the physogastric queens do not contribute significantly to the offspring of a colony, i.e. they do not reproduce. This would mean that the colonies are not functionally polygynous. However, in light of the high number of workers, this scenario might not be as likely. Furthermore, we found that many thorns containing queen(s) also harboured high numbers of eggs (personal observation). Future studies should be conducted to fully resolve the course of colony growth in *P. peperi*.

Mating among alates of the same colony might allow colonies to grow continuously and spread over clusters of host plants via budding. Colonies could easily follow the growth of host plants because acacias largely reproduce vegetatively via subterranean stolons, producing new trees close to the parent. However, swarming (that is flying away of queens to found new colonies) appears essential for the colonisation of distant plants, because we found single queens of *P. peperi* in individual, newly developed thorns on ant-free branches (Fig. 4).

We do not know what the genetic diversity within the gene pool is and therefore we cannot give any estimate of the probability that two queens drawn at random would share any particular number of alleles. However, at six of eight loci at least one of the foundresses carried three alleles. This suggests strong available genetic diversity. What is obviously more surprising is the loci with three alleles for one queen and only one for the other (Table 2 and Table S2). This could suggest that the founding queens were derived from different gene pools. Aggression assays also confirmed that *P. peperi* is not unicolonial, as has been described for invasive ants (e.g. Tsutsui *et al.* 2000; Cremer *et al.* 2008). Thus, we chose 'supercolony' to describe the colonies of *P. peperi*, because the species forms large aggregations of nests that are nonaggressive to each other, although aggression among different colonies does occur in this species (Fig. 3).

We reconstructed ancestral states of the colony structure 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 (Keller 1995; Tsuji & Tsuji 1996; but see Schrempf & Heinze 2007). Among the taxa included in this study, polygyny apparently evolved twice. This finding is consistent with the predictions of Helanterä *et al.* (2009), that unicoloniality is an evolutionary dead-end and unicoloniality arising from a unicolonial ancestor is unlikely.

In conclusion, we interpret our findings as evidence for further evolution towards an extreme specialisation as an obligate mutualist. In the obligate acacia-ant *P. peperi*, intranidal mating allows for the formation of large supercolonies with a high degree of relatedness among nestmates, which appears to be a strategy that allows an inferior colony founder to be competitive. We hypothesize that this exceptional life history could be the consequence of a directed co-evolutionary process, because the growing pattern of the ant colony matches exactly the growth of its host plant and thus allows establishment of large and constantly growing colonies on a constantly growing host. The type of colony structure and breeding system described here for *P. peperi* could play an important role in species coexistence in a dynamic and competitive habitat of ecologically successful plant–ants.

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This study was part of Stefanie Kautz's doctoral research during which she investigated adaptations of obligate plant–ants to their lifestyle as mutualists, including specific colony structures and mating systems. Steffen Pauls is a postdoctoral fellow of the German Academy of Sciences Leopoldina. He uses genetic markers to study the phylogeography, population structure and evolution of various insect groups. Daniel Ballhorn's postdoctoral research interests include analyses of plant defence syndromes both in nature and under laboratory conditions. Mutualistic acacia-ants, which function as indirect defence mechanism to their hosts, are among his interests. Associate Curator and Chair of the Botany Department at the Field Museum, Thorsten Lumbsch is primarily interested in phylogeny, taxonomy and phylogeography of symbiotic systems, focusing on lichen-forming fungi. Martin Heil was head of the Department of General Botany and Plant Ecology (University of Duisburg-Essen) and now is at CINVESTAV Irapuato, where he studies induced resistances of plants to pathogens and herbivores and the physiological mechanisms that underlie the stabilisation of defensive plant–ant mutualisms.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Species and specimens included in the phylogenetic study, with voucher specimen, and GenBank Accession Nos. CS = Costa Rica; GT = Guatemala; LT = San Andres Tuxtla area; MR = Matias Romero area; MX = Mexico; PM = Panama; PTO = Puerto Escondido area; ZP = Zipolite area; P = pseudomyrmex; LWC = Lars W. Clement

Table S2 Allele frequencies and genotypes observed within each colony of *Pseudomyrmex peperi*

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