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Breeding systems and genetic diversity in tropical carpenter ant colonies: different strategies for similar outcomes in Brazilian Cerrado savanna

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Eusocial insects tend to present low genetic diversity (GD) within colonies, which can increase with the co-occurrence of multiple queens (polygyny) or with multiple mating by a single queen (polyandry). Therefore, it is important to elucidate how these strategies influence GD, which in turn mediate population ecology and how organisms respond to their environment. We studied two carpenter ant species from the Brazilian savanna, *Camponotus renggeri* and *C. rufipes*. Using microsatellites, we evaluated the number of breeders, the genetic relatedness and the contribution of polygyny and polyandry to GD within colonies. Both species exhibited facultative polygyny. In *C. renggeri*, low related queens formed colonies jointly and present low mating frequency. In this species, colony GD increased with the number of queens. Contrastingly, closely related queens of *C. rufipes* formed polygynous colonies, exhibiting high mating frequency. In *C. rufipes*, both queens and males contributed to colony GD. Despite the differences, the two species have similar GD at the colony scale. Under low mating frequency, our data support that polygyny has evolutionary importance for increasing GD in ant colonies, a mechanism mainly conferred to polyandry. Although the impact of GD in variable ecological and adaptive contexts remains uncertain, this study highlights how distinct reproductive strategies may generate similar patterns of GD in ants.

ADDITIONAL KEYWORDS: breeding system strategy – *Camponotus* – colony genetic structure – polyandry, polygyny.

INTRODUCTION

Eusocial insects have long been a riddle for evolutionary biology, posing a 'special difficulty' for Darwin's theory of natural selection (Ratnieks *et al.*, 2011). Accordingly, their typical colonial life and particular parentage relationships are of persistent interest to researchers on eusocial insects, with special concern as to how such organisms are capable of maintaining their intraspecific genetic variation (Hughes *et al.*, 2008a; Seppä, 2008). Intraspecific genetic diversity at distinct biological levels is a key aspect of every living species, with direct implications for population productivity, longevity and ability to respond to ecosystem changes and natural selection (Hughes *et al.*, 2008b; Nair, 2014). Recent studies have aimed to uncover the determinants of intraspecific genetic diversity across different taxa; they assert that life-history traits, such as longevity, productivity and mating system,

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are the most important factors influencing genetic polymorphisms (Leffler *et al.*, 2012; Romiguier *et al.*, 2014; Ellegren & Galtier, 2016). Among these traits, the reproductive strategy seems to have the strongest effect by directly influencing the effective population size (N_e), which in a panmictic population can be interpreted as the number of individuals in the population contributing to offspring (Romiguier *et al.*, 2014). Consequently, an increment in N_e would also increase neutral genetic diversity (Romiguier *et al.*, 2014; Ellegren & Galtier, 2016).

In ants, the haplodiploid sex-determination system leads to a naturally lower effective population size compared with diploid organisms (N_e haplodiploid = ¾ N_e diploid; see Hedrick & Parker, 1997). In particular, the few breeders generally observed in ant colonies (only queens and male mates contributing to the numerous sterile offspring) also cause this limited molecular variation (Seppä, 2008). The breeding system is defined by the number of mothers (queens) and fathers (male mates) in the colony, the genetic relationship between same-sex and different-sex breeders, and the reproductive partitioning among breeders (reproductive skew) (Ross, 2001). Thus, reproductive strategy is also expected to influence genetic variation in ant colonies. To increase intracolonial genetic diversity, it is believed that eusocial hymenopterans can increase the co-occurrence of multiple queens (polygyny) and multiple mates by a single queen (polyandry), these two strategies not being mutually exclusive (Hughes *et al.*, 2008a; Nonacs, 2017). In ants, many queens living together may improve the naturally low N_e in populations (Wilson, 1971), which may be a response to ecological pressures such as resource scarcity of food or nesting sites (Briese, 1983; Hölldobler & Wilson, 1997; Hughes *et al.*, 2008a; Oliveira *et al.*, 2011; Rubin *et al.*, 2013; Avila & Fromhage, 2015). Moreover, polygyny may increase worker numbers and colony survival. Therefore, polygynous ants are expected to survive better in the face of risky or stressful environmental conditions or under demographic constraints (Hölldobler & Wilson, 1977). Similarly, the genetic diversity improvement provided by polyandry may also increase colony tolerance and adaptability to ecosystem changes (Crozier & Page, 1985). Mating with many males is also hypothesized to confer additional benefits to the colony, including (1) improvement of the division of labour (e.g. Evison & Hughes, 2011), (2) reduced chances of parasitic or pathogenic infections (Sherman *et al.*, 1988; Hughes & Boomsma, 2004), (3) reduced deleterious effects of genetically incompatible matings (Zeh & Zeh, 1997; Simmons, 2001) and the production of unusual diploid males (Crozier & Page, 1985) and (4) increased amount of colony sperm storage (Cole, 1983).

Although polygyny and polyandry have been recorded for different ant species, these two reproductive strategies are not consistent across all species. Indeed, polygyny and polyandry are negatively correlated (Hughes *et al.*, 2008a), and reproductive strategies across species may comprise a range in numbers of breeders. For instance, extreme polygyny is found in some *Formica* species (Pamilo *et al.*, 2016) and the army ant *Neivamyrmex carolinensis* (Emery, 1894) (Kronauer & Boomsma, 2007), while high mating frequencies (polyandry) are commonly reported for monogynous species such as other army ants (Kronauer *et al.*, 2006; Barth *et al.*, 2014) and leaf-cutter ants (Boomsma *et al.*, 1999; Evison & Hughes, 2011). Many ant species present mixed strategies along this continuum, with intermediate and variable numbers of queens and mates, such as in species of *Camponotus* (Akre *et al.*, 1994; Goodsman & Hahn, 2004, 2005; Mersch *et al.*, 2017), *Cataglyphis* (Cronin *et al.*, 2016a) and *Myrmecia* (Qian *et al.*, 2011).

Undoubtedly, the breeding system is a key aspect of ant life-history and genetic variation. Research aiming to contrast patterns between closely related species would then be highly informative regarding the factors that explain the genetic diversity in such groups (Leffler *et al.*, 2012). We here comparatively evaluate the impact of the breeding system (i.e. the contribution of polygyny and polyandry) on genetic diversity in the colony of eusocial hymenopterans. As biological systems, we studied two closely related Neotropical ant species, *Camponotus renggeri* (Emery, 1894) and *C. rufipes* (Fabricius, 1775) (Fig. 1A, B). Specifically, we characterize (1) the matrilines and patrilines, (2) the genetic relatedness within and between reproductive and worker castes and (3) the contribution of multiple matrilines and patrilines to the genetic diversity of *C. renggeri* and *C. rufipes* colonies. Both species are highly abundant in the Brazilian Cerrado savanna and frequently attend extrafloral nectaries and honeydew-producing hemipterans on foliage (Fig. 1A, B; Oliveira & Freitas, 2004), but present contrasting natural history traits (Table 1). *Camponotus rufipes* builds various types of nests with high spatial persistence and has an aggregated nest distribution that suggests a polydomous habit (i.e. a single colony physically divided in more than one nest). Contrastingly, nests of *C. renggeri* are underground or in dead trunks and randomly distributed, and colonies show frequent nest relocation behaviour (Ronque *et al.*, 2016, 2018). Both species may present colonies with one or more than one wingless queen, suggesting the occurrence of monogyny and polygyny in *C. renggeri* and *C. rufipes* colonies, a condition known as facultative polygyny

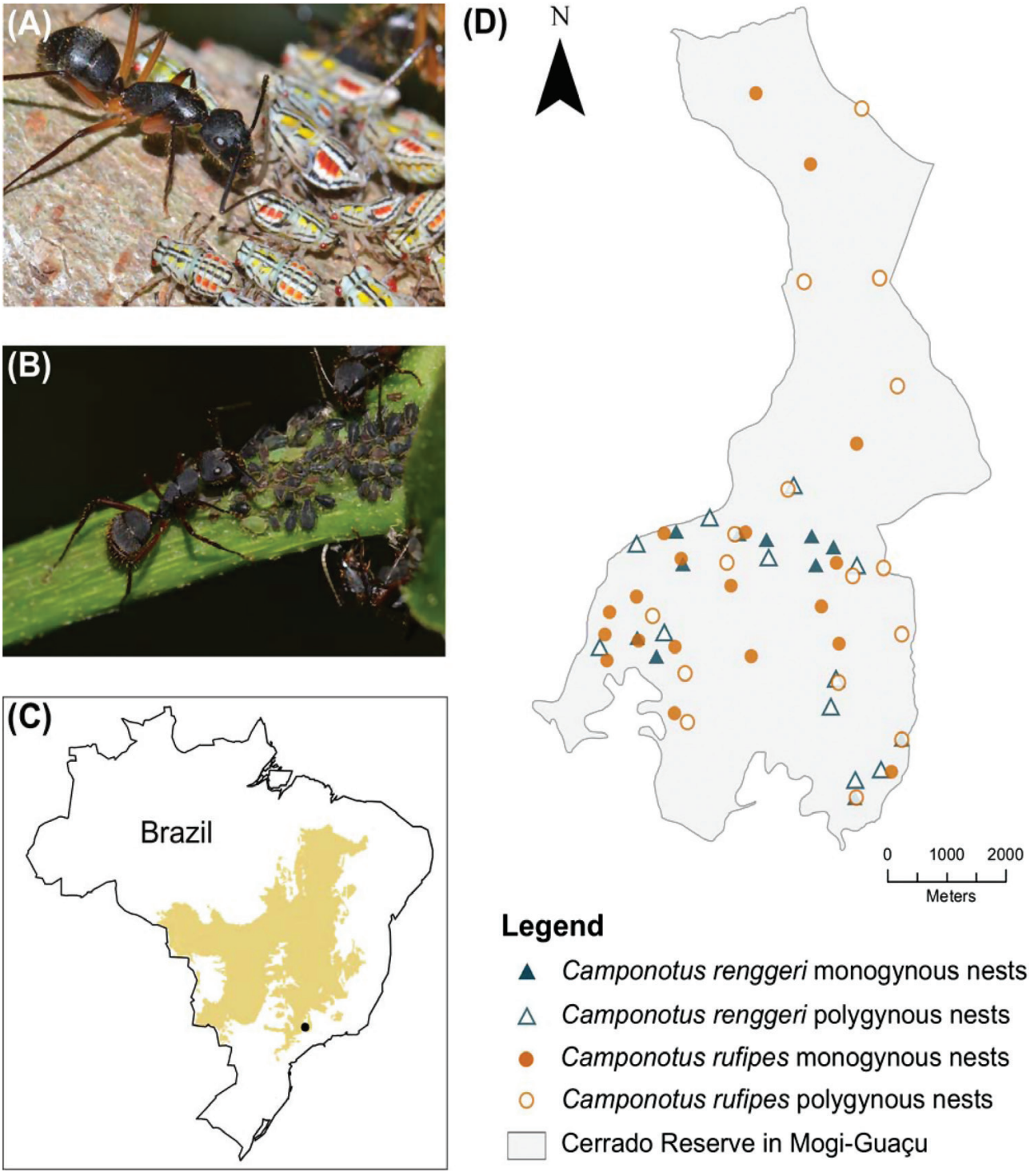

Figure 1. Carpenter ants under study and sampling site. A, *Camponotus renggeri* and (B) *C. rufipes* tending honeydew-producing hemipterans (photographs courtesy of Luisa Mota). C, Cerrado distribution area in Brazil (in yellow) and study site location in the state of São Paulo, southeast Brazil. D, distribution of *C. renggeri* and *C. rufipes* nests in the cerrado reserve. See also Supporting Information, Table S1.

Table 1. Contrasting nesting habitats of *Camponotus renggeri* and *C. rufipes* (after Ronque *et al.*, 2016, 2018)

	Camponotus renggeri	*Camponotus rufipes*
Activity rhythm	mainly nocturnal	mainly nocturnal
Nest architecture	underground; dead trunk	dry straw; dry straw and trunk; underground; dead trunk
Nest persistence	lower	higher
Nest distribution	random	aggregated
Polydomy	absent	present
Number of workers/colony	105–340	251–3654
Number of queens/colony	1–7	1–2

(Table 1; Ronque *et al.*, 2016, 2018). Therefore, closely related *C. renggeri* and *C. rufipes* are interesting model species to evaluate how contrasting natural history traits can be associated with discrepancies in breeding strategies, a trait directly impacting genetic variation of the colony. For instance, because *C. renggeri* relies on less versatile and more vulnerable nesting habitats, this species would present high levels of polygyny so as to increase survival chances of the colony (Steiner *et al.*, 2010). In this work we show that *C. renggeri* and *C. rufipes* indeed present differences in their breeding systems. However, regardless of reproductive strategy, both species exhibited similar patterns of colony genetic diversity. Our study provides new evidence on the genetics and breeding systems in *Camponotus* ants, an aspect still poorly explored in tropical environments.

MATERIALS AND METHODS

Fieldwork

Fieldwork was carried out in the Cerrado reserve near Mogi-Guaçu (22°18S, 47°11'W), São Paulo state, south-eastern Brazil (Fig. 1C). The vegetation in the area consists of a mosaic of tree plantations (including *Pinus* and *Eucalyptus*) and two main Cerrado physiognomies: (1) the 'cerrado *sensu stricto*' consisting of dense scrub of shrubs and trees up to 3–8 m tall, with a fair amount of herbaceous vegetation and (2) the 'cerradão' consisting of a closed woodland with crown cover of 50–90%, made up of 8–14 m tall trees casting considerable shade on a much reduced ground layer [further details in Oliveira-Filho & Ratter (2002)]. In December 2014 and February 2015, colonies were indentified by looking for the characteristic nest architectures of *Camponotus renggeri* and *C. rufipes*, as previously described by Ronque *et al.* (2016, 2018). For two weeks, each species was searched for nearly 60 h in the study area; 22 colonies of *C. renggeri* and 35 of *C. rufipes* were collected (Fig. 1D). We selected colonies at least 100 m apart from one another. This distance is sufficiently large to avoid resampling the same colony, since the home ranges of the two species are up to 10 m^2 in this Cerrado reserve (Ronque *et al.*, 2018). We confirmed the genetic differentiation among colonies by using 10 000 permutations G-test implemented in the package 'hierfstat' (Goudet, 2005), in the R software (R Development Core Team, 2013) (G-test: $P = 0.0001$ for both *C. renggeri* and *C. rufipes*, indicating that colonies are more differentiated than expected by chance). The geographic coordinates of tagged nests were recorded using a global positioning system (GPSmap 60CSx, Garmin International Inc., Olathe, KS, USA, WGS 1984 UTM Zone 23S; Supporting Information, Table S1). The sampling of *C. renggeri* and *C. rufipes* workers was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio; licenses 45550–1 and 45550–3). Ant workers were preserved in 99.5% absolute ethanol and stored at –20 °C.

DNA extraction

Total genomic DNA of *C. renggeri* workers was extracted with DNeasy Blood & Tissue Kit (QIAGEN), following the manufacturer's protocol for insects. The DNA of *C. rufipes* workers was obtained following a modified cetyltrimethyl ammonium bromide extraction protocol (Saghai-Maroof *et al.*, 1984). For both extraction protocols, we used the whole body of the workers.

Microsatellite analyses

Overall, 389 workers of *C. renggeri* and 677 of *C. rufipes* (nine to 22 workers per colony of each species) were genotyped using different sets of 17 highly polymorphic microsatellite loci each (Supporting Information, Table S2). The markers used were previously developed by Azevedo-Silva *et al.* (2015), and amplifications followed the PCR protocols proposed by those authors with modifications for multiplexing (i.e. two to three microsatellite loci were amplified in a single PCR reaction; Sint *et al.*, 2012; Table S3). At each forward primer 5′ end a M13 tail (5′-CACGACGTTGTAAAACGAC-3′) was added. The

same M13 sequence tagged with Infrared dye (IRDye 700 and IRDye 800, Li-Cor Biosciences, Licoln, NE, USA) was added to PCR (Schuelke, 2000), which enabled us to score the amplified microsatellite fragments sizes using Li-Cor 4300 DNA analyser (Li-Cor Biosciences, Lincoln, NE, USA) and Saga software (Li-Cor Biosciences). To estimate genotyping errors, we randomly sampled 30 individuals of each species to re-amplify all markers and rescore them. The genotyping error was expressed as the percentage of loci genotype difference between the first and the second amplification.

Statistical analyses

Descriptive loci analyses

For microsatellite descriptive analyses, we considered all colonies from each species as a single population. For each microsatellite locus we estimated its adherence to Hardy–Weinberg equilibrium (HWE) using the program GENEPOP 4.7 (Rousset, 2008). Allelic richness and expected heterozygosity were estimated using the excel Microsatellite Toolkit (Park, 2001). Linkage disequilibrium (LD) was evaluated using the software FSTAT v.2.9.3.2 (Goudet, 1995). To avoid biased results due to non-independent genotypes in ant colonies, we randomly sampled one individual per colony and this subset was used for the HWE and LD analyses. Bonferroni correction for multiple comparisons was employed at the significance level of 0.05. Null allele frequencies were estimated for each locus in both species by using the Expectation Maximization algorithm (Dempster *et al.*, 1977) implemented in the software FreeNa (Chapuis & Estoup, 2007).

Component 1 of the breeding system: number of queens and mating frequency

We assessed the number of breeding queens and males in *C. renggeri* and *C. rufipes* colonies using the genotypes of the workers to reconstruct parental genotypes (Qian *et al.*, 2011; Cronin *et al.*, 2016a). We adopted this approach considering that it is non-destructive and mainly because queens were often not found during excavations of *C. renggeri* and *C. rufipes* nests (M. Azevedo-Silva, personal observation). We used the program COLONY v.2.0 (Jones & Wang, 2010), which implements a full likelihood method to assign parentage among individuals accounting for deviations from HWE. We set the females as polygamous for COLONY analyses because we have previously found multiple queens inside *C. renggeri* and *C. rufipes* nests (Table 1; Ronque *et al.*, 2016), and also because functional polygyny is known to occur in other *Camponotus* species (e.g. Akre *et al.*, 1994; Goodisman & Hahn, 2004). In contrast, the males were considered monogamous due to limited amount of stored sperm, assuming they copulate once and die right after mating (Hölldobler & Wilson, 1990; but see: Heinze, 2016). Moreover, we considered the previously estimated genotyping errors and null allele frequencies and allowed for inbreeding. From COLONY sibship and parentage inferences, we estimated the number of queens per colony, the number of male mates per queen, their genotypes and assigned workers to their reconstructed parents (Jones & Wang, 2010). The parental genotypes with the highest likelihood were used in the subsequent analyses.

The approach we used may underestimate the number of queens and their mates if non-sampling occurs. Additionally, non-detection error can lead to underestimated numbers of multiple mating per queen if two males have identical multilocus genotypes. Thus, we carried out analyses to evaluate if the sampling effort was sufficient to avoid these errors (Supporting Information, Appendix S1). Results indicate that we successfully detected all matrilines and patrilines in the colonies of both *Camponotus* species (see Supporting Information, Appendix S1).

Because males may contribute unequally to the offspring, the inferred numbers obtained in COLONY sibship analyses were corrected to effective number of matings per queen ($M_{e,p}$), following the equation described by Nielsen *et al.* (2003):

$$M_{e,p} = \frac{(n-1)^2}{\sum_{i=1}^{k} P_i^2\,(n+1)\,(n-2) + 3 - n}$$

where n is the total number of offspring of the M queen, k is the total number of males that copulated with this queen and P_i is the relative contribution of each i^{th} male to the offspring of the queen. Although this estimator was first developed to assess the effective number of mating events per queen, we used the same approach to calculate the effective number of queens per polygynous colony. The estimated number of queens and their mates inferred by COLONY (Jones & Wang, 2010) did not differ from the effective numbers obtained by the formula of Nielsen *et al.* (2003) (differences tested using likelihood ratio test; see Supporting Information, Table S4). Thus, we used the effective numbers for both species in all subsequent analyses. We also evaluated if the number of effective queens per colony and effective mates per queen differed between *C. renggeri* and *C. rufipes*, using likelihood ratio tests implemented in the package 'lmtest' (Zeileis & Hothorn, 2002) in R.

Component 2 of the breeding system: genetic relationship in colonies

The number of mothers and fathers in colonies, and the genetic relationship (relatedness) between same-sex and different-sex breeders, define the level of relatedness between the workers (Ross, 2001). The genetic relationship between sisters in haplodiploid species can vary from 0.75, in the scenario of a single queen inseminated by a single male, to nearly zero in cases of extreme polygyny and polyandry (Crozier & Pamilo, 1996). We estimated the pairwise relationship based on Queller and Goodnight's statistics implemented in the software RELATEDNESS v.5.0 (Queller & Goodnight, 1989). All genotyped individuals of each nest were included as background allele frequencies. Relatedness values were obtained between different levels of social relationship: (1) between nestmate workers in colonies (R_{ww}), (2) between queens from polygynous colonies (R_{qq}), (3) between males that copulated with different queens in polygynous colonies (R_{mm_all}), (4) between males that copulated with a single queen in monogynous and polygynous colonies (R_{mm}) and (5) between queens and their mates (R_{qm}). For these analyses, individuals were weighted equally, which means that all individuals contribute equally to population allele frequency. Standard errors were estimated by jackknifing over loci. Relatedness values were compared with theoretical expectations for full (0.75) and half-sisters (0.25) and for full brothers (0.5) using two-tailed z test in R, unless specified. We also tested for statistically significant differences of relatedness estimates between monogynous and polygynous colonies, and between *C. renggeri* and *C. rufipes*, using likelihood ratio tests implemented in the package 'lmtest' (Zeileis & Hothorn, 2002) in R.

Component 3 of the breeding system: reproductive skew

The reproductive partitioning (skew) of queens in polygynous colonies (S_q) and males that copulated with a single queen (S_m) were calculated based on Nonacs' B index, using the software SKEW CALCULATOR (Nonacs, 2000). The program computes the minimum B value (expected in case of equal reproductive distribution among same-sex breeder), the maximum B value (expected in case of offspring monopolization by a single individual) and a 95% confidence interval (CI) around these metrics. If the CI includes zero, the partitioning of maternity or paternity is not significantly different from random. If the minimum B falls within CI, then an equal partitioning of reproduction cannot be excluded. On the other hand, if the maximum B is equal to the upper CI, monopolization cannot be rejected. Given that we obtained parental genotypes from sampled workers, skew estimates refer to queens and males that indeed contributed to offspring.

Relationship between breeding system and colony genetic diversity

The genetic diversity of *C. renggeri* and *C. rufipes* colonies was characterized based on allelic richness (A) and private allelic richness (pA). Because the colonies have distinct sample sizes, we calculated both statistics based on the rarefaction method proposed by Kalinowski (2004), implemented in the program HP-Rare (Kalinowski, 2005). Expected heterozygosity (H_E) and the Weir & Cockerham's inbreeding coefficient (1984; F_{IS}) were also estimated at the colony level using the package 'hierfstat' (Goudet, 2005) in R. We calculated the colony effective size (N_e) following the method proposed by Wang (2009) and implemented it in the program COLONY v.2.0 (Jones & Wang, 2010). This method is based on the sibship assignment and can be employed to populations with substantial deviations from HWE, which is the case of ant colonies whose workers tend to be highly related. We tested for statistically significant differences of these genetic diversity parameters between *C. renggeri* and *C. rufipes* using likelihood ratio tests with the package 'lmtest' (Zeileis & Hothorn, 2002) in R.

We modelled each genetic variable (A, pA, H_E, N_e) and average relatedness between workers (R_{ww}) in response to the effective number of queens in colonies and the average number of effective mates per queen using linear models in R. A null model was also built, and the best-fitted model was chosen by carrying out a model selection. We kept the effective number of queens and mates as explanatory variables in our model selection of *C. renggeri* and *C. rufipes,* because there was no evidence of collinearity between them (Supporting Information, Appendix S2). Model selection was performed in R with the package 'bbmle' (Bolker & R Development Core Team, 2020), using the Akaike Information Criteria corrected for small samples ($AICc$). The differences between each model and best model ($\Delta AICc$), as well as the Akaike's weight of evidence ($wAICc$, i.e. the relative power of explanation of each model), were estimated among the competing models (Burnham & Anderson, 2002). Models with $\Delta AICc < 2$ and $wAICc > 0.1$ were considered the most plausible among candidates (Zuur *et al.*, 2009). For each explanatory variable, the estimated parameters (and respective confidence interval) of the best-fitted models were plotted to verify significance.

RESULTS

Descriptive loci analyses

For *Camponotus renggeri* we recorded a total of 171 alleles for 17 microsatellite loci, ranging from three to

19 alleles per locus. The H_E per locus ranged from 0.214 to 0.9, with an average of 0.66. The frequencies of null alleles and genotype errors were low, with an average of 0.023 and 0.03, respectively. For *C. rufipes*, 240 alleles were identified across the 17 loci, with a range from 4 to 29 alleles per locus. The mean H_E was 0.81, ranging from 0.523 to 0.944 per locus. The frequency of null alleles and genotype error mean values were, respectively, 0.031 and 0.054. We identified one and four loci that deviated significantly from HWE for *C. renggeri* and *C. rufipes*, respectively. For both species we did not detect LD between any pair of loci. Characterization by locus is described in Supporting Information, Table S2.

Component 1 of the breeding system: number of queens and mating frequency

COLONY sibship and parentage analyses revealed the occurrence of one to eight queens per *C. renggeri* colony, totalling 51 queens across the 22 colonies (Supporting Information, Table S5), half of which were monogynous and half polygynous. Overall, considering monogynous and polygynous colonies, mating frequency for *C. renggeri* ranged from one to four matings per queen. After correcting for breeder contribution to offspring, the effective number of queens in polygynous colonies ranged from 1.11 to 5.62, while the mean effective mating frequency ($M_{e,p}$) was from 1 to 4.37.

We also found monogynous (N = 19) and polygynous (N = 16) colonies of *C. rufipes*. COLONY estimated that the number of queens varied from one to five, totalling 63 queens across all 35 colonies. The corrected effective number of queens in polygynous colonies ranged from 1.11 to 5.21 (Supporting Information, Table S5). The number of effective queens per colony did not differ between *C. renggeri* and *C. rufipes* (χ^2 = 1.3209, P = 0.2504). The mating frequency estimated from sibship inferences ranged from one to ten mates per queen (one to 22.23 after correction for effective frequency, $M_{e,p}$). Thus, considering monogynous and polygynous colonies, *C. rufipes* queens copulated with more males than *C. renggeri* queens (χ^2 = 5.5781, P = 0.0182). The number of queens, the mean number of mates per queen and the total number of males per colony are presented in Supporting Information, Table S5.

Component 2 of the breeding system: genetic relationship in colonies

For both species, the relatedness between workers in monogynous colonies ($Rww_{monogynous}$) was high, but significantly different from the theoretical expectation for full sisters (0.75; Fig. 2; Supporting Information, Table S6). In polygynous colonies ($Rww_{polygynous}$), the relatedness was higher than expected for half-sisters (0.25) (Fig. 2; Supporting Information, Table S6). Specifically, the relatedness (described here in terms of mean ± standard error) among workers in *C. renggeri* monogynous colonies (0.716 ± 0.016) was significantly higher than in polygynous colonies (0.421 ± 0.022)

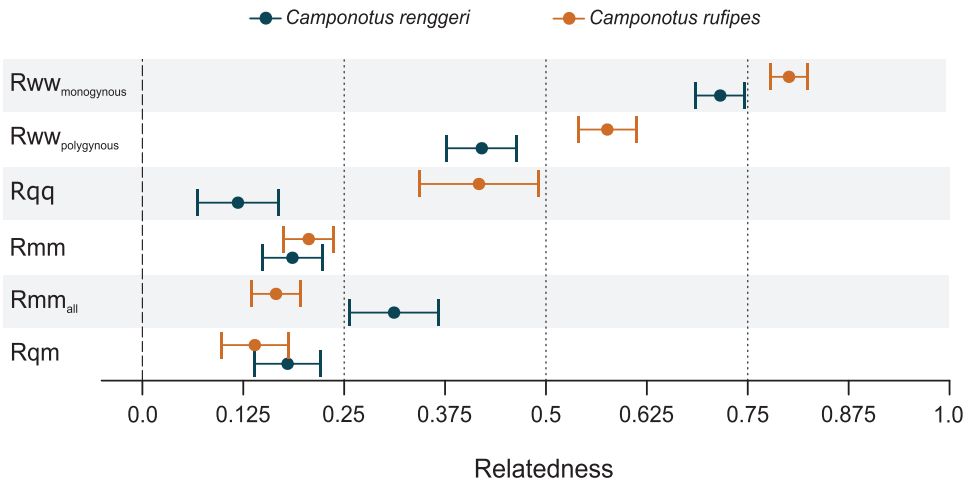

Figure 2. Relatedness estimates for *Camponotus renggeri* and *C. rufipes* nests. Relatedness estimates for *Camponotus renggeri* and *C. rufipes*, with mean and 95% confidence intervals based on Queller & Goodnight's (1989) estimates of genetic relationship between nestmate workers in monogynous colonies ($Rww_{monogynous}$); workers in polygynous colonies ($Rww_{polygynous}$); queens in polygynous colonies (Rqq); males that copulated with a single queen in mono and polygynous colonies (Rmm); all males that copulated with the same and different queens in polygynous colonies (Rmm_{all}), and queens and their mates (Rqm). Dotted lines indicate theoretical values of relatedness expected for full (0.75) and half (0.25) sisters and full brothers (0.5).

(χ^2 = 13.856, P = 0.0002). The same pattern was observed among *C. rufipes* workers in monogynous (0.801 ± 0.012) and polygynous (0.576 ± 0.018) colonies (χ^2 = 13.327, P = 0.0003). However, in general, relatedness among *C. rufipes* workers was higher than among *C. renggeri* workers, considering both monogynous and polygynous colonies (χ^2 = 4.844, P = 0.0278; Fig. 2).

Relationship among queens differed between *Camponotus* species. Queens in polygynous colonies (*Rqq*) of *C. renggeri* were less related than the theoretical values of 0.25 expected for half-sisters in haplodiploid organisms (0.118 ± 0.025; Supporting Information, Table S6). Queens of *C. rufipes* were significantly more related than *C. renggeri* (0.417 ± 0.038) (χ^2 = 7.4362, P = 0.0064) and significantly more related than half-sisters (Fig. 2; Supporting Information, Table S6).

For males that copulated with the same queen (*Rmm*), we found that mean relatedness did not differ between *C. renggeri* (0.312 ± 0.028) and *C. rufipes* (0.166 ± 0.016) (χ^2 = 0.0984, P = 0.7537) and mean relatedness values were significantly below the 0.5 value expected for brothers (Fig. 2; Table S6). Similarly, we found low genetic relationship among all males in polygynous colonies (Rmm_{all} was 0.186 ± 0.019 for *C. renggeri* and 0.206 ± 0.016 for *C. rufipes*) (Fig. 2; Table S6), with no significant difference between species (χ^2 = 1.0124, P = 0.3143).

For both *Camponotus* species, relatedness between queens and their male mates (*Rqm*) was significantly higher than zero, but lower than 0.25 predicted by theory for siblings (0.18 ± 0.021 for *C. renggeri*, and 0.139 ± 0.021 for *C. rufipes*; Fig. 2; Supporting Information, Table S6). Also, mean relatedness between queens and male mates did not differ between *C. renggeri* and *C. rufipes* (χ^2 = 2.4172, P = 0.12). Estimated relatedness per colony is described in Supporting Information, Table S5 and detailed z tests between estimated and theoretical relatedness values are in Supporting Information, Table S6.

Component 3 of the breeding system: reproductive skew

Maternity and paternity reproductive partitioning were different for both *Camponotus* species. We observed significant maternity skew in four of the 11 *C. renggeri* polygynous colonies, but only one tended to monopolization by a single queen. From a total of 51 queens, 16 mated with more than one male, but only two of these queens presented significant skew among multiple mates, with no evidence of monopolization. Of the 16 *C. rufipes* polygynous colonies, ten presented significant maternity skew, out of which five tended to monopolization by a single queen. From a total of 63 estimated *C. rufipes* queens, 30 copulated with more than one male and only three presented significant reproductive skew. As in *C. renggeri*, we did not detect evidence of monopolization among *C. rufipes* males. Estimated reproductive skews per colony are described in Supporting Information, Table S5.

Relationship between breeding system and colony genetic diversity

The inbreeding coefficient (F_{IS}) in colonies was negative for almost all colonies of both *C. renggeri* and *C. rufipes* (Table 2; Supporting Information, Table S5), which agrees with the low relatedness between queens and their male mates in both ant species. Similar estimated values of genetic diversity at colony level were recorded for *C. renggeri* and *C. rufipes* (Table 2). In fact, the genetic diversity parameters we measured (H_E, A, pA and N_e) were statistically undistinguishable between *Camponotus* species (Fig. 3A–D), revealing that *C. renggeri* and *C. rufipes* present similar levels of genetic variation in colonies. However, estimates of genetic diversity and worker relatedness responded differently to the effective number of queens and mates. Relatedness among *C. renggeri* workers is best explained by the effective number of queens (*wAICc* = 0.81; Table 3), with a negative and significant association (Supporting Information, Fig. S1). The number of queens also best explains A (*wAICc* = 0.81), pA (*wAICc* = 0.79), H_E (*wAICc* = 0.82) and *Ne* (*wAICc* = 0.8) (Table 3), all with a positive and significant relationship (Supporting Information, Fig. S1). Contrastingly, relatedness among workers of *C. rufipes* (*wAICc* = 0.99), A (*wAICc* = 1) and H_E (*wAICc* = 0.81) (Table 3) are best explained by both the effective number of queens and their mates. In all models, these associations are significant and positive, except for relatedness among workers, whose coefficients are negative (Supporting Information, Fig. S1). In *C. rufipes*, pA was positively associated with the number of mates (*wAICc* = 0.61;

Table 2. Mean ± standard errors of inbreeding coefficient (F_{IS}), expected heterozygosity (H_E), rarified allelic richness (A), rarified private allelic richness (pA) and effective colony size (*Ne*) at colony level of *Camponotus renggeri* and *Camponotus rufipes*

Genetic diversity estimates	*C. renggeri*	*C. rufipes*
F_{IS}	-0.354 ± 0.0444	-0.444 ± 0.0183
H_E	0.473 ± 0.0186	0.492 ± 0.0183
A	2.745 ± 0.1654	2.809 ± 0.1719
pA	0.098 ± 0.0183	0.069 ± 0.0134
Ne	3.5 ± 0.4779	3.657 ± 0.38

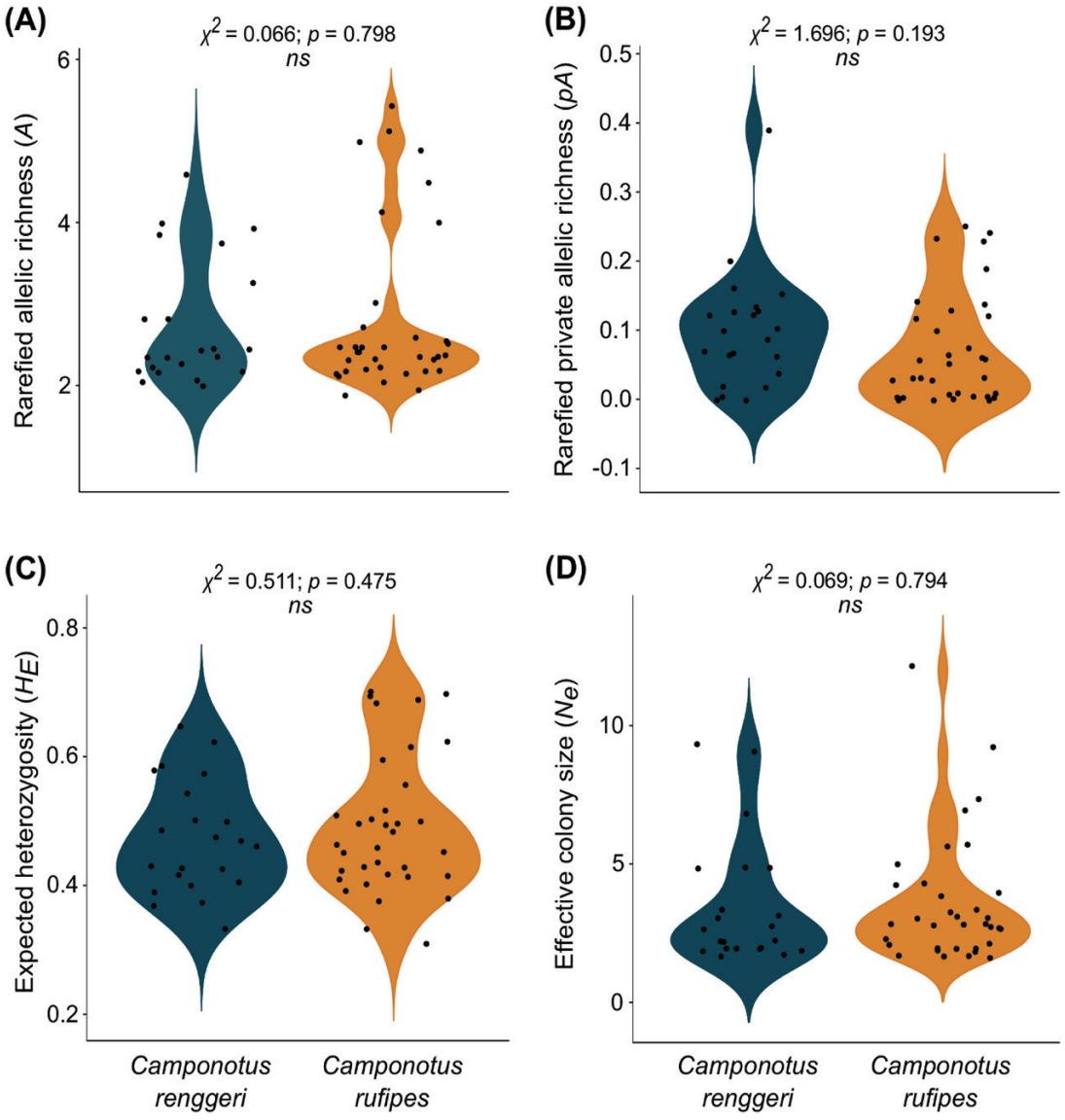

Figure 3. Genetic diversity estimates for *Camponotus renggeri* and *C. rufipes*. A, rarefied allelic richness; B, rarified private allelic richness; C, expected heterozygosity; D, effective colony size. Comparison between species based on the results of likelihood ratio tests are shown. *ns* denotes non-significant difference between *C. renggeri* and *C. rufipes* genetic estimates.

Table 3; Supporting Information, Fig. S1), whereas N_e presented a positive and significant relationship with the number of queens (*wAICc* = 0.75; Table 3; Supporting Information, Fig. S1).

DISCUSSION

In this study, we compared the breeding system of two related ant species and showed that, despite differences between *Camponotus renggeri* and *C. rufipes* in terms of number of breeders, genetic relatedness and reproductive skew, the species present similar levels of genetic diversity in colonies. Although there was no significant difference between species in the number of queens per colony, polygynous colonies of *C. renggeri* exhibited low related nestmate queens mating with one or a few males, and with low reproductive partitioning (skew) among them. In this species, queen number accounts for the genetic variability in colonies. In contrast, polygynous colonies of *C. rufipes* frequently had closely related queens, with higher levels of polyandry than *C. renggeri*, and increased reproductive skew among nestmate queens. Both queens and males contributed to colony genetic diversity in *C. rufipes*.

Table 3. Models for genetic diversity statistics in *Camponotus renggeri* and *Camponotus rufipes* colonies, in response to effective number of queens (Q) and their mates (M). Values in bold indicate most plausible models among the candidates

		C. renggeri				*C. rufipes*			
		Model				*Model*			
		Q	M	Q + M	Null	Q	M	Q + M	Null
Rww	*K*	**3**	3	4	3	3	3	**4**	3
	ΔAICc	**0.0**	41	2.9	40.4	12.3	27.7	**0.0**	60.6
	wAICc	**0.81**	<0.001	0.19	<0.001	0.0021	<0.001	**0.9979**	<0.001
	β	**-**	-	- / -	+	-	-	- / -	-
A	*K*	**3**	3	4	3	3	3	**4**	3
	ΔAICc	**0.0**	43.3	2.9	42.7	18.4	35.5	**0.0**	71.6
	wAICc	**0.81**	<0.001	0.19	<0.001	<0.001	<0.001	**1**	<0.001
	β	**+**	+	+ / +	-	+	+	+ / +	+
pA	*K*	**3**	3	4	3	3	**3**	4	3
	ΔAICc	**0.0**	14.8	2.6	14.5	3.0	**0.0**	2.5	4.2
	wAICc	**0.79**	<0.001	0.21	<0.001	0.137	**0.615**	0.174	0.075
	β	**+**	+	+ / +	-	+	+	- / +	+
H_E	*K*	**3**	3	4	3	3	3	**4**	3
	ΔAICc	**0.0**	22.5	3.0	21.5	3.0	11.5	**0.0**	33.2
	wAICc	**0.82**	<0.001	0.18	<0.001	0.1822	0.0026	**0.8152**	<0.001
	β	**+**	+	+ / +	-	+	+	+ / +	+
Ne	*K*	**3**	3	4	3	**3**	3	4	3
	ΔAICc	**0.0**	58.7	2.8	58.7	**0.0**	57.6	2.2	74.2
	wAICc	**0.8**	<0.001	0.2	<0.001	**0.75**	<0.001	0.25	<0.001
	β	**+**	+	+ / +	-	+	+	+ / +	+

Notes: *Rww*, relatedness among workers in colonies; *A*, rarefied allelic richness; *pA*, rarified private allelic richness; H_E, expected heterozygosity; N_e, effective colony size; *K*, number of estimated parameters in each model; *ΔAICc*, difference between Akaike Information Criteria corrected for small samples between each model and the best model; *wAICc*, Akaike's weight of evidence of each model; *β*, beta coefficient direction of each variable in the model. Null model represents absence of effect.

Previous studies classified the ant genus *Camponotus* as predominantly monogynous due to life-history features such as high aggressiveness, caste polymorphism and independent colony foundation (Hölldobler & Wilson, 1990; Crozier & Pamilo, 1996). Despite the evidence for polygyny based on colony genetic structure in *Camponotus* species such as *C. novaeboracensis* (Fitch, 1855) (Gadau *et al.*, 1998), *C. ocreatus* Emery, 1893 (Goodisman & Hahn, 2004) and *C. festinatus* (Buckley, 1866) (Goodisman & Hahn, 2005), the frequency of colonies headed by multiple queens was considered low in these studies. In contrast, we observed that *C. renggeri* and *C. rufipes* exhibited high frequency of polygyny in the populations studied. Ronque *et al.* (2016) reported the occurrence of multiple queens in *C. renggeri* and *C. rufipes* nests, but only by using molecular tools are we now able to confirm that multiple queens indeed contribute to offspring, a costly trait that would be compensated by increased worker production in immature colonies (Tsuji & Tsuji, 1996). Facultative polygynous ant species are commonly associated with saturated habitats with scarce nesting locations, which represent an ecological pressure capable of promoting cooperation among breeders (Hölldobler & Wilson, 1977; Heinze & Foitzik, 2009; Avila & Fromhage, 2015). Distinct ecological pressures can be associated with queen numbers in ant colonies. For instance, monogyny occurs more frequently in tropical and temperate areas with low to intermediate levels of competition, and in colonies presenting high longevity and reproduction in late life-stages (Heinze & Foitzik, 2009). On the other hand, polygyny may be associated with extreme climates with intermediate to high levels of competition, and with colonies presenting low longevity and higher reproduction in early life-stages (Heinze & Foitzik, 2009). Although our study was conducted in the Neotropics, the high number of *C. rufipes* and *C. renggeri* polygynous colonies can be explained by the typical seasonal climate in Cerrado savanna, with remarkable hot/rainy and cold/dry seasons, with natural fires in the latter period (Oliveira-Filho & Ratter, 2002). Facultative polygyny also occurs in other Neotropical Biomes where seasonal changes during the year are less drastic (Medeiros *et al.*, 1992).

The number of queens did not differ significantly between colonies of *C. renggeri* and *C. rufipes*. However, workers in *C. rufipes* colonies presented higher relatedness values than in *C. renggeri*. The

underlying causes for these findings are probably the differences between these species in ecological traits. *Camponotus renggeri* queens in polygynous colonies were less related than expected to half-sisters. The association among unrelated foundresses in new colonies is thought to increase colony survival chances due to higher quality and faster production of workers (Steiner *et al.*, 2010). This condition is generally limited to immature colonies; since only one queen survives, polygyny becomes an uncommon feature of established ant colonies (Steiner *et al.*, 2010). However, it is also known that social structure may vary along time, with replacement of queens (related or not) in some ant species (Purcell & Chapuisat, 2013). Determining colony age during sampling was not viable because it demands colony monitoring, a task especially challenging for *C. renggeri* due to low nest persistence (Ronque *et al.*, 2016). Even though we did not evaluate polygyny continuity over time, this life-history trait is likely associated with the predominant use of fallen dead trunks as nests by *C. renggeri*. Such fragile material is more likely to be destroyed by environmental conditions and may become a limitation for the establishment of new nests (Ronque *et al.*, 2016). This limitation most likely favours polygyny in *C. renggeri*. In contrast, *C. rufipes* queens in polygynous colonies were genetically related (with values higher than expected for half-sisters). Even though it was not possible to evaluate if polygyny persists as colonies mature, this finding brings new insights on adult dispersal and colony foundation in *C. rufipes*. Ant queens that fly away in nuptial flights are unlikely to return to their natal nests or to join their sisters to form a new colony (Crozier & Pamilo, 1996; Peeters & Molet, 2010). In *C. rufipes*, gynes probably copulate close to their natal sites and inseminated sister queens remain together to form a new colony, or even return to their natal nests, which would suggest the occurrence of secondary polygyny in this species. Additionally, polygyny is also associated with polydomy in many ants (Crozier & Pamilo, 1996), a trait also exhibited by *C. rufipes* (Matta *et al.*, 2013; Ronque *et al.*, 2016). Thus, new nests would be formed by budding polygynous nests that remain interconnected (Steiner *et al.*, 2010), a dispersal strategy widely distributed in eusocial insects and subject to variation under different environmental conditions (Cronin *et al.*, 2013, 2016b). Such a strategy is associated with higher mortality risk in immature colonies and low competition among nests at intermediate levels of environmental disturbance, but less favourable in spatial structured populations in environments under spatially-wide scaled disturbance (Nakamaru *et al.*, 2007, 2014). We do not discard that multiple queens in *C. renggeri* and *C. rufipes* may coexist in the same nest but remain well apart from one another (i.e. oligogyny), as already reported for other *Camponotus* species (Hölldobler, 1961; Hölldobler & Wilson, 1990). Thus, we encourage further investigation on polygyny persistence along time in both *Camponotus* species. This would help to clarify if queens indeed form new colonies together (primary polygyny) or if new queens are accepted and/or replaced in mature colonies (secondary polygyny).

Our results also showed that *C. renggeri* and *C. rufipes* copulated with few males (less than three), which partially agrees with expectations for *Camponotus*, whose queens are generally single mated (Hasegawa, 1995; Gadau *et al.*, 1996; Gadau *et al.*, 1998; Mersch *et al.*, 2017). Given this low mating frequency, polygyny would be a strategy to increase colony sperm storage in both species, which would guarantee a high fertilization rate to produce large numbers of workers during the colonies lifespan (Cole, 1983). This is supported by the low queen monopolization in *C. renggeri* and *C. rufipes*, with distinct queens contributing to offspring in polygynous colonies.

The degree of relatedness between reproductive castes (males and queens, males that copulated with a single queen and all males in nests) was consistently lower than the theoretical expectations for sibs. In addition, we observed low values of inbreeding in nests. Altogether, these results suggest that individuals from different colonies mate to form a new colony, and that *C. renggeri* and *C. rufipes* probably have efficient mechanisms to avoid inbreeding. In ants, such mechanisms can involve avoidance by individuals with similar nest pheromones, or males and queens flying out of colonies at different times (Hölldobler & Wilson, 1990).

Another remarkable difference between *C. renggeri* and *C. rufipes* was the importance of polygyny and polyandry for colony genetic diversity. For both species we observed an expected positive association between the colony effective size (N_e) and queen numbers. Such a finding puts *C. renggeri* and *C. rufipes* in agreement with the hypothesis that (at least at the colony level) polygyny improves the naturally low effective size of ants, which would reduce their risk of extinction (Wilson, 1971). For *C. renggeri*, the number of queens was the most important variable determining the genetic relatedness among workers and all the evaluated genetic diversity parameters. Probably, the low mating frequency of this species (1.41 on average) makes polyandry less relevant and, consequently, confers to the joining queens the most likely way of incrementing genetic variation in colonies. In contrast, for *C. rufipes*, which presented a higher mating frequency (2.69 on average), polygyny and polyandry were associated with reduced worker relatedness and increased expected heterozygosity and allelic richness. In this species, only polyandry led to an increase of

private alleles inside the colonies. This result suggests that the males are responsible for bringing new alleles for *C. rufipes* colonies, which is reasonable since closely related queens were found coexisting. Such findings may support the hypothesis that polyandry evolves in species in which polygyny is not sufficient to lead to increased genetic variation in ant colonies (Hughes *et al.*, 2008a; Qian *et al.*, 2011). Despite discrepancies in reproductive traits, we did not find distinguishable levels of genetic diversity between colonies of *C. renggeri* and *C. rufipes*. It is important to emphasize that similar outcomes observed at the colony level may change through higher organization scales. For instance, at the population level (i.e. considering the set of colonies in a given area), *C. renggeri* and *C. rufipes* exhibited different patterns of genetic variation and distribution across space (Ronque *et al.*, 2016).

CONCLUSION

The assessment of breeding systems of *C. renggeri* and *C. rufipes* reveals important details regarding their social organizations, and enhances their relevance in determining the genetic diversity in colonies of these two tropical species. Traditionally, polygyny is believed to occur in response to ecological pressures, and polyandry is regarded as a main mechanism increasing colony genetic diversity (Briese, 1983; Crozier & Page, 1985; Hölldobler & Wilson, 1997; Hughes *et al.*, 2008a; Rubin *et al.*, 2013; Avila & Fromhage, 2015; Nonacs, 2017). Although we could not assess the ecological pressures leading to polygyny in *C. renggeri* and *C. rufipes*, our results suggest that multiple queens also have an evolutionary importance for increasing genetic variation in these species. Therefore, we think that for species with low mating frequency and with one to a few queens in colonies, polygyny does affect colony genetic diversity (as shown for *C. renggeri* and *C. rufipes*). At the same time, a small increment in the number of mates per queen greatly influences colony genetic variation in these species. It is known that higher genetic diversity increases colony productivity and longevity in social insects (Mattila & Seeley, 2007), reducing colony susceptibility to pathogenic infestations (e.g. van Baalen & Beekman, 2006) and influencing ant communities (e.g. Tsutsui *et al.*, 2003; Steiner *et al.*, 2010). Colony genetic structure may change geographically, an aspect well described for some ants in the temperate zone (e.g. Pamilo *et al.*, 2016) but still poorly explored for tropical ants. Although we did not assess genetic diversity of colonies under variable environmental contexts, current analyses on landscape genetics of *C. renggeri* and *C. rufipes* should elucidate how molecular polymorphism at the population scale varies across different physiognomies of Cerrado savanna (M. Azevedo-Silva, M. C. Côrtes, C. S. Carvalho, G. M. Mori, A. P. Souza and P. S. Oliveira, unpubl. data). The present work adds important information for further investigation on ant breeding systems and genetic diversity of ant colonies in the tropics.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Detailed description of nests' location:

Table S1. Nest identification codes (ID) for *Camponotus renggeri* and *C. rufipes*, and nest geographic coordinates in decimal degrees, in WGS 84 Geographic Coordinate System.

Table S2. Multiplex, amplification conditions and characteristics of microsatellite markers genotyped for *Camponotus renggeri* and *C. rufipes* in this study. Notes: A_{locus}, allelic richness; H_E, expected heterozygosity; fA_{null}, null allele frequency; *Std*, standard PCR protocol. *TD range of temperature for touchdown PCR amplification. † Locus with significant departure from Hardy–Weinberg equilibrium

Table S3. Volume of chemicals (μL) for each PCR modification, for multiplexing microsatellite amplification in *Camponotus renggeri* and *C. rufipes.* PCR were carried out at a final volume of 10 μL. For both *C. renggeri* and *C. rufipes* microsatellite fragments amplification, PCR were carried out following procedures described in Azevedo-Silva *et al.* (2015), with four different modifications for multiplexing, depending on primer set (Table S2). All loci were amplified using touchdown thermocycling conditions: 94 ºC for 4 min; 10 × [94ºC for 45 s, 60 ºC or 57 ºC (–0.5ºC/cycle) for 1 min and 72 ºC for 1 min 15 s]; 25 × (94ºC for 45 s, 50 ºC for 1 min and 72 ºC for 1 min 15 s); and 72 ºC for 10 min.

Notes. * Primer pair of microsatellite locus with the largest size in the multiplex.

Detailed description of sampling effort estimates:

Appendix S1. Sampling effort.

Table S4. Likelihood ratio for testing significant differences between the number of queens (and their mates) estimated in COLONY (Jones & Wang, 2010), and effective number of queens (and their mates) according to Nielsen *et al.* (2003) for *Camponotous renggeri* and *C. rufipes*. Notes: χ^2, likelihood ratio chi-squared statistics; *P*, *P*-value.

Table S5. Characteristics of *Camponotus renggeri* and *C. rufipes* colonies from Mogi-Guaçu, state of São Paulo, Brazil. Nest codes initialized with 'G' and 'F' indicate *C. renggeri* and *C. rufipes* nests, respectively. Notes: n_w, number of workers sampled; n_q, number of queens; n_m, mean number of mates per queen estimated by COLONY v.2.0 (Jones & Wang, 2010); $M_{e,p(q)}$, effective number of queens; $M_{e,p(m)}$, effective mates per queen following Nielsen *et al.* (2003); R_{ww}, relatedness between workers; R_{qq}, nestmate queens in polygynous colonies; R_{mm_all}, all males in polygynous colonies; R_{mm}, mates of a single queen; R_{qm}, queens and their male mates calculated using the algorithm of Queller & Goodnight (1989), implemented in the software RELATEDNESS v.5.0; S_q, queen reproductive skew; S_m, mates reproductive skew (of queens that copulated more than once) calculated in the software SKEW CALCULATOR (Nonacs, 2000); * significant skew; † significant skew that tended to monopolization; F_{IS}, Weir & Cockerham's inbreeding coefficient (1984); H_E, expected heterozygosity; F_{IS} and H_E were calculated using the package 'hierfstat' (Goudet, 2005) in R software (R Development Core Team, 2013); *A*, allelic richness and *pA*: private allelic richness by rarefaction method proposed by Kalinowski (2004) and implemented in the program HP-Rare (Kalinowski, 2005); *Ne*, effective colony size according Wang (2009) and implemented in the program COLONY v.2.0 (Jones & Wang, 2010); '-': not applicable.

Table S6. Z tests between inferred relatedness in *Camponotus renggeri* and *C. rufipes* colonies and theoretical expectations (T. exp.) for full sisters (0.75), half-sisters (0.25), full brothers (0.5) and siblings (0.25). *Z*: z-score

and *P*: p-value obtained for z tests in R. Notes: $R_{ww\text{monogynous}}$, relatedness between workers in monogynous colonies; $R_{ww\text{polygynous}}$, relatedness between workers in polygynous colonies; R_{qq}, relatedness between nestmate queens in polygynous colonies; R_{mm}, relatedness between mates of a single queen; R_{mm_all}, relatedness between all males in polygynous colonies; R_{qm}, relatedness between queens and their male mates. Relatedness estimates were calculated using the algorithm of Queller & Goodnight (1989), implemented in the software RELATEDNESS v.5.0.

Appendix S2. Model selection – predictor variables. To avoid collinearity between predictor variables (effective number of queens and effective number of mates), we used Spearman correlation to test the association between them. We did not find correlation between the predictor variables for *C. renggeri* (Spearman correlation: *rho* = 0.262, *N* = 22 and *P* = 0.238). In contrast, this correlation was positive and significant for *C. rufipes* (Spearman correlation: *rho* = 0.584, *N* = 35 and *P* = 0.0002), but because it was less than 0.7, we kept both variables (effective number of queens, and mates) in our model selection of this species.

Figure S1. Beta coefficient plots of the best models for the relationship between number of breeders ('Queens' and 'Mates') and genetic diversity estimates. For model selection, we used Akaike Information Criteria corrected for small samples (AICc). A–E are beta coefficient plots for *Camponotus renggeri* and F–J for *C. rufipes* best models. R_{ww} is relatedness between nestmate workers; *A* is allelic richness and *pA* private allelic richness estimated by rarefaction method; H_E is expected heterozygosity and *Ne* effective colony size. Dots represent the regression coefficients and lines the 95% confidence intervals.