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RESEARCH ARTICLE

Polygyny in the nest-site limited acacia-ant *Crematogaster mimosae*

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Abstract Polygyny is common in social insects despite inevitable decreases in nestmate relatedness and reductions to the inclusive fitness returns for cooperating non-reproductive individuals. We studied the prevalence and mode of polygyny in the African acacia-ant *Crematogaster mimosae*. These ants compete intensively with neighboring colonies of conspecifics and with three sympatric ant species for resources associated with the whistling-thorn acacias in which they all obligately nest. We used the genotypes of alate males at ten microsatellite loci to reconstruct queen genotypes and found that *C. mimosae* colonies are frequently secondarily polygynous, in that they include multiple closely related (and sometimes full-sib) queens, and (more rarely) unrelated queens. We also found that individual queens in both monogynous and polygynous colonies had mated with multiple males, making *C. mimosae* an interesting example of simultaneous polygyny and polyandry. The presence of polygyny in *C. mimosae* and the intense competition for nest-sites between *C. mimosae* and its conspecifics support the association between nest-site limitation and polygyny. Polygyny may allow for increased worker populations and a competitive advantage, as inter-colony conflicts are typically won by the colony with the larger number of workers.

Keywords *Crematogaster mimosae* · Acacia-ants · Microsatellite · Polygyny · Kenya · *Acacia drepanolobium*

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Introduction

Despite the expectation that kin selection will favor single-queen systems within eusocial insect colonies (Hamilton, 1964), many ant species are known to be polygynous (Crozier and Pamilo, 1996). The presence of multiple reproductive queens often decreases the relatedness of colony members and may reduce the inclusive fitness returns for non-reproductive individuals by encouraging their cooperation with less related nestmates (Hamilton, 1964; Nonacs, 1988; Ross, 1988; Keller, 1995). Factors that have been shown to promote polygyny despite these costs include intraspecific brood raiding during colony founding (Rissing and Pollock, 1987; Herbers, 1993; Sommer and Hölldobler, 1995; Bernasconi and Strassmann, 1999), nest-site limitation due to habitat saturation (Herbers, 1986; Seppä et al., 1995; Puntilla, 1996; Pedersen and Boomsma, 1999a; Feldhaar et al., 2005), frequent habitat disturbance (Hölldobler and Wilson, 1977), low queen lifespan compared to colony survivorship (Nonacs, 1988), and the

advantages of increased genetic diversity within colonies (Hughes and Boomsma, 2004, 2006; Wiernasz et al., 2004, 2008; Hughes et al., 2008), although this can also be accomplished through multiple matings. Primary polygyny, the founding of colonies by multiple, usually unrelated queens, can increase initial worker production, potentially strengthening colony defenses soon after colony founding (Rissing and Pollock, 1987; Sommer and Hölldobler, 1995; Bernasconi and Strassmann, 1999). Nonetheless, primary polygyny rarely persists through colony development, as initially polygynous foundress queens usually become aggressive as colonies mature (Rissing and Pollock, 1987; Sommer and Hölldobler, 1995; Bernasconi and Strassmann, 1999). Secondary polygyny, via the adoption of newly mated queens into an already established colony, is more common and often involves recruiting queens into their natal colonies, leading to comparatively high relatedness among nest-mate workers (Crozier and Pamilo, 1996, Table 4.7; Kautz et al., 2009).

The East African acacia-ant, *Crematogaster mimosae*, nests obligately in whistling-thorn *Acacia drepanolobium* trees, where it faces intense intra- and inter-specific competition for nesting space with three other acacia-ants—*C. nigriceps, C. sjostedti,* and *Tetraponera penzigi*—that are also dependent on *A. drepanolobium* (Young et al., 1997; Palmer et al., 2000; Stanton et al., 2002; Palmer, 2004). Less than one percent of *A. drepanolobium* trees are typically unoccupied by ants, leading to intense competition among nuptial queens for the few available trees (Palmer et al., 2000; Stanton et al., 2002). Substantial competition continues throughout the life of *C. mimosae* colonies which actively expand to additional adjacent trees through aggressive interactions with neighbors of all four ant species (Palmer et al., 2000; Palmer, 2004).

Ant species diversity in this system is maintained by competition-colonization trade-offs between species (Stanton et al., 2002). Subordinate species are far more successful at colonizing unoccupied plants but are displaced by dominants in established, and more crowded, territory (Stanton et al., 2002). Intense nest-site limitation such as this is positively associated with polygyny in a number of other ant species (Herbers, 1986; Seppä et al., 1995; Sommer and Hölldobler, 1995; Puntilla, 1996), including another *Crematogaster* plant-ant (Feldhaar et al., 2005) and the neotropical acacia-ant *Pseudomyrmex peperi* (Kautz et al., 2009). The competitively subordinate but superior colonizers in the Kenyan acacia-ant community, *C. nigriceps* and *T. penzigi*, are monogynous, while the most dominant species, *C. sjostedti*, is polygynous, suggesting a similar pattern of nest structure succession in this community from monogyny in less nest-site limited areas to polygyny in areas with intense competition (Stanton et al., 2002). *C. mimosae* falls between the most subordinate and most dominant species in the dominance hierarchy (Stanton et al., 2002).

In this study, we use patterns of microsatellite variation to infer the degree and mode of polygyny in *C. mimosae* colonies and to test for multiple mating by individual queens. We also explore the association between the number of queens in colonies and nest-mate worker relatedness. These results enhance our understanding of the otherwise well-studied population and community ecology of this multifaceted ant-acacia interaction.

Materials and methods

Sample collection

Ant samples were collected at the Mpala Research Centre, in the Laikipia District of central Kenya, from 25 June to 10 July 2008 and 1–20 January 2009. Ants were collected from *Acacia drepanolobium* trees, which dominate the overstory on sites characterized by heavy clay ("black cotton") soil in this area (Palmer, 2004). Samples were collected from the same general study area where many previous investigations of this acacia-ant community have been conducted (Stanton et al., 1999, 2002; Palmer et al., 2000, 2002, 2008; Palmer, 2003, 2004).

We used the general method outlined in Palmer (2004) to determine colony boundaries. Colonies may occupy a single tree or span up to ten adjacent trees. For each colony, we chose a focal tree occupied by *C. mimosae* and collected workers and males from that tree for genetic analysis. We then transferred workers in clipped swollen thorns from neighboring trees to the focal tree and assessed whether these workers fought with the focal tree workers, indicating the ants were from different colonies. All transfers were done to the focal tree and no reciprocal transfers were performed to prevent contaminating the non-focal trees with foreign ants before genetic samples were obtained.

We used the number of trees occupied by a colony as a measure of colony size. Palmer (2004) used total tree height occupied as a measure of the number of workers in a colony of *C. mimosae*. The stands of trees examined were relatively uniform in height, making the number of trees occupied an appropriate approximation of colony size.

Once colony limits were determined, we clipped and opened swollen thorns to collect workers and male alates from each colony. At least 20 workers were collected from each tree, and at least 20 males were collected from all trees where they were present. All ants were initially placed directly into 95 % ethanol, and later either transferred to 70 % ethanol, or into 1.5 mL microcentrifuge tubes containing dried silica gel and dried for 2–3 weeks before storage at −20 °C.

Genotyping

We extracted whole genomic DNA from individual workers and males using the tissue extraction protocol of the Qiagen DNeasy Blood and Tissue Kit (Qiagen), with an elution volume of 100 µl. Wings were removed from males and gasters removed from workers before extraction to reduce the concentration of potential PCR inhibitors (Feldhaar et al., 2003). DNA was stored at −80 °C.

Individual genotypes were determined at ten microsatellite loci developed for this species (RRB106, RRB470, RRB476, RRB518, RRB547, RRB610, RRB703, RRB732, RRB751, RRB767, Rubin et al., 2009). All reverse primers included a six-base 'pigtail' (5′GTTTCT) at the 5′ end to ensure complete adenylation of products and to help standardize allele sizes (Brownstein et al., 1996). Forward primers were modified at the 5′ end with one of four fluorescent labels (PET, 6-FAM, VIC or NED; Applied Biosystems). Two multiplexed polymerase chain reactions (PCR) were performed for each individual, one with RRB476, RRB518, RRB547, RRB751, and RRB767 primer pairs, and the other with RRB106, RRB470, RRB610, RRB703, and RRB732. Each 10 µl reaction consisted of 10–100 ng DNA, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 3.25 mM $MgCl_2$, 0.25 U *Taq* polymerase (Sigma), and primer concentrations between 80 and 240 pM to obtain equal fluorescent signals. PCR was carried out using a Dyad thermal cycler (Bio-Rad Laboratories) with a cycling profile of 2 min at 95 °C followed by 35 cycles of 50 s at 95 °C, 1 min at 62 or 60 °C (for the first and second multiplex mix, respectively), 1 min at 72 °C, and a final extension cycle of 30 min at 72 °C. PCR products were resolved on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems), and allele sizes estimated with the GeneMapper version 3.7 software (Applied Biosystems).

Inference of queen genotypes

We used male ant genotypes as a proxy for queen genotypes because dissection of entire *C. mimosae* colonies to find and collect queens is destructive and impractical. Our goal was to determine queen genotypes from relatively small samples of haploid offspring, a problem for which many pedigree analysis programs are not suited. We, therefore, implemented a C++ program exclusively for this purpose. In general, our method of determining queen genotypes assumes that (1) we were able to collect at least some male progeny from all reproducing queens within colonies, and (2) non-queen *C. mimosae* workers produce no or very few male alates. To infer the genotypes of queens, we first identified groups of the sampled males that could have been produced by a single diploid queen. Male ants develop asexually from haploid eggs laid by queens. Therefore, groups of haploid males produced by the same diploid queen can have no more than two alleles at each locus. We termed these groups of males *feasible groups*. The alleles found in each feasible group give the genotype of a queen that could have potentially produced these males. If each sampled male is in at least one feasible group, then we have potential genotypes of queens that produced these males. We assumed the existence of the fewest possible queens that could have produced the observed male genotypes. Thus, we needed to find the minimum number of feasible groups where each male appears at least once. To ease computation, we defined a feasible group to be *maximal* if we could not create a new feasible group by adding another male. We minimized the number of groups necessary, and inferred queen genotypes from the resulting groups. This minimization problem is a special case of the classical computer science problem, Set Cover, which is traditionally difficult to solve. In this case, computation time is an increasing function of the number of males sampled and the total number of maximal feasible groups. As our sample sizes of males were relatively small within a given colony, the problem can be solved quickly using an integer programming formulation. A short paper describing the algorithm and the accompanying source code can be found at http://rmozone.com/ross/.

We genotyped between 11 and 36 workers per colony based on the number of trees in the colony and between 13 and 60 males per colony based on the number of trees in the colony and the number of males collected. For several colonies, the initial samples of males did not give robust inferences of queens, i.e. there were a large number of mismatches with worker genotypes. Therefore, we added more males to the analysis for these colonies. This method of choosing the number of males per colony may have led to sampling bias as we generally sampled more males from larger colonies and the larger the number of males analyzed, the greater the likelihood that additional queens would be found. To guard against this potential bias, we ran our program on subsets of males from each colony. For every colony, we randomly selected every number of males between two and the final number of males genotyped and ran the analysis on these subsets. Thirty random subsets of every number of males were analyzed to produce means and confidence intervals for each size subset. Through this analysis, we saw the progression of inferred queen number as a function of random groups of males of every possible size and were able to determine when adding additional males ceased to increase the number of inferred queens. Occasional production of males by workers is known from many ants (Bourke, 1988) and could lead to overestimation of the number of queens in a colony using our method. Heinze et al. (2000) found some evidence for worker reproduction of males in the related *Crematogaster smithi,*

but the frequency of this type of reproduction does not appear to be common in *Crematogaster*. Low rates of worker reproduction are unlikely to seriously influence our results, but in light of our assumptions we checked for genotype mismatches between inferred queen genotypes and worker genotypes. We considered mismatches to be any situation in which a worker did not share at least one allele at every locus with an inferred queen. These mismatches gave us a measure of the precision and robustness of our inferences of queen genotypes.

Mating frequency was determined as the minimum number of mates required for the observed patterns in worker genotypes given inferred queen genotypes. We were only able to confidently make this inference for the four colonies with the lowest number of reproductive queens, as the minimum number of mates for each queen was not clear in the highly polygynous colonies.

Our method for determining queen genotypes determines the minimum number of queens required, assuming that the male genotype data are perfect. However, we also used COLONY v1.2 (Jones and Wang, 2010) to get additional estimates of queen mating frequency and incorporate the presence of genotyping errors in our analyses. COLONY uses a likelihood approach and genotype data from all worker and male members of a colony to infer the most likely genotypes of queens and their mates, rather than the minimum number, as is determined by our method. We analyzed all data from each colony individually. Genotyping error rates are unknown for these loci so we allowed a 5 % frequency of error.

Statistical analyses

Population allele frequencies were calculated using a subsample of 1–3 worker ants from each of 25 different colonies around the study area using the program RELATEDNESS 5.0.8 (Queller and Goodnight, 1989). These 25 colonies include the eight studied in depth here as well as 17 others. Using RELATEDNESS, we corrected for bias across colonies to account for the relatedness of nestmates. These additional colonies made our estimates of the true population allele frequencies more accurate. Mean colony relatedness coefficients were also calculated in RELATEDNESS 5.0.8. Standard errors and 95 % confidence intervals were calculated by jackknifing over individuals for worker relatedness values. Two colonies had only two inferred queens making jackknifing over individuals impossible. Therefore, confidence intervals for mean queen relatedness were calculated by jackknifing over loci. Pairwise relatedness coefficients between all inferred queens, workers, and males and probabilities of sibling relationships between all queens and workers within colonies were calculated with KINGROUP (Konovalov et al., 2004). Relatedness coefficients calculated in KINGROUP can vary from −1 to 1 with positive values suggesting kin relationships and negative values suggesting non-kin. Pedigree analysis in KINGROUP uses the population allele frequencies and the genotypes of the individuals being analyzed to determine the likelihood that the individuals could be related. This likelihood is specified by the probabilities that individuals share alleles through their mothers (r_m) and fathers (r_p). In the haplodiploid situation, r_m is 0.5 and r_p is 1 for full sibling workers or queens, r_m is 0.5 and r_p is 0 for maternal half sibling workers or queens, and r_m is 1 and r_p is 0 for parent offspring queens and workers. We used KINGROUP to test for each of these relationships versus all other possibilities among workers and inferred queens. Expected and observed heterozygosities at the microsatellite loci were calculated with GenePop (Raymond and Rousset, 2004).

We used a permutation test (Manly, 1991) to determine whether queens within colonies were more closely related than would be expected by a random distribution of queens across colonies. We inferred the genotypes of 26 queens and calculated the relatedness for every pair of these queens, giving 55 pairwise within-colony relatedness values and 270 pairwise between-colony relatedness values. To generate a null distribution of the expected within-colony queen relatedness, we took the means of 55 random pairwise queen relatedness values from the 325 total comparisons, with 1,000 iterations. The mean relatedness value for the observed 55 within-colony comparisons was compared to the resulting null distribution.

Effective queen number

The genetically effective number of queens contributing to haploid offspring in each colony was estimated using the method of Kümmerli and Keller (2007) given by: $N_{e,hc} = (2r_{hs} - r_{fb})/(2r_{hc} - r_{fb})$ where r_{hs} is the relatedness among haploid siblings (0.5), r_{fb} is the relatedness among female breeders, and r_{hc} is the relatedness among haploid offspring. The genetically effective number of breeders contributing to diploid offspring was calculated as: $N_{e,dc} = 3/(4r_w)$ where r_w is the relatedness among diploid worker offspring.

Results

Microsatellite loci

We genotyped 185 workers and 297 males from 8 colonies at all ten microsatellite loci. The number of alleles per locus varied from 4 to 17 (mean = 7.8). Expected heterozygosity ranged from 0.442 to 0.896 (mean = 0.659) and observed heterozygosities from 0.451 to 0.967 (mean = 0.661).

Queen number and relatedness

In our sample of eight colonies, three colonies had single queens and five had multiple queens (2, 2, 5, 6, and 8 queens, respectively). Across all colonies, we inferred 26 queen genotypes. Pairwise relatedness coefficients of queens within colonies ranged from −0.15 to 0.84, with a mean value across all pairwise within-colony queen comparisons of 0.37 ± 0.01 (mean ± SE). Pairwise between-colony queen relatedness coefficients ranged from −0.42 to 0.46, with a single outlier at 0.65 (this outlier was the result of a comparison between queens from adjacent colonies) and a mean of −0.01 ± 0.04. The distribution of the within-colony comparisons is bimodal, with most relatedness values centered around either 0.6 or 0.1 (Fig. 1), whereas the distribution of pairwise between-colony queen relatedness coefficients was centered around zero (Fig. 1). The mean within-colony relatedness deviated significantly from the null expectation of no association between queen relatedness and colony membership (Permutation test, $z = 11.1$, $P < 0.001$).

The higher relatedness seen in the within-colony comparisons is likely a result of the presence of sister queens. Pedigree analyses using KINGROUP (Konovalov et al., 2004) suggest that three of the five polygynous colonies had at least one pair of full-sister queens, and four had at least one pair of half-sister queens ($P < 0.01$ for full-sib comparisons; $P < 0.05$ for half-sibs; Table 1). One colony included two queens that were putatively a parent and offspring pair ($P < 0.05$), though this was non-significant after Bonferroni correction.

Colonies 2 and 6 each had single queens with mean relatedness values to other queens in their colonies of 0.026 and −0.021, respectively, while across all queens in these colonies mean relatedness was 0.41 and 0.40, respectively. These two queens share no alleles with any nestmate queens

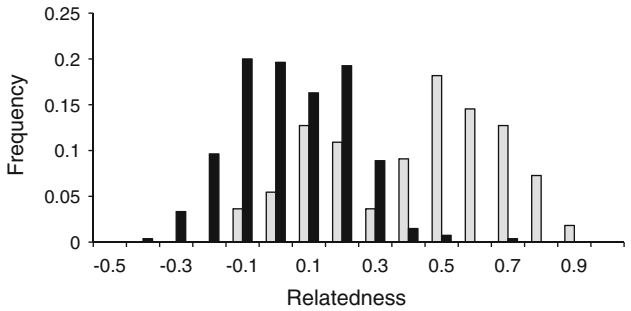

Fig. 1 Frequencies of all observed pairwise relatedness coefficients between queens from different colonies (*dark bars*) and queens from the same colony (*light bars*). Note the bimodal distribution of within-colony queen relatedness. Most queens from the same colony were closely related, explaining the higher relatedness peak, but two queens were unrelated to their nestmates, leading to the lower peak in the distribution

Table 1 Characteristics of the eight colonies examined

Colony	#Inferred queens	Mean queen *r*	Mean worker *r*	Mean male *r*	Sib workers	Sib queens	#Inferred mates	#COLONY inferred mates	#Males genotyped	#Workers genotyped	#Trees	Range of males/queen	$N_{e,dc}$	$N_{e,hc}$	#Mismatches
1	1	–	0.63 ± 0.12	0.53 ± 0.07	48/55	–	2	1	13	11	4	–	1.19	–	2
2	5	0.41 ± 0.21	0.26 ± 0.05	0.27 ± 0.05	47/190	6/10	–	1–2	51	20	5	9–15	2.88	4.81	0
3	1	–	0.31 ± 0.08	0.50 ± 0.05	199/435	–	3	1–3	21	30	3	–	2.42	–	6
4	1	–	0.74 ± 0.04	0.48 ± 0.01	120/120	–	1	1	28	16	4	–	1.01	–	0
5	2	0.45 ± 0.29	0.53 ± 0.12	0.43 ± 0.08	88/153	1/1	4[a]	1–3	48	18	3	15–32	1.41	1.36	1
6	8	0.40 ± 0.25	0.17 ± 0.07	0.21 ± 0.05	119/630	21/28	–	1–4	60	36	8	5–21	4.41	29.12	1
7	6	0.29 ± 0.36	0.18 ± 0.10	0.32 ± 0.07	38/276	5/15	–	1–5	54	24	3	3–24	4.16	2.01	7
8	2	0.49 ± 0.46	0.56 ± 0.18	0.43 ± 0.05	342/435	0/1	–	1–3	22	30	3	11–11	1.34	1.41	1

r indicates the relatedness coefficient ±95 % confidence intervals. Sib Workers and Sib Queens indicate the proportion of all within-colony comparisons that showed significant full or half sibling relationships ($P < 0.05$). #Inferred mates are the minimum number of mates for each queen based on the queen genotypes inferred by our method. #COLONY inferred mates are the most likely number of mates for each queen as determined by COLONY. Males/queen is the number of genotyped males that were used to infer a queen's genotype. $N_{e,hc}$ is the genetically effective number of queens in the colony contributing to haploid offspring (i.e., males). $N_{e,dc}$ is the genetically effective number of queens in the colony contributing to diploid offspring

[a] The inferred #mates for colony 5 is the total number of mates among both queens in the colony

at two or more microsatellite loci and are, therefore, not close kin with their nestmate queens. The unrelated queen from colony 2 was related to the single adjacent colony 3 queen by 0.65 and was likely her full sister.

The results of the random subset tests show that we generally used an appropriate number of male ants to infer queen number (Fig. 2). The number of queens inferred asymptotes at the final inferred queen number as males are added to the analysis for all colonies except one. Even with 60 males included in the analysis, the inferred number of queens in colony 6 never asymptotes and our inference for this colony is likely an underestimate. These results indicate that adding more males to the analyses of any colonies other than colony 6 would have been unlikely to increase the number of queens estimated.

COLONY (Jones and Wang, 2010) yielded similar numbers of queens for all colonies but inferred three queens as opposed to two in colonies 5 and 12 and two queens instead of one in colony 3. Of the 29 queens inferred to exist by COLONY, nine genotypes were reconstructed exactly as with our method. All other reconstructed queens differed in at least one allele at one locus.

The genetically effective number of queens contributing to worker and male offspring were sometimes very different from each other (Table 1). Both of these estimates generally underestimated the minimum number of queens required to produce the genotyped males compared to the other two methods based directly on offspring genotypes.

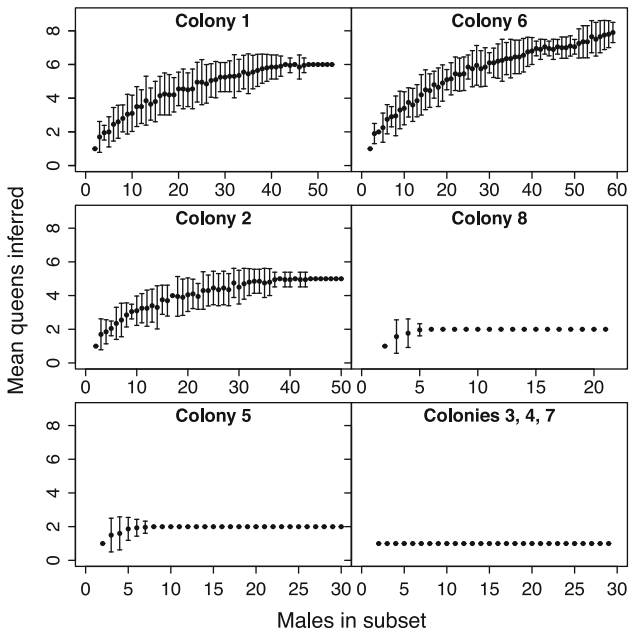

Fig. 2 Means and 95 % confidence intervals of the inferred number of queens in a colony based on subsampling of male genotypes

Worker relatedness and mismatches

Mean within-colony worker relatedness coefficients across all pairwise comparisons ranged from 0.18 to 0.74 (Table 1). KINGROUP pedigree analysis of these workers yielded proportions of worker pairs within colonies that were significantly likely to be full siblings from 38/276 (13.8 %) to 120/120 (100 %) ($r_m = 0.5$, $r_p = 1$, $P < 0.05$; Table 1).

Mismatches between workers and their inferred mothers occurred for one worker in three colonies and 2, 6, and 7 workers in three other colonies (Table 1). Mismatches between potential mothers and daughters always occurred at only a single locus, but loci differed for different pairs of mismatched mothers and daughters. The two cases with more than two worker mismatches could be explained by incorrect inference of a homozygous queen locus (colony 3) or one missing queen from inferred queens (colony 7).

Mating frequency

Mating frequencies could only be determined with confidence using the method developed here in single-queen colonies (colonies 1, 3, and 4) and the two-queen colony (5). Inferred numbers of mates for queens in single-queen colonies were 2, 3, and 1 male, respectively. The two queens in colony 5 had a total of 4 mates between them (Table 1).

COLONY was unable to reconstruct meaningful mating frequencies for ten queens, because one or no diploid offspring were genotyped for these queens. Mating frequencies for more queens were inferred by COLONY than by our method though those mating frequencies that were inferred by both methods were similar (Table 1). The number of mates inferred for a single queen by COLONY ranged from one to five.

Queen number, worker relatedness, and colony size

A positive, though non-significant, trend existed between queen number and the number of trees occupied by a colony (two-tailed Spearman correlation; $P = 0.486$, $\rho = 0.290$; Fig. 3). As predicted, queen number was negatively correlated with mean within-colony worker relatedness (two-tailed Spearman correlation; $P = 0.008$, $\rho = -0.847$; Fig. 3). The number of trees occupied by a colony is an index of colony size; hence worker relatedness decreases with colony size.

Within-colony genetic diversity was used to estimate both colony relatedness and queen number, making these two measures inherently related. However, queen number was estimated directly from male genetic diversity, whereas colony relatedness was measured using exclusively worker

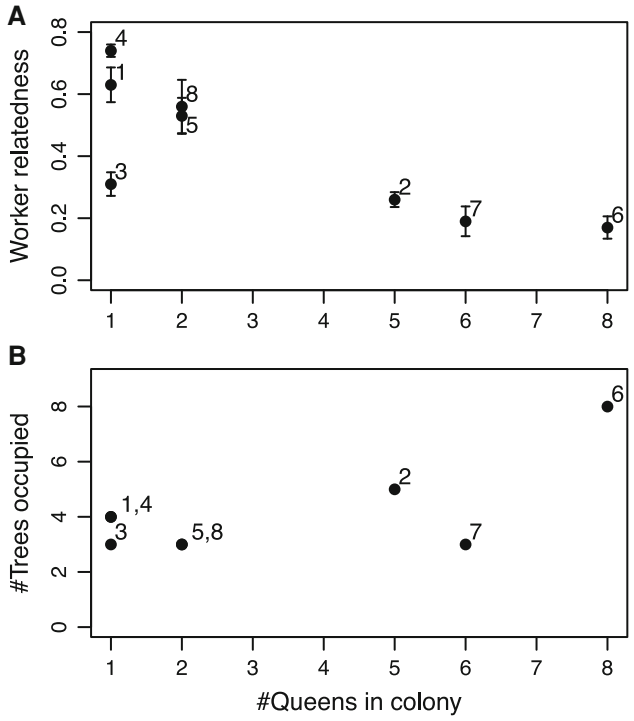

Fig. 3 Relationship of the number of laying queens in a colony with **a** mean (±SE) within-colony worker relatedness and **b** the number of trees occupied by a colony. Colonies are labeled as in Table 1

genotypes. This distinction in measurements based on castes gives us confidence in our estimates.

Discussion

Using microsatellite data, we show the presence of both polygyny and polyandry in the acacia-ant, *Crematogaster mimosae*. Polygyny involving closely related queens appears to be the most common situation in this species, but we also find examples of colonies containing unrelated queens and single-queen societies. As expected, nestmate relatedness decreases as the number of queens present increases, and colonies with greater numbers of queens are generally larger. Nest-site limitation is characteristic for *C. mimosae* and competing acacia-ants and has been associated with polygyny in a number of other ants, although this hypothesis has not been tested here. An increased ability to produce large worker populations for colony defense may also be partly responsible for the evolution of polygyny in *C. mimosae*.

Estimating queen number

The program we describe determines the absolute minimum number of queens required to produce the given male genotypes, whereas COLONY incorporates probabilistic modeling to determine the likely queen genotypes (Jones and Wang, 2010). In this case, both methods performed similarly. Although situations in which many more haploid male genotypes are available than diploid worker genotypes may be unusual, our method performs at least as well as COLONY. Perhaps the most useful aspect of the method described here is its immunity to queen mating frequencies. When mating frequency is unknown, high levels of polyandry could mislead likelihood methods into reconstructing more queens than are actually present.

Our method of reconstructing queen genotypes from the genotypes of haploid males requires a low error rate in allelic scoring, particularly for males. These genotypes were only scored if allelic peaks were clear, and worker genotypes that resulted in mismatches were subject to the same scrutiny. The rarity of genotype mismatches supports our application of this method for inferring queen genotypes. The mismatches that did occur illustrate the potential shortcomings of this approach. Incorrect inference of queen homozygosity could occur if all males collected from a single queen had only one of her alleles. Missing queens could result if we did not collect males from all laying queens or two queens had such similar genotypes that we could not distinguish between them. It is also possible that the queens that produced the few mismatched workers had died, and all of her alate male descendents had already dispersed. In general, however, the overall rarity of mismatches between worker genotypes and the corresponding queen genotypes inferred from within-colony males suggests that these queen genotype inferences are robust.

Polygyny and relatedness

Reproductive queens in *C. mimosae* colonies are much more closely related than would be expected if these associations were a result of primary polygyny involving queens from unrelated natal colonies. Some of these within-colony queens were full sisters. This pattern likely results from adoption of natal queens, as found in a number of other ant species (e.g. Bourke and Franks, 1995; Keller, 1995; Chapuisat et al., 1997; Sundström, 1997; Dalecky et al, 2005). The recruitment of natal queens may serve to increase rates of worker production in *C. mimosae* colonies while maintaining a relatively high degree of within-colony relatedness. Stanton et al. (2002) found that *C. mimosae* foundresses are more common in areas with many *C. mimosae* colonies, whereas the likely monogynous sympatric species *C. nigriceps* and *T. penzigi* dispersed much more readily. The active return and recruitment of *C. mimosae* queens to their natal colonies could partially explain their low rate of dispersal to unoccupied trees.

Despite potentially severe decreases on within-colony relatedness, polygyny with multiple unrelated queens has

been found in some other species of ants (Heinze et al., 2001; Fournier et al., 2003; Kellner et al., 2007). Whereas most polygynous colonies in *C. mimosae* are likely formed by related queens, we found one queen in each of two colonies that was not related to the remaining queens in the colony. These unrelated queens were responsible for the lower mean within-colony relatedness and the bimodal relatedness distributions for these two colonies (Fig. 1).

Little is known about the nuptial flight of *C. mimosae* or how queens choose nesting sites after mating. However, the near-complete habitat saturation that renders unoccupied trees very rare means that most alate queens must settle on occupied trees. Virgin queens of the extremely polygynous Central American acacia-ant *Pseudomyrmex veneficus* rarely disperse themselves, instead perching on the branches of their natal trees and attracting extra-colonial males via pheromone release (Janzen, 1973). The similar ecology of *C. mimosae* suggests that these queens may be mating in an analogous fashion. However, some alates must successfully nest in trees already occupied by other colonies, resulting in polygynous partnerships between unrelated queens. Primary polygyny involving related or unrelated queens is also possible if foundresses initially nest on the same tree, and eventually join nests to become one colony, though aggressiveness between queens makes this unlikely (Stanton et al., 2002).

We found one case in which queens from different but adjacent colonies were very closely related, most likely as full sibs. This situation could result from mated queens attempting to return to their natal colony but settling on nearby trees instead. Alternatively, the close relatedness of queens in neighboring colonies may be evidence of colony budding, a relatively common phenomenon in ants (Beye et al., 1998; Pirk et al., 2001; Debout et al., 2007).

The differences in the genetically effective numbers of queens based on worker and male relatedness suggest that reproductive skew may occur in this species. The sometimes large differences in the numbers of males collected from each queen and the absence of worker offspring from particular queens both support this possibility (Table 1). However, our sampling was insufficient to draw definitive conclusions.

Multiple mates

We found clear evidence of polyandry in three of the five queens examined. Of the 29 queens reconstructed by COLONY, 10 were inferred to be polyandrous. Simultaneous polygyny and polyandry have been shown in some ant species (Pamilo, 1993; Pedersen and Boomsma, 1999b; Kellner et al., 2007; Trontti et al, 2007), but Keller and Reeve (1994) suggested that this condition is rare because potential benefits of genetic variability could be fully achieved by either polyandry or polygyny. Although Schmid-Hempel and Crozier (1999) showed that ants of this type are not unusual, Hughes et al. (2008) and Kronauer and Boomsma (2007) found strong negative relationships between polygyny and mating frequency in ants. Based on direct observation of colony formation, Stanton et al. (2002) suggested that *C. mimosae* colonies begin with a single queen, and hence queens may mate multiply to ensure within-colony genetic diversity in the initial stages of colony development. Even in polygynous colonies, queens are often highly related, providing little additional genetic diversity.

Colony size and worker relatedness

As expected for polygynous situations, as the number of queens in a colony increases, the relatedness of workers decreases (Fig. 3). This decreasing relatedness may set an upper bound on the number of queens that can be recruited into a colony before cooperative behavior is selected against. Such a limit could explain why *C. mimosae* colonies rarely span more than ten trees.

Adaptive implications of polygyny

Though non-significant, we found a positive trend between colony size and the number of queens in the colony (Fig. 3). Komene et al. (1999) and Dalecky et al. (2005) found that colony size correlated with the number of reproductives in colonies of *Rhytidoponera aurata* and *Petalomyrmex phylax*, suggesting that polygyny may allow for the maintenance of larger worker populations. In general, larger *C. mimosae* colonies have a substantial competitive advantage over smaller colonies as the outcome of conflicts is determined by the size of the respective worker populations of the colonies involved (Palmer et al., 2000; Palmer, 2004). This suggests that the occurrence of polygyny in *C. mimosae* contributes to the production of more workers and is an adaptive strategy that may increase the likelihood of colony survival and expansion, though we have not tested this directly.

C. mimosae colonies able to produce greater numbers of ants are at a substantial competitive advantage for both territory expansion and colony defense, particularly early in colony development when competing foundresses may attempt to usurp host plants. Primary polygyny is potentially advantageous for *C. mimosae* as it could help increase initial worker reproduction and colony defense in new colonies. While such pleometrotic associations have not been observed, the rarity of founding *C. mimosae* queens could be to blame (Stanton et al., 2002). Secondary polygyny, through recruitment of mated queens to their natal nests, would not provide advantages to founding colonies but

could increase total worker populations while maintaining relatively high nestmate relatedness.

While the possible adaptive significance of creating larger worker populations should not be discounted, several other explanations for polygyny in these ants are likely. Nest-site limitation has been repeatedly associated with polygyny in ants (Herbers, 1986; Seppä et al., 1995; Sommer and Hölldobler, 1995; Puntilla, 1996). Plant-ants, in particular, are often subject to severe nest-site limitation and, apparently, adapt to these conditions through polygyny (Feldhaar et al., 2005). Similarly, acacia-nesting *Pseudomyrmex* ants in the neotropics can be highly polygynous (Kautz et al., 2009) and nest-site competition is so intense that many colonies of the non-polygynous species *P. gracilis* will share hosts to prevent other species from invading (Kautz et al., 2012). And while mature colonies of *Cecropia* nesting *Azteca* ants do not have multiple queens, they do found colonies pleometrotically (Longino, 1991), possibly to boost initial worker production and defense. A similar relationship between nest-site limitation and polygyny appears to have evolved within the Kenyan *Crematogaster* and *Tetraponera* acacia-ant community. The monogynous *C. nigriceps* and *T. penzigi* are far more successful at founding colonies in unoccupied fringe habitats than the polygynous *C. mimosae* and *C. sjostedti,* providing a community level example of the trend towards polygyny in nest-limited areas (Palmer et al., 2000; Stanton et al., 2002).

In several other cases of plant-ant polygyny, the life histories of the host plants are associated with similar changes in queen number during colony maturation. Feldhaar et al. (2003) found that two species in a guild of *Crematogaster* plant-ants maintain associations with their *Macaranga* host plants beyond the lifespan of a single ant queen by adopting new queens as the colony ages. We show evidence for another case where ant-plant growth is mirrored by life history changes in the associated guild of plant-ants. Young *A. drepanolobium* are much more likely to be occupied by the monogynous *C. nigriceps* and *T. penzigi* but as trees age their residents switch to the polygynous *C. mimosae* and *C. sjostedti* (Palmer et al., 2000). Although there is ample evidence for aggressive interactions and a stable dominance hierarchy being responsible for this change, the monogynous colonies may have an insufficient lifespan to survive the duration of the plant's life. Long-term studies of isolated colonies would be necessary to examine this directly.

Janzen (1973) hypothesized that polygyny in *Pseudomyrmex* acacia-ants is due simply to the need for acacia-ant queens to be able to move out of old, soon to be dead, thorns into freshly grown spaces. The extreme physogastry characteristic of ant queens that produce very large numbers of offspring is, therefore, not an option for acacia-ants. Recruitment of additional more streamlined queens may be the most straightforward way of increasing worker populations in these ants while still maintaining the mobility required for queen survival, although we have not examined this hypothesis here.

Colony structure in *Crematogaster*

Our results suggest that polygyny in *C. mimosae* is adaptive for the nest-site limited environment in which it lives. However, we have not addressed the evolutionary history of this group of ants. If the ancestors to *C. mimosae* were also polygynous then the trait may be due to phylogenetic inertia rather than adaptation to the current environment. The genus *Crematogaster* is highly diverse, with over 450 described species as well as hundreds of subspecies (Blaimer, 2012a) making systematic studies difficult. Despite recent successes in *Crematogaster* phylogenetics (Blaimer, 2012a, b), the exact relationships between *C. mimosae* and the rest of the genus remain unknown.

However, queen numbers are known to vary substantially across *Crematogaster.* Some species are strictly monogynous (e.g. *C. brasiliensis,* AntWeb, 2013; *C. smithii,* Heinze 2000) but many are polygynyous or have variable colony structures (e.g. *C. carinata, C. crinosa, C. erecta, C. limata, C. longispina, C. sumichrasti,* AntWeb, 2013; *C.* (*Decacrema*) msp. 4, Feldhaar et al., 2000; *C.* (*Decacrema*) msp. 2, Feldhaar et al., 2005; *C. pygmaea,* Quinet et al., 2009), suggesting that the trait is quite plastic, at least within *Crematogaster*, and that the behavior of *C. mimosae*'s ancestors may have little bearing on its own behavior.

Regardless of any impact of evolutionary history, the *Crematogaster* acacia-ant interaction includes three species of related ants with different colony structures. Our findings here show that a continuum in queen number exists between species within this system from the monogynous *C. nigriceps* to the variable *C. mimosae* to the highly polygynous *C. sjostedti*. The dominance hierarchy and pattern of succession follow the same order. *T. penzigi* and *C. nigriceps* are the first to colonize new habitat, but *C. mimosae* and *C. sjostedti* dominate during nest conflict and takeover. The three-way interaction between queen number, colony size and dominance status among these ants makes them useful examples for studies of colony structure variation in nest-site limited areas. Further studies should test the hypothesis that polygyny is related to nest-site limitation by examining *C. mimosae* colonies in both densely populated and more recently colonized areas. A correlation between nest-site competition and frequency of polygyny would be highly suggestive that polygyny in this system occurs in response to lack of available nest-sites. Additional studies of these ants integrating phylogenetics and samples from a variety of

habitats will be invaluable for understanding the evolution of polygyny in ants as a whole.

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