



Decoupled evolution of mating biology and social structure in *Acromyrmex* leaf-cutting ants

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Abstract

Insect societies vary greatly in their social structure, mating biology, and life history. Polygyny, the presence of multiple reproductive queens in a single colony, and polyandry, multiple mating by females, both increase the genetic variability in colonies of eusocial organisms, resulting in potential reproductive conflicts. The co-occurrence of polygyny and polyandry in a single species is rarely observed across eusocial insects, and these traits have been found to be negatively correlated in ants. *Acromyrmex* leaf-cutting ants are well-suited for investigating the evolution of complex mating strategies because both polygyny and polyandry co-occur in this genus. We used microsatellite markers and parentage inference in five South American *Acromyrmex* species to study how different selective pressures influence the evolution of polygyny and polyandry. We show that *Acromyrmex* species exhibit independent variation in mating biology and social structure, and polygyny and polyandry are not necessarily negatively correlated within genera. One species, *Acromyrmex lobicornis*, displays a significantly lower mating frequency compared to others, while another species, *A. lundii*, appears to have reverted to obligate monogyny. These variations appear to have a small impact on average intra-colonial relatedness, although the biological significance of such a small effect size is unclear. All species show significant reproductive skew between patriline, but there was no significant difference in [reproductive skew](#) between any of the sampled species. We find that the evolution of social structure and mating biology appear to follow independent evolutionary trajectories in different species. Finally, we discuss the evolutionary implications that mating biology and social structure have on life history evolution in *Acromyrmex* leaf-cutting ants.

Significance statement

Many species of eusocial insects have colonies with multiple queens (polygyny), or queens mating with multiple males (polyandry). Both behaviors generate potentially beneficial genetic diversity in ant colonies as well as reproductive conflict. The co-occurrence of both polygyny and polyandry in a single species is only known from few ant species. Leaf-cutting ants have both multi-queen colonies and multiply mated queens, providing a well-suited system for studying the co-evolutionary dynamics between mating behavior and genetic diversity in colonies of eusocial insects. We used microsatellite markers to infer the socio-reproductive behavior in five South American leaf-cutter ant species. We found that variation in genetic diversity in colonies was directly associated with the mating frequencies of queens, but not with the number of queens in a colony. We suggest that multi-queen nesting and mating frequency evolve independently of one another, indicating that behavioral and ecological factors other than genetic diversity contribute to the evolution of complex mating behaviors in leaf-cutting ants.

Keywords Attini · Fungus-growing ants · Polyandry · Polygyny · Social evolution · Social parasitism

Introduction

The evolution of mating behavior and reproductive biology is central to the evolution of complex animal societies (Clutton-Brock 1985; Bourke 2014). Extensive research has been conducted linking mating biology evolution and social

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evolution in mammals, birds, and arthropods (Rubenstein and Wrangham 1986), notably identifying trait associations involving life history, social organization, parental care, and mating systems (Maynard Smith 1977; Clutton-Brock 1985; Choe and Crespi 1997; Stanford 1998; Lukas and Clutton-Brock 2012a). Mating systems in particular have been associated with cooperative breeding and social evolution in insects, birds, and mammals, and monogamy was identified as one important trait associated with the evolutionary origin of cooperative breeding in animal societies (Hughes et al. 2008a; Cornwallis et al. 2010; Lukas and Clutton-Brock 2012b).

The eusocial insects have been used as a model for studying the evolution of complex societies and cooperative breeding because of their complex social organization. Eusocial insects are characterized by overlapping generations, cooperative brood care, and an irreversible reproductive division of labor (West-Eberhard 1975; Wilson 1975). Lifetime monogamy is considered a precondition for the evolution of eusociality, as a strictly monogamous mating biology allows for maximum relatedness between individuals of the same colony (Hughes et al. 2008a; Boomsma 2009; Cornwallis et al. 2010; West and Gardner 2010; Lukas and Clutton-Brock 2012b). However, secondary deviations from lifetime monogamy have been observed in many eusocial insects. Interestingly, the evolution of mating biology appears asymmetric in different eusocial insect lineages. Polygyny, the presence of multiple egg-laying queens in a colony, has primarily been observed in ants and termites, but is only observed in a small proportion of social bees and wasps (Hartke and Baer 2011; Boomsma et al. 2014). In contrast, polyandry, multiple mating by females, has frequently been observed in honeybees, but is only known from few examples in ants and vespine wasps, and is virtually unknown from bumblebees, stingless bees, polistine wasps, and termites (Strassmann 2001; Hartke and Baer 2011). Only very few species, all of which are ants, are known to display both polygyny and polyandry simultaneously (Keller and Reeve 1994; Pedersen and Boomsma 1999a; Schmid-Hempel and Crozier 1999; Kellner et al. 2007).

The factors underlying the evolution of polygyny and polyandry in eusocial insects have been extensively debated. Many hypotheses have been proposed to explain both phenomena, particularly in eusocial Hymenoptera. Two main hypotheses for the evolution of polyandry in eusocial insects are widely accepted (Ratnieks and Boomsma 1995; Boomsma and Ratnieks 1996; Crozier and Fjerdingstad 2001; Strassmann 2001; Boomsma et al. 2009): (1) the sperm-limitation hypothesis posits that multiple mating events may be required for queens to acquire enough sperm to reproduce throughout their lifetimes (Fjerdingstad and Boomsma 1998; Kraus et al. 2004; Kronauer and Boomsma 2007). (2) The genetic-diversity hypothesis proposes that

increased genetic variation within colonies is beneficial and adaptive due to an increased resistance of genetically variable colonies to pathogens and parasites (Kraus and Page Jr. 1988; Baer and Schmid-Hempel 1999; Seeley and Tarpay 2007), and/or higher colony productivity due to a more efficient partitioning of task specializations among workers, through genetic polyethism (Page et al. 1989; Crozier and Fjerdingstad 2001; Hughes et al. 2003; Julian and Fewell 2004; Oldroyd and Fewell 2007; Waddington et al. 2010).

On the other hand, the evolution of polygyny is addressed by two principal explanatory frameworks: (1) ecological factors, which associate high potential cost to dispersal and/or independent nest founding due to unfavorable environmental conditions, both biotic and abiotic (Bourke and Franks 1995). Newly mated females may be selected to attempt rejoining their natal colonies (or a nearby conspecific colony), resulting in secondary polygyny from queen adoption. Queen adoption is beneficial if the likelihood of successful dispersal and colony founding is low, under pressures such as competition for resources, low nest site availability (in the case of high population density), and high predation (Bourke and Franks 1995; Seppä et al. 1995; Banschbach and Herbers 1996; Pedersen and Boomsma 1999a; Ingram 2002; Purcell et al. 2015). Similarly, primary polygyny, resulting from the association of colony-founding queens, may result from ecological pressures where solitary colony foundation is unfavorable, and co-founding results in a higher probability of a successful colony being established (Cahan and Julian 1999; Haney and Fewell 2018). (2) Social/genetic factors may promote the evolution of polygyny (both primary and secondary) by allowing for, and sometimes promoting, the co-existence of queens in colonies. The proposed benefits are aligned with the genetic diversity benefits for the evolution of polyandry, including more efficient division of labor in a diverse workforce, greater resistance to disease and parasites, and overall more productive colonies (Bourke and Franks 1995; Keller 1995; Schmid-Hempel and Crozier 1999; Oldroyd and Fewell 2007).

The main difference of polygyny versus polyandry for intracolony relatedness is that polygyny introduces more genetic variation into a colony compared to polyandry because females produced by a single, multiply mated queen are half siblings, with a minimum relatedness of 0.25, while females produced by different unrelated mothers should not be more related than any two individuals from that population (i.e., they are only as related as the inbreeding coefficient in their population) (Pedersen and Boomsma 1999a). In some cases the increased genetic diversity introduced by polyandry is alleviated or cancelled out by inbreeding (Trontti et al. 2007), and by the fact that males mating with a single female are often related (Pedersen and Boomsma 1999b; Kellner et al. 2007). Similarly, relatedness between nestmate queens (for example, if nestmate queens are sisters

or mother/daughters) would alleviate some of the negative effects of polygyny on relatedness. Empirical evidence has shown that experimentally combining workers from different colonies (which should then be as related as two workers produced by unrelated nestmate queens) increased disease resistance in socially polymorphic species across eusocial insects (Baer and Schmid-Hempel 1999; Tarpay 2003; Hughes and Boomsma 2004; Reber et al. 2008).

In contrast to these benefits of polygyny and polyandry, reduced colony relatedness also increases the potential for conflict in eusocial insect colonies. Nepotism (the preferential rearing of related brood by workers) is an expected consequence of multiple reproductive lineages coexisting in a colony. While it has not been demonstrated clearly in cases of polyandry (Page et al. 1989; Breed et al. 1994), empirical evidence has demonstrated occurrences of nepotism in polygynous species (Hannonen and Sundström 2003). While this phenomenon increases the inclusive fitness of nepotistic lineages, it may result in an overall reduction of colony productivity (Page et al. 1989).

Polygyny itself introduces conflict over reproduction between nestmate queens, particularly for the production of reproductive females (Reeve and Ratnieks 1993; Bourke and Franks 1995). In extreme cases, this conflict can result in the evolution of obligate inquiline social parasites, as has been documented for inquiline social parasites that originated as reproductive cheaters and speciated directly from their hosts (Savolainen and Vepsäläinen 2003; Rabeling et al. 2014b, 2018; Leppänen et al. 2015; Nehring et al. 2018). These obligately parasitic species are often workerless or nearly-workerless, and “infiltrate” colonies of their hosts, taking advantage of host workers to rear their offspring (Buschinger 2009; Rabeling 2020). Secondary polygyny has been associated with the evolution of inquiline social parasitism, as it would not only provide an opportunity for socially parasitic nest foundation, but also provide the opportunity for reproductive cheating to appear and be accepted inside the host colony (Bourke & Franks, 1991; Buschinger, 2009; Dahan & Rabeling *in Review*).

Among ants, the colony and social structures of leaf-cutting ants are known to be particularly diverse (Boomsma et al. 1999), and five obligate social parasite species are known in *Acromyrmex* leaf-cutting ants and the monotypic social parasite genus *Pseudoatta* (Rabeling et al. 2019). *Acromyrmex* leaf-cutting ants are of particular interest for studying the evolution of mating behavior and life history traits (Hölldobler and Wilson 1990; Diehl-Fleig and de Araújo 1996; Boomsma et al. 1999; Villesen et al. 1999, 2002; Murakami et al. 2000; Diehl et al. 2001; Rabeling et al. 2015, 2019; Nehring et al. 2018). All free-living (i.e., non-socially parasitic) leaf-cutting ant species investigated so far, which includes at least seven *Acromyrmex* species, have been reported to be obligately polyandrous. In contrast,

the queens of fungus-growing ants in distantly related genera outside the leaf-cutting ants are predominantly singly mated (Villesen et al. 1999, 2002). Additionally, some species of *Acromyrmex* (*A. heyeri*, *A. echinator*, *A. subterraneus bruneus*, and *A. subterraneus molestans*), as well as *Amoimyrmex striatus*, are reported to be facultatively polygynous (Delabie 1989; Bekkevold et al. 1999; Diehl et al. 2001; Souza et al. 2004; Nehring et al. 2018), whereas others, such as *A. landolti* (Keller and Reeve 1994) and *A. octospinosus* (Boomsma et al. 1999), as well as most species of *Atta* are monogynous, with the exception of the primarily polygynous *Atta texana* (see Mintzer 1987). Therefore, *Acromyrmex* is one of the rare ant genera with different species exhibiting a mosaic of mating behaviors and social structures, which allows for comparative investigations of both the evolutionary causes and consequences of complex mating behaviors in eusocial Hymenoptera, especially with respect to the co-evolution between multiple mating and polygyny, and its consequences on the evolution of life history traits in these species, such as the emergence of social parasitism. Here, we investigate the mating biology and population genetic colony structure of five South American *Acromyrmex* species. We conduct a comparative evolutionary analysis of mating biology and colony structure, and we provide an analysis of the co-evolutionary dynamics between polyandry and polygyny in *Acromyrmex* leaf-cutting ants.

Methods

Species collection and sampling

To study the mating biology and social structure in South American leaf-cutting ants, we investigated five species: *Acromyrmex ambiguus*, *A. crassispinus*, *A. heyeri*, *A. lobicornis*, and *A. lundii*. All but one of these species are known hosts of the obligate inquiline social parasites *Pseudoatta argentina* and *A. charruanus*, and the social structure and mating biology of these host species, except for *A. heyeri*, remain unknown so far (Gallardo 1916; Diehl et al. 2001; Rabeling et al. 2015). *Acromyrmex lobicornis* has not been observed to host a social parasite species, but it was inferred as the closest relative to a clade including three social parasite species (Rabeling et al. 2018). Therefore, the biology of both *A. lobicornis* and the known hosts is relevant for discussing the association between social structure and the evolution of social parasitism in leaf-cutting ants (Dahan & Rabeling, *in review*). Workers, and when present, sexual reproductives (alates), of each species were collected from nests in their native ranges in Uruguay and Southern Brazil, between March 2012 and November 2013 (Fig. 1; Table S1). All samples were preserved in 100% ethanol and identified visually with a Leica M205 C stereomicroscope,

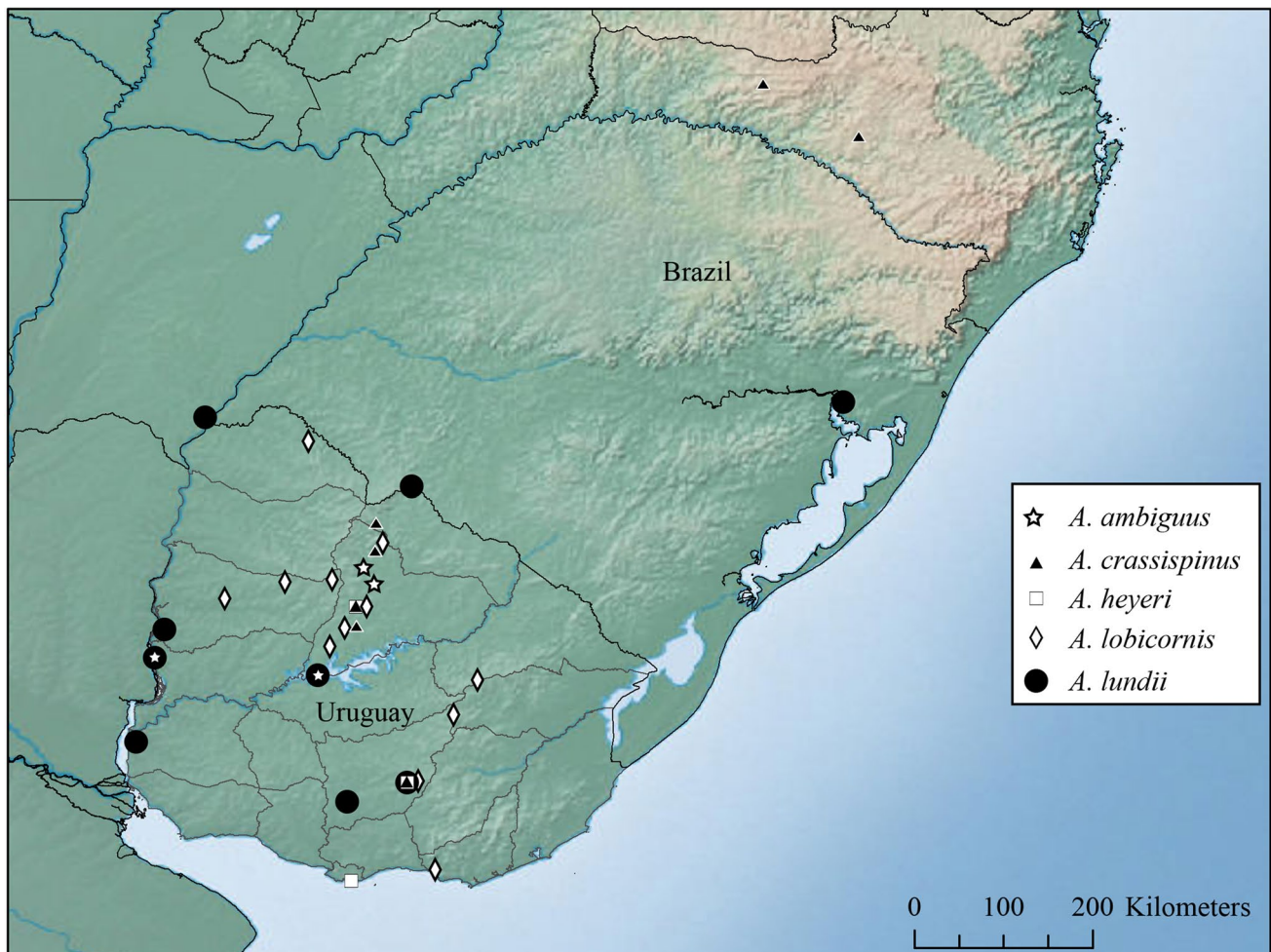


Fig. 1 Sampling map depicting field study sites where *Acromyrmex* colonies were collected

using published taxonomic keys (Gonçalves 1961; Fowler 1985, 1988). Voucher specimens were deposited in the Social Insect Biodiversity Repository (SIBR) at Arizona State University in Tempe, Arizona, and in the entomological collection of Martin Bollazzi's laboratory at the Facultad de Agronomía of the Universidad de la República in Montevideo, Uruguay.

Microsatellite selection and colony genotyping

To infer individual genotypes, six microsatellite loci were selected out of 22 loci characterized for *A. lundii* (Rabeling et al. 2014a), based on allele diversity and range overlap in each locus, to obtain the most informative sampling (Table S1). The selected markers were used in three duplex PCR reactions. For each screened colony, we sampled between 16 and 48 individuals, with a minimum of 16 workers and a maximum of 16 males. Colony samples were maximized based on field sampling, and a total of 89 colonies were analyzed for *A. crassispinus* ($n = 18$), *A. heyeri*

($n = 25$), *A. lobicornis* ($n = 18$), and *A. lundii* ($n = 28$). Additionally, we genotyped four colonies of the related species *A. ambiguus*, but additional sampling could not be completed (see SI). If sampling 48 individuals per colony was impossible, sampling was reduced by increments of 8 individuals, to a minimum of 16 workers. In all but two species, all genotypes available were from workers. For *A. crassispinus*, female alates were available in six colonies, in which we sampled 5–24 alates; in *A. heyeri*, both female and male alates were sampled from five colonies (8–12 female alates, 10–16 males). In total, 4,614 individuals were screened, generating 27,684 genotyped loci. DNA was purified using the Qiagen PureGene blood & tissue kit. Markers were amplified in duplex PCR reactions, each containing 1–10 ng of template DNA. Genotyping was conducted at each locus by fragment analysis, and genotypes were visually scored against a Genescan LIZ-500 standard using 6-Fam and NED tagged primers (Applied Biosystems). All scoring of genotypes was conducted in Geneious 8.1.4 (Kearse et al. 2012), and all genotypes were scored blindly by a single

observer (RAD) without knowledge of colony of origin of each samples.

Inference of parental genotypes

Parental genotypes were inferred for each colony, first by using the COLONY software package (Jones and Wang 2010), followed by manual resolution using the COLONY output as a starting point. The number of queens in a colony was inferred from the results of the parental inference. Offspring with three missing loci or more in the final dataset (i.e., loci that failed to amplify after at least two PCR attempts) were discarded. A high frequency of missing loci within colonies may be indicative of a high frequency of null alleles or undetectable mutations, which would introduce bias and error in our analyses. Thus, whole colonies where more than 25% of the offspring had two missing loci or more were excluded from further analyses. Whole colonies with more than 25% of discarded workers were also excluded. COLONY was run using entire species-specific datasets, updating the allele frequencies between each run. The software is equipped to handle such missing loci: the input allelic dropout rate, representing the frequency with which alleles change in offspring due to mutations, was set as 10^{-4} , as this is the fast limit of microsatellite mutation rate observed in arthropods (Bhargava and Fuentes 2010). The genotyping error rate, accounting for extrinsic factors resulting in genotyping errors (null alleles, human error, etc.), was also set at 10^{-4} for each locus, as the default for the software. The results for each colony were then visually inspected to confirm the validity of the inferred maternal genotypes, and manually rechecked, correcting the inference to produce the most conservative estimate, and correcting any genotyping error. In many cases, several resolutions could account for the observed genotypes within a single colony. In these cases, resolutions were selected based on several criteria, in order of importance:

1. Parental inferences with the least number of queens (maternal genotypes) were favored;
2. If genotypes of multiple queens differed at a single locus within a colony, a single queen was inferred as mother of all the offspring of these queens, and the discordant offspring would be accounted for as genotyping error;
3. Inferences approaching a 1:1 Mendelian ratio for maternal alleles were favored (*sensu* Kronauer et al. 2004);
4. Inferences with the least number of sires (paternal genotypes) were favored;
5. Inferences minimizing the number of discarded individuals were favored;
6. Inferences minimizing the genotyping error rate were favored.

These criteria of selection ensured that the most conservative resolution was selected (i.e., resulting in the lowest possible number of queens and sires) for the genotypes obtained from a single colony. Due to our sampling and inference approach, it was possible to underestimate the number of queens and sires, but much less likely to overestimate these values. After manual resolution, maternal genotypes contributing less than four offspring (for $n = 48$), or two offspring (for $n \leq 24$) were ignored and their offspring discarded. This threshold was selected not only to be able to calculate the Mendelian segregation ratios of queens, but also to minimize false positives (offspring clustering to an additional matriline due to mutation, migration from a neighboring colony, or genotyping error), while maintaining a high probability of sampling offspring of all queens in a colony, even with moderately high reproductive skew between queens (see SI).

Inference of mating frequencies and intra-colonial relatedness

The effective number of mates per queen was inferred using the formula

$$m_e = \frac{(N - 1)^2}{\sum p_i^2(N + 1)(N - 2) + 3 - N}$$

where N represents the total number of successfully assigned offspring, and p_i represents the proportion of offspring produced by the i th father (Nielsen et al. 2003). We then calculated inter-individual relatedness (r) within all colonies following the Queller & Goodnight method (Queller and Goodnight 1989). In order to avoid pseudoreplication, the average within-colony relatedness was calculated for each colony and considered in all statistical analyses.

Reproductive skew

We investigated reproductive skew (describing inter-individual differences in offspring production) between the different patrilines mated to each single queen, using the Pamilo-Crozier skew index (Pamilo and Crozier 1996). In all cases, only workers were used to determine reproductive skew. The index represents the deviation between the number of patrilines observed mated to a queen and the effective mating frequency of a queen, standardized to the maximum variance in offspring contribution:

$$S = \frac{M - m_e}{M - 1}$$

Here, M is the inferred number of males mated to a queen and m_e is the effective mating frequency of that queen (see above). This index varies between 0, representing no skew,

with equal contribution between all sires mated to a queen, and 1, representing a case where the reproductive skew is absolute and a single sire is producing all offspring. Note that the inference method presented here means that the value of S can never be exactly 1, since we are relying on patriline detection in the offspring generation to infer the males represented in the colony.

Statistical analyses

We investigated variation in observed mating frequency (number of males mated to each queen), effective mating frequency, reproductive skew, and average intra-colonial relatedness for all five species. Observed mating frequency, effective mating frequency, and skew index were compared between species using Kruskal–Wallis tests, performing post hoc pairwise Mann–Whitney U tests where appropriate, applying a false discovery rate correction in all cases (FDR = 0.05). The response variables (number of mates, effective mating frequency, and reproductive skew) can be expected to violate assumptions of normality; therefore, non-parametric tests were favored. In order to assess the roles of effective mating frequency and social structure on average intra-colonial relatedness between species, a linear regression was fit across all species with relatedness as a response variable, and number of queens, effective mating frequency, and their interaction, as well as species, as predictor variables. Species was included as a predictor variable in order to account for any intrinsic between-species differences in relatedness. Effective mating frequency was log-transformed for this analysis. Assumptions of residual normality and homoscedasticity were met for the regression (Fig. S1). We excluded *A. ambiguus* from all interspecific analyses to avoid statistical issues involving low power and uneven sampling, as we were only able to analyze four colonies. For the intra-specific estimates of mating behavior, we chose to report the data for the four *A. ambiguus* colonies; however, these results should be viewed as preliminary. Within species, we compared both the average observed relatedness (r) and the effective mating frequency between polygynous and monogynous colonies. Because of our sampling, these comparisons were only possible for *A. heyeri* and *A. lobicornis*. In these cases, we used Student's t -tests (with equal variance) to compare the groups, except to compare effective mating frequency in *A. heyeri*, where a Welch two-sample t -test was more appropriate to account for unequal variance. In all cases, we used the average value for each colony as a replicate within a group to avoid pseudoreplication (averaging the effective mating frequency of all queens in a colony for polygynous colonies). In all cases, we set the significance threshold at $\alpha = 0.05$. All analyses were performed in R

4.1.0, using the packages 'related' and 'ggplot2' (Wickham 2009; Pew et al. 2015; R Core Team 2021).

Results

Social structure of *Acromyrmex* leaf-cutting ants

We investigated the social structure of five South American species of *Acromyrmex* leaf-cutting ants based on 27,684 genotypes among six loci generated for 4,614 individuals. We detected different social structures and mating behaviors among these species. All 28 colonies of *Acromyrmex lundii* were inferred to be monogynous. In contrast, *A. crassispinus* (2/18 polygynous; 11%), *A. heyeri* (5/25 polygynous, 20%), and *A. lobicornis* (5/18 polygynous; 28%) were socially polymorphic, consisting of both monogynous and polygynous colonies (Table 1). Among the facultatively polygynous species, most polygynous colonies comprised two matriline, with the exception of one colony of *A. crassispinus* in which three queens were inferred, and one colony of *A. lobicornis* in which four queens were inferred (Table 1). One out of four *A. ambiguus* colonies was polygynous with two matriline, whereas the other three colonies were monogynous, suggesting that *A. ambiguus* is also facultatively polygynous.

Multiple mating and effective mating frequency of *Acromyrmex* queens

In all five species, queens used sperm from multiple males to produce offspring, although there were marked differences between species (Table 1; Kruskal–Wallis test: $\chi^2_3 = 21.66$, $p = 7.67 \times 10^{-5}$). Specifically, the observed mating frequency (i.e., the actual number of detected males mated to a queen) of *A. crassispinus* queens (average observed mating frequency $M = 6.48$) is higher than that of three of the other species, although it is statistically indistinguishable from that of *A. heyeri* queens ($M = 5.50$; Table 1; Fig. 2a). Conversely, queens of *A. lobicornis* are mated to significantly fewer males compared to the other species ($M = 2.88$). It is worth noting that queens of *A. crassispinus* and *A. heyeri* also mate with a higher maximum number of mates (max. 12 mates) compared to *A. lobicornis* (max. 4 mates) and *A. lundii* (max. 6 mates) (Table 1). Preliminary analysis suggests that queens of *A. ambiguus* mate with a high number of males ($M = 6.00$, max 10 mates), appearing comparable to queens of *A. crassispinus* and *A. heyeri*.

Interestingly, in spite of the higher observed mating frequency in *A. crassispinus*, this species had a similar effective mating frequency (i.e., the effective number of mates contributing to a queen's offspring) ($m_e = 4.51$)

compared to *A. heyeri* ($m_e = 4.43$) and to *A. lundii* ($m_e = 3.33$; Table 1). Similar to the pattern seen for the observed mating frequency, the effective mating frequency of queens of *A. lobicornis* ($m_e = 2.36$) was significantly lower than in the other species (Kruskal–Wallis test: $\chi^2_3 = 17.81$, $p = 4.81 \times 10^{-4}$; Fig. 2b). In all cases, the observed number of mates was much lower than the number of sampled offspring, ensuring unbiased estimation of m_e following the assumptions of the estimator (Nielsen et al. 2003). In *A. ambiguus*, the effective mating frequency of queens was comparatively high ($m_e = 5.92$).

Reproductive skew

We detected reproductive skew between patriline in all species (Fig. 3). While the values recovered varied between ~0.1 and ~0.45 (a value of 0 indicates an equal reproductive share between males, whereas a value of 1 indicates that all offspring are sired by a single male), no significant differences were found between species (*A. crassispinus*: $S = 0.41$; *A. heyeri*: $S = 0.26$; *A. lobicornis*: $S = 0.26$; *A. lundii*: $S = 0.26$; Kruskal–Wallis test: $\chi^2_3 = 6.82$, $p = 0.08$; Table 1, Fig. 3).

Intra-colonial relatedness

Based on the multiple regression, we found no evidence for a significant difference in average intra-colonial relatedness between species (ANOVA: $F_{2,72} = 3.10$, $p = 0.0513$; Table 1, Fig. 4). When scrutinizing the regression parameters, it appears that *A. heyeri* colonies show a slightly lower

intra-colonial relatedness ($r \pm \text{std. error} = 0.36 \pm 2.8 \times 10^{-2}$) compared to *A. lundii* ($r = 0.46 \pm 2.6 \times 10^{-2}$), *A. crassispinus* ($r = 0.46 \pm 3.2 \times 10^{-2}$), and *A. lobicornis* ($r = 0.46 \pm 3.2 \times 10^{-2}$). Effective mating frequency had a highly significant effect on intra-colonial relatedness (estimated regression parameter $b_{\text{mating}} = -0.234$, $F_{1,72} = 44.61$, $p = 4.86 \times 10^{-9}$; Fig. 4). We also found a significant difference in how effective mating frequency affected relatedness in monogynous vs. polygynous colonies ($F_{1,72} = 4.054$, $p = 0.0478$; Fig. 4). On the other hand, social structure alone had no detectable effect on relatedness ($F_{1,72} = 0.0193$, $p = 0.890$, Fig. 4). Relatedness in colonies of *A. ambiguus* appeared indistinguishable from that inferred in colonies of the other species ($r = 0.42 \pm 0.10$).

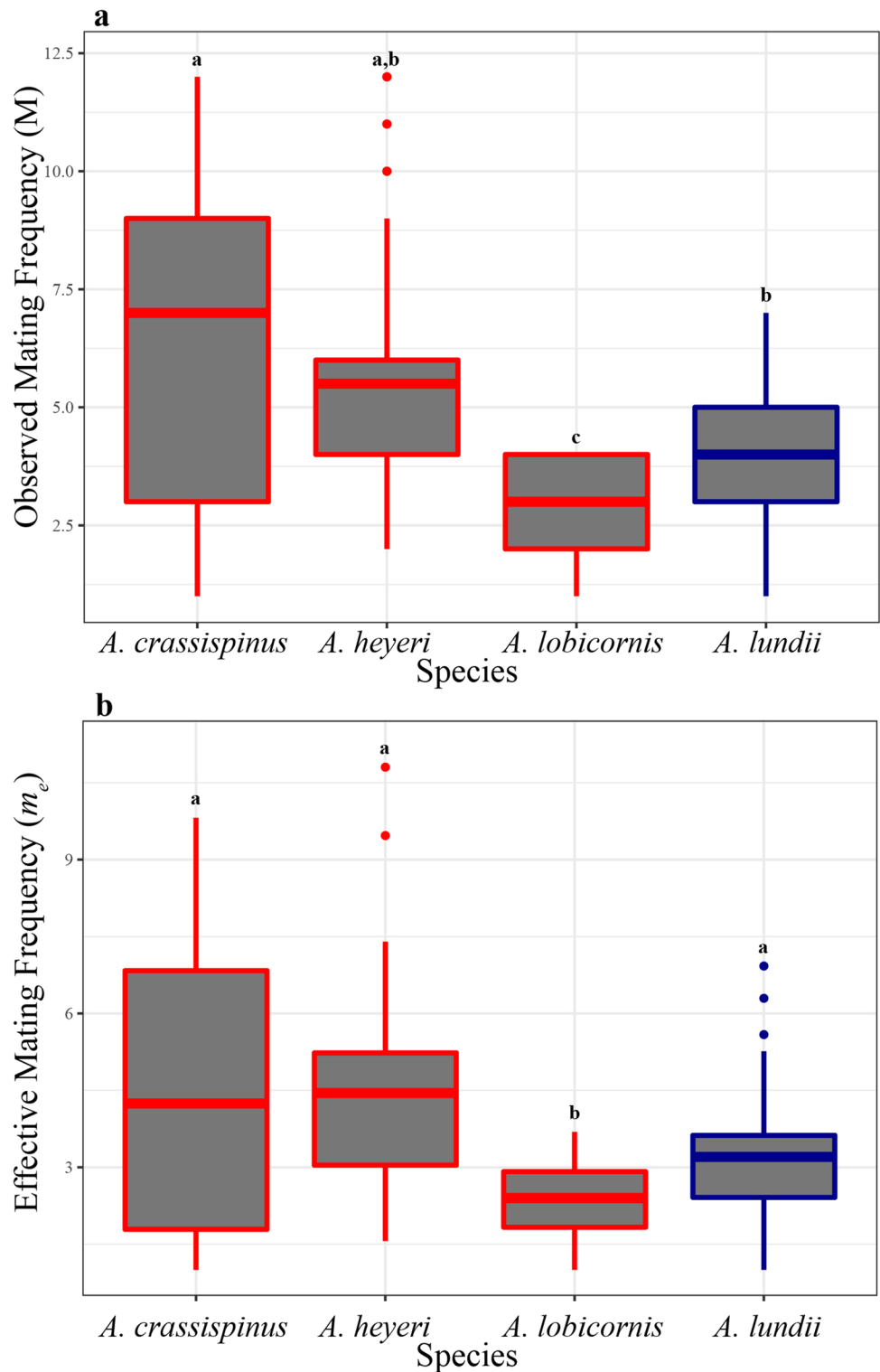
Relatedness and effective mating frequency variation within species

Comparing the effective mating frequency and intra-colonial relatedness between polygynous and monogynous colonies yielded contrasting results in *A. heyeri* and *A. lobicornis*. In *A. heyeri*, the average effective mating frequency of queens in polygynous colonies (average $\bar{m}_e \pm \text{std. error} = 3.17 \pm 0.41$) was significantly lower in comparison to the effective mating frequency of queens in monogynous colonies ($m_e = 4.92 \pm 0.51$; Welch t -test: $t_{14.35} = 2.65$, $p = 0.0187$; Fig. 5a). On the other hand, we detected no significant difference between the intra-colonial relatedness of monogynous ($r \pm \text{std. error} = 0.38 \pm 0.02$) and polygynous ($r = 0.26 \pm 0.07$) colonies of *A. heyeri* (t -test: $t_{23} = 2.00$, $p = 0.057$; Fig. 6a).

Table 1 Summary of social structure in colonies of five *Acromyrmex* leaf-cutting ants. The numbers in parentheses in the top row represent the number of polygynous colonies over the total sampling for the corresponding species

	<i>A. ambiguus</i>	<i>A. crassispinus</i>	<i>A. heyeri</i>	<i>A. lobicornis</i>	<i>A. lundii</i>
Number of colonies sampled	4	18	25	18	28
Frequency of polygyny	25.00% (1/4)	11.00% (2/18)	16.00% (4/25)	22.22% (4/18)	0.0% (0/28)
Maximum number of queens	2	3	2	4	1
Minimum number of mates	4	1	2	1	1
Maximum number of mates	10	12	12	4	6
Average number of queens per colony (std. deviation)	1.25 (0.50)	1.17 (0.51)	1.15 (0.37)	1.39 (0.78)	1 (N/A)
Observed mating frequency M (std. deviation)	6.000 (2.345)	6.476 (3.737)	5.500 (2.570)	2.875 (1.076)	4.179 (1.634)
Effective mating frequency m_e (std. deviation)	5.919 (2.405)	4.510 (2.849)	4.433 (2.197)	2.356 (0.796)	3.329 (1.395)
Reproductive skew S (std. deviation)	0.091 (0.135)	0.414 (0.231)	0.257 (0.150)	0.258 (0.206)	0.256 (0.213)
Intra-colony relatedness r (std. error)	0.423 (0.096)	0.459 (0.036)	0.355 (0.024)	0.461 (0.036)	0.464 (0.025)

Fig. 2 Mating frequency in four species of *Acromyrmex* leaf-cutting ants. (A) Observed mating frequency, the total number of males mated to a single queen. (B) Effective mating frequency, the effective number of males contributing equally to a queen's offspring production. Letters above the boxes represent the significance groups recovered from pairwise Mann–Whitney *U* test with FDR correction ($\alpha=0.05$). Red (gray) boxes represent polygynous species; blue (black) boxes represent monogynous species. (Parenthesis colors refer to grayscale equivalences)



Conversely, in *A. lobicornis* there was no significant difference in the effective mating frequency of polygynous colonies ($m_e = 2.24 \pm 0.39$) compared to that of monogynous colonies ($m_e = 2.53 \pm 0.21$, *t*-test: $t_{16} = 0.65$, $p = 0.525$; Fig. 5b). However, we found that average relatedness was significantly lower in polygynous colonies

of *A. lobicornis* ($r = 0.33 \pm 5.90 \times 10^{-2}$) compared to monogynous colonies ($r = 0.51 \pm 3.66$; *t*-test: $t_{16} = 2.67$, $p = 0.0167$; Fig. 6b). These results suggest relatedness may be affected in different ways by variation in mating frequency and queen number in different species of leaf-cutting ants, likely depending on the relatedness of

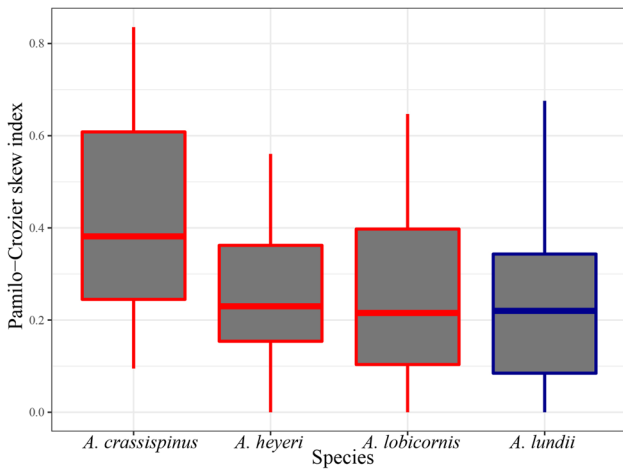
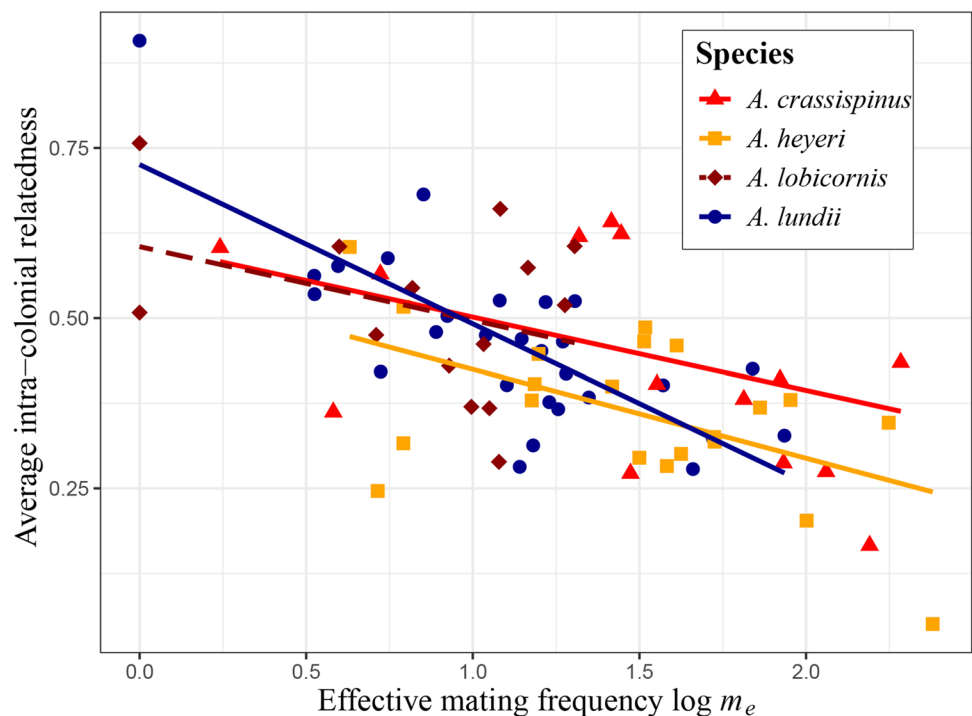


Fig. 3 Pamilo-Crozier skew index in workers of four species of *Acromyrmex* leaf-cutting ants. Red (gray) boxes represent polygynous species; blue (black) boxes represent monogynous species. (Parenthesis colors refer to grayscale equivalences)

reproductively active queens and their mates. Interestingly, relatedness appears to be affected primarily by either mating frequency or queen number in different species, as opposed to a combination of both.

Fig. 4 Scatterplot of average intra-colonial relatedness vs. effective mating frequency (natural log) in four species of *Acromyrmex* leaf-cutting ants. Warm (gray) colors represent facultatively polygynous species; the blue (black) line and circles represent monogynous species. *A. lobicornis* (a polygynous species, diamonds) is represented with a dashed line for convenience in grayscale. (Parenthesis colors refer to grayscale equivalences)



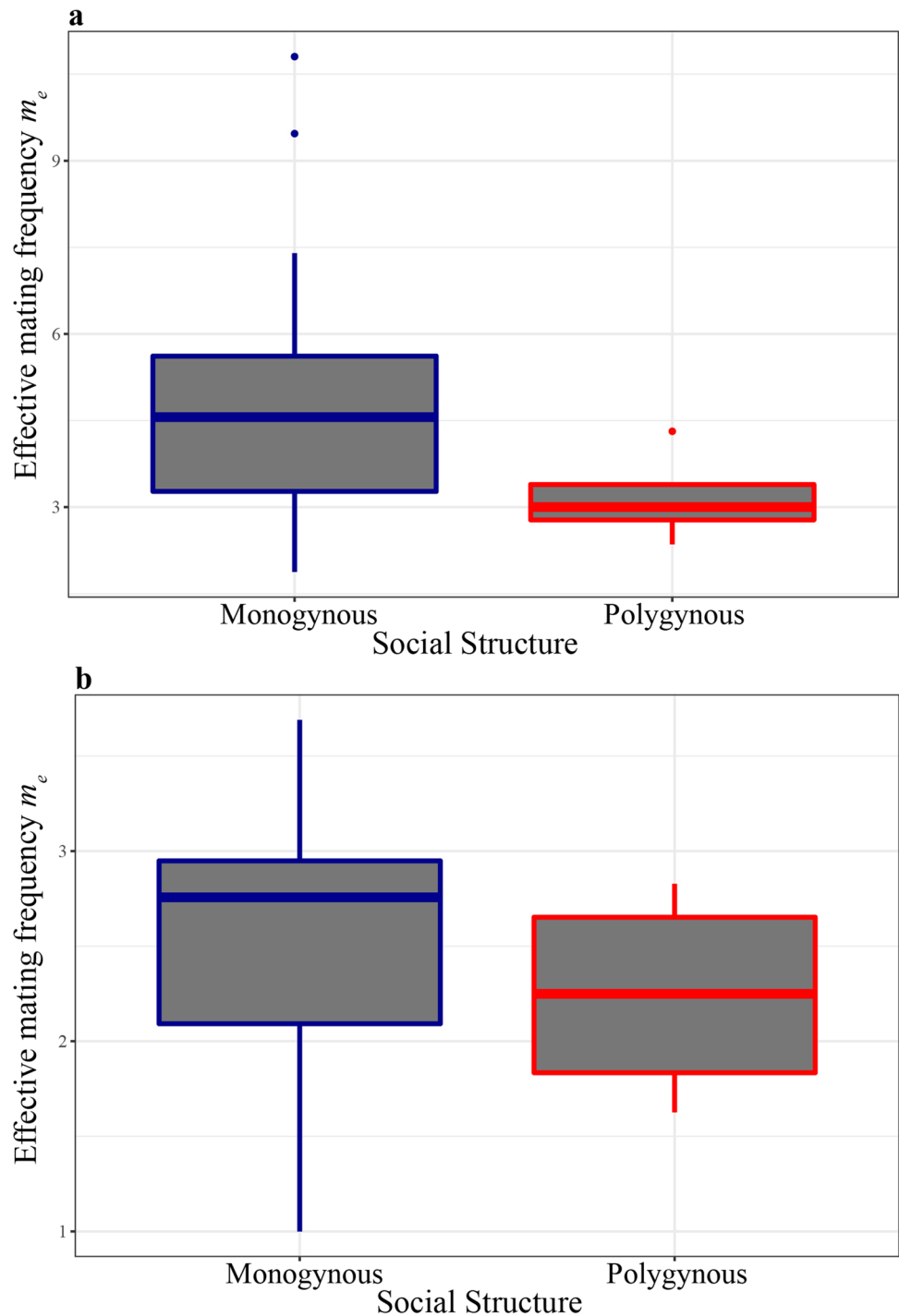
Discussion

We characterized the social structure and mating biology of five species of South American *Acromyrmex* leaf-cutting ants. Our analysis infers one species, *A. lundii*, as monogynous, while the other four species, *A. ambiguus*, *A. crassispinus*, *A. heyeri*, and *A. lobicornis* are facultatively polygynous. All five species are polyandrous. Our findings suggest that variation in relatedness is affected differently by variation in mating biology in different species, and that species have no appreciable differences in reproductive skew among males mated to the same queens. Finally, we find that variation in mating biology and social structure have little effect on the intra-colonial relatedness.

Patterns of association between mating biology and social structure in ants

Our results reveal that mating biology is variable among South American species of *Acromyrmex* leaf-cutting ants and that mating behavior and social structure occur in different combinations across leaf-cutting ants. Polygyny and a relatively high mating frequency ($m_e \geq 3.50$) were repeatedly observed, with variation in both traits occurring independently of one another between species. One of the studied species, *A. lundii*, appears to have reverted to obligate monogyny, while another, *A. lobicornis*, showed a significantly lower effective mating frequency compared

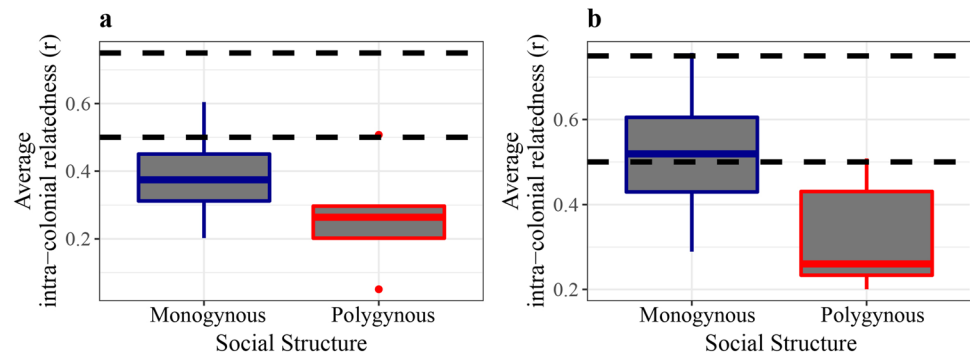
Fig. 5 Effective mating frequency in monogynous and polygynous colonies of (a) *Acromyrmex heyeri* and (b) *A. lobicornis*. Red (gray) boxes represent facultatively polygynous colonies; blue (black) boxes represent monogynous colonies. (Parenthesis colors refer to grayscale equivalences)



to the others. It is theoretically possible that the inferred monogyny of *A. lundii* could stem from the non-sampling of polygynous colonies. However, the frequency of polygynous colonies in the other sampled species ranged from 11 to 28%, and the number of sampled colonies was larger for *A. lundii* than for any other species in our study. Calculating the probability of not sampling a polygynous colony out of 28 total colonies based on a binomial distribution, and assuming a conservative rate of polygyny of 10%, the binomial

probability of sampling no polygynous colonies is 0.05, suggesting that our results are unlikely to stem from sampling error. Interestingly, these inter-specific variations in mating biology appear to have little impact on average intra-colonial relatedness in species (Fig. 4). The only noteworthy variation seems to be seen in colonies of *A. heyeri*, which have slightly lower relatedness on average (Fig. 4). Variation in [intra-colonial relatedness](#) across species is not influenced by species-level social structure (i.e., whether a species

Fig. 6 Average intra-colonial relatedness in monogynous and polygynous colonies of *Acromyrmex heyeri* (a) and *A. lobicornis* (b). Red (gray) boxes represent polygynous colonies; blue (black) boxes represent monogynous colonies. Parenthesis colors refer to grayscale equivalences)



was facultatively polygynous or obligately monogynous), but was significantly affected by effective mating frequency (Fig. 4, Table 1). This result was unsurprising given the limited number of monogynous colonies in the sample, and the fact that polygynous colonies were excluded from the regression analysis, thus showing only the predictable negative effect of mating frequency on relatedness. On the other hand, the effect of effective mating frequency on intra-colonial relatedness was itself dependent on the species social structure, with a larger effect in the strictly monogynous *A. lundii* compared to the other, socially polymorphic species (Fig. 4). This result suggests that relatedness responds differently to variations in mating biology between monogynous and polygynous species, even in monogynous colonies of species that are facultatively polygynous. The overall general pattern of trait association between social structure, mating biology, and relatedness appears to be unrelated to the phylogenetic relationship between the investigated species (Fig. 7).

Previous studies investigated the mating biology of four Central and South American *Acromyrmex* leaf-cutting ant species. Diehl and colleagues (Diehl et al. 2001) conducted a qualitative investigation of mating biology and colony structure in the South American species *A. heyeri* and *Acromyrmex striatus*, finding facultative polygyny and prevalent polyandry in both species, which is consistent with our results. Boomsma and colleagues (Boomsma et al. 1999) investigated the mating biology of the monogynous Central American species *A. octospinosus*, while Bekkevold and colleagues (Bekkevold et al. 1999) as well as Nehring and colleagues (Nehring et al. 2018) investigated both mating biology and social structure in its close relative *A. echinator*. The monogynous *A. octospinosus* was found to have a high effective queen mating frequency ($m_e = 3.93$; $n = 22$), and a mean pedigree relatedness (calculated from effective mating frequency rather than from genotypic data) of 0.38. *Acromyrmex echinator* was found to be facultatively polygynous (Bekkevold et al. 1999; Nehring et al. 2018) and reproductive queens were found to have a relatively lower effective mating frequency ($m_e = 2.33$; $n = 20$), although the estimate was based on monogynous colonies only ($n = 13$) (Bekkevold et al. 1999). Average intra-colonial relatedness

in these *A. echinator* colonies was estimated from the effective mating frequency to be 0.41. Based on these results, *A. echinator* appears similar to *A. lobicornis* with a low effective mating frequency, facultative polygyny, and high intra-colonial relatedness, while *A. octospinosus* presents a unique pattern of trait association with high effective mating frequency, monogyny, and low intra-colonial relatedness.

Overall, among the six *Acromyrmex* species with detailed sampling for mating biology and social structure, we cannot identify any consistent pattern of trait association between social structure and mating biology. In fact, only *A. echinator* and *A. lobicornis* appear to have converged on a common association phenotype (with polygyny, low effective mating frequency, and relatively high mean intracolony relatedness). All other species were found to display unique patterns of trait associations (Table 2). Evolutionary changes in social structure and mating frequency also appear to be phylogenetically unrelated (Fig. 7). Interestingly, polygyny and polyandry are not consistently negatively correlated in these *Acromyrmex* leaf-cutting ants.

In all instances, the mating behavior of leaf-cutting ants was characterized as snapshots in time, rather than characterizing the actual lifetime reproductive behavior of a colony and the queen(s) within it. Lifetime variation in sperm usage and colony structure (resulting, for example, from the adoption of new queens) could lead to significant variation in intracolony relatedness, effective mating frequency, or queen number during a colony's lifespan, of which we can only document a limited view. A full investigation of parentage and relatedness through a colony's lifetime would be fascinating but problematic in leaf-cutting ants given the long lifespan of queens. However, some evidence suggests that the composition of sperm used by queens does not change over long periods of time, suggesting that such snapshots may indeed provide an accurate view of paternity in a colony (Stürup et al. 2014). Furthermore, this limitation in time may also explain the importance of species-level social structure (i.e., whether a species is characterized a polygynous vs. monogynous), as lifetime variation in queen number in colonies can have a significant impact on the relatedness and population structure of these species. In our sampling, the

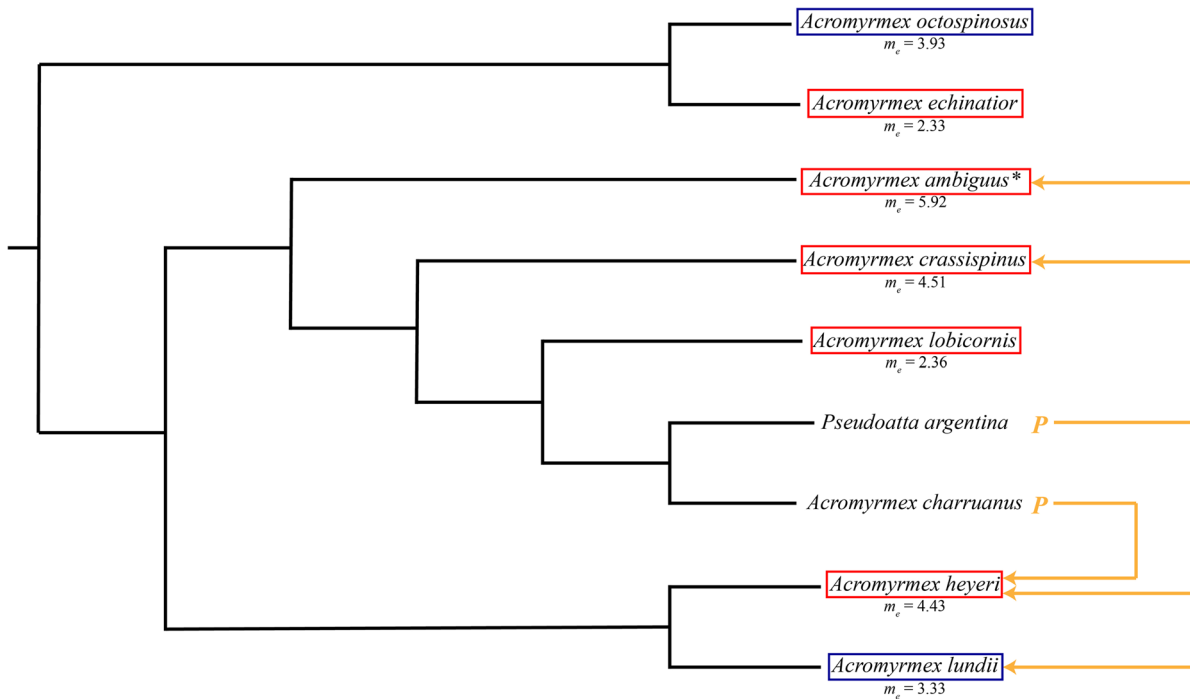


Fig. 7 Phylogenetic distribution of mating biology and social structure in *Acromyrmex* leaf-cutting ants. Species boxed in red (gray) are socially polymorphic (i.e., facultatively polygynous); species boxed in blue (black) are obligately monogynous. A yellow (gray) P denotes inquiline social parasites, and arrows show host-parasite relationships. Data for *A. ambiguus*, denoted by an asterisk, is based on low

sample size. The *Acromyrmex* phylogeny is modified from Rabeling et al. 2018. Information about the social structure and mating frequency of the Central American leaf-cutting ant species *A. echinator* and *A. octospinosus* were generated by Boomsma et al. 1999 and Bekkevold et al. 1999. (Parenthesis colors refer to grayscale equivalences)

species-level classification was shown to significantly affect the relationship between mating frequency and relatedness, implying that a higher effective mating frequency in obligately monogynous species had a larger impact on colony relatedness compared to species with facultative polygyny (Fig. 4).

Functional polygyny in ants may be either primary, resulting from multiple queens co-founding colonies (pleometrosis), or secondary, resulting from the adoption of new queens in established mature colonies. While many examples of pleometrotic species are known, only a few

have been documented to actually result in functional primary polygyny (Bourke & Franks, 1995; Johnson, 2004; Mintzer, 1987; Rissing et al. 1986; Trunzer et al., 1998). In contrast, secondary polygyny appears to be more common in ants (Boomsma et al., 2014; Bourke & Franks, 1995; Hölldobler & Wilson, 1977). The mode of polygyny in the *Acromyrmex* species sampled here is unknown. Pleometrosis has been observed in *A. crassispinus*, but in all cases but one queen died before colony maturation (Diehl-Fleig & de Araújo, 1996). In contrast, primary polygyny has been well characterized in the North American desert

Table 2 Summary of patterns of trait association between mating biology and social structure among six species of *Acromyrmex* leaf-cutting ants. High effective mating frequency was defined as a

value of $m_e = 3.50$ or above. High mean intracolony relatedness was defined as a value of $r = 0.40$ or above. Thresholds were selected as the approximate averages of variables

	m_e	Social structure	Mean intracolony relatedness	Source
<i>A. crassispinus</i>	High	Polygyny	High	This study
<i>A. echinator</i>	Low	Polygyny	High	Bekkevold et al. 1999
<i>A. heyeri</i>	High	Polygyny	Low	This study
<i>A. lobicornis</i>	Low	Polygyny	High	This study
<i>A. lundii</i>	Low	Monogyny	High	This study
<i>A. octospinosus</i>	High	Monogyny	Low	Boomsma et al. 1999

species *A. versicolor* (Rissing et al., 1986; Rissing et al., 1989), and has been observed under laboratory conditions in the South American species *Amoimyrmex striatus* (Diehl-Fleig & de Araújo, 1996). On the other hand, queen adoption has been observed in the laboratory in a single species, the South American *A. subterraneus molestans* (Souza et al. 2005). Whether the observed polygyny in our sampled species is primary or secondary is unknown. Circumstantial evidence in *A. crassispinus* would indicate that the observed polygyny may be secondary (Diehl-Fleig & de Araújo, 1996).

Independent evolution of polygyny and polyandry

When comparing *Acromyrmex* species to other fungus-growing ant species, and to other species across the ant tree of life, the co-occurrence of polygyny and polyandry in the same species is relatively rare, or at least a rarely documented phenomenon. If polygyny and polyandry evolve chiefly to increase the genetic variability within a colony, the prediction follows that polygyny and polyandry should be negatively correlated in eusocial insects; that is, polygynous species should be primarily monandrous, and monogynous lineages polyandrous (Keller and Reeve 1994). This association has been identified across ants (Schmid-Hempel and Crozier 1999; Hughes et al. 2008b). In fungus-growing ants, this pattern is weak, and for *Acromyrmex* leaf-cutting ants, we do not observe this negative association. Outside of *Acromyrmex* only few fungus-growing ant species in the genera *Mycocepurus* and *Sericomyrmex* are known to display facultative polygyny (Villesen et al. 2002; Rabeling et al. 2007a, b, 2014b). The evolutionary transition from monandry to multiple mating has been well documented as a single evolutionary transition at the origin of the so-called higher attines (Villesen et al. 1999, 2002; Nygaard et al. 2016), comprising the leaf-cutting ants, *Acromyrmex*, *Amoimyrmex*, and *Atta*, as well as the genera *Mycetomoellerius*, *Paratrachymyrmex*, *Sericomyrmex*, *Trachymyrmex*, and *Xerolitor* (Sosa-Calvo et al. 2018; Solomon et al. 2019). Thus, it appears that facultative polygyny evolves independently of polyandry in fungus-growing ants. Notably, functional secondary polygyny is absent in the closely related leaf-cutting ant genus *Atta*, despite the fact that *Atta* species are known to be polyandrous (Fjerdingstad et al. 1998; Fjerdingstad and Boomsma 2000; Helmkamp et al. 2008; Evison and Hughes 2011). When both traits co-occur in a clade, selection may act on both independently, modulating each trait individually to reach an optimum based on the individualized ecological and social niche occupied by a species (e.g., pathogen resistance, worker caste polymorphism, etc. See “Introduction”). Alternatively, these traits may not be under strong enough selection to overcome drift, and trait associations could be the result of random variation

in these traits in these species. However, based on the strong evolutionary implications of both polygyny and multiple mating, it seems unlikely that drift only would drive the evolution of these traits. Our results show remarkable consistency in average intra-colonial relatedness in Central and South American species of *Acromyrmex* (Table 2, Figs. 4 and 7). We found that relatedness varies significantly with effective mating frequency and social structure, both within and between species (Fig. 4), and we found significant variation in effective mating frequency and queen number both between and within species (Fig. 2). In combination, these results suggest that species may converge toward an optimum intra-colonial relatedness. This convergence appears to be mediated via complex and dynamic combinations of multiple mating and multi-queen breeding. Interestingly, the patterns of association appear not to be limited to a straightforward prediction of negative association between mating frequency and queen number, possibly implying that other unidentified ecological factors may play a role in the mediation of intra-colonial relatedness. An alternative explanation may be that relatedness is not under selection in these colonies, and the observed similarities are simply the result of chance convergence due to the trait associations in different species and other, unaccounted factors.

Outside of the fungus-growing ants, studies investigating the mating biology of *Formica*, *Myrmecia*, and *Myrmica* species, in which polyandry and polygyny co-occur, have produced different and often contradicting results (Pamilo 1993; Pedersen and Boomsma 1999a; Hannonen et al. 2004; Qian et al. 2011). These findings further support the hypothesis that, while polyandry and polygyny are negatively correlated in general, when both traits co-occur in groups of closely related species, they can evolve independently at shallow evolutionary timescales, dynamically filling species-specific ecological and social niches.

Ecological factors promoting polygyny and polyandry

Two major hypotheses have been proposed to explain the evolution of polyandry in eusocial insects. The sperm limitation hypothesis suggests that multiple males are needed to provide enough sperm to the queen of large colonies. The hypothesis has been widely tested in the literature, and has received support in honeybees (Kraus et al. 2004), as well as in the leaf-cutting ant *Atta colombica* (Fjerdingstad and Boomsma 1998) and army ants (Kronauer and Boomsma 2007). In this case, polyandry is expected to evolve more readily in association with the evolution of large colony sizes. In *Acromyrmex*, investigated species form large colonies (Burchill and Moreau 2016 and references therein). Interestingly, in the fungus-growing ants, the transition to large colony size is observed in the ancestor of the

leaf-cutting ants, after the inferred origin of polyandry in “higher” attines (Villesen et al. 2002; Burchill and Moreau 2016). Most notably, the hypothesis predicts that polyandrous species with larger colonies should exhibit significant reproductive skew among patriline following natural variation in sperm contribution from males, because the queen has to use the entirety of the sperm stored in her spermatheca (Kraus and Moritz 2010; Barth et al. 2014). This pattern was observed in the Panamanian species *A. echinator* (Stürup et al. 2014), and was inferred for South American *Acromyrmex* species in this study. Paternal skew was observed for all multiply mated queens, thus providing circumstantial support for this prediction of the sperm limitation hypothesis.

The workers of leaf-cutting ants are highly polymorphic with large workers accomplishing foraging and exploration tasks outside the nest, while small workers remain inside the nest tending to the brood and the fungus garden. In *A. echinator*, worker morph determination is complex and has been inferred to be in part genetically determined (Hughes et al. 2003; Hughes and Boomsma 2007). In this system, high mating frequency by the queen was interpreted to be favorable because it increases the genetic diversity of workers and allows for a better optimization of colony efficiency through the ratio of large vs. small workers (Hughes and Boomsma 2007). More generally in leaf-cutting ants, worker task specialization is associated with variability in size, contrasting with the rest of the fungus-growing ants, in which task specialization typically depends on age-dependent factors (Murakami et al. 2000). This could represent a shift in the determination of division of labor in colonies, in which genetic factors affecting worker size determination are linked with factors determining worker task specialization. Our data does not allow us to directly address any potential genetic factors in the size determination of workers in *Acromyrmex* colonies. Future studies may benefit from investigating any paternal differences in the size distribution of workers.

To conclude, our results are consistent with the hypothesis that relatedness may be the ultimate subject of selection, resulting from the trade-off between kin selection and the ecological benefits of genetic diversity outlined above. Multiple mating and polygyny appear to evolve along independent trajectories, and their respective effect on relatedness is dynamic and inconsistent between strictly monogynous and socially polymorphic species, as well as between socially polymorphic species themselves.

Evolutionary consequences of mating biology and social structure

The dynamic evolution of relatedness, mating behavior, and social structure is expected to promote the evolution of

alternative life history strategies, such as the emergence of reproductive cheating and social parasitism. A reduction in relatedness has been documented to promote the emergence of cheating lineages in many social taxa (Kuzdzal-Fick et al. 2011). Furthermore, social structure in ants plays an important role in the emergence and maintenance of inquiline social parasitism, in which a parasite queen takes advantage of the host’s workers to reproduce. Polygyny itself is thought to be a pre-condition for the evolution of social parasitism in many species (Bourke and Franks 1991; Nonacs and Tobin 1992; Dahan and Rabeling, in review). Once evolved, social parasitism can become an important source of evolutionary pressure on host species, as co-evolutionary dynamics would be expected between host and parasites. Factors promoting nestmate recognition and policing in hosts would thus experience rapid evolution, influencing relatedness, mating behavior, and social structure (Brandt et al. 2005).

Our study shows that the known host species of two inquiline social parasites, *A. charruanus* and *P. argentina*, are facultatively polygynous, which is consistent with the hypothesis that polygyny is important for the origin and maintenance of inquilinism in ants (Fig. 7) (Bourke and Franks 1991; Dahan and Rabeling, in review). Interestingly, *A. lundii* was inferred to be monogynous and future studies will be needed to resolve the evolutionary history of social parasitism relative to the host’s reversal from poly- to monogyny. In general, our results add to the growing body of evidence that the complex social structures of *Acromyrmex* leaf-cutting ants provide a fertile breeding ground for the parallel evolution of social parasitism.

Conclusions

In summary, we found a complex and dynamic association between colony structure and mating biology in leaf-cutting ants of the genus *Acromyrmex*. Our findings suggest that *Acromyrmex* leaf-cutting ants are socially polymorphic, and the association between polygyny and polyandry seems to be governed by species-specific fitness optima that may be related to ecological conditions.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00265-021-03113-1>.

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Author contribution CR designed the study; CR and MB planned and conducted field work; CR developed molecular markers; RAD, NKG, and BG conducted laboratory work; RAD and NKG analyzed molecular data; RAD conducted statistical analyses; RAD and CR wrote the manuscript. All authors commented on and approved the manuscript.

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Data availability All genotype data and sampling information are deposited on Dryad data repository and are available at https://datadryad.org/stash/share/FN8RkYyO6MPwNzYOCwUtkRIVTPHDKOQhTU3Ueepy_50

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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