



Three ways to distinguish species: using behavioural, ecological, and molecular data to tell apart two closely related ants, *Camponotus renggeri* and *Camponotus rufipes* (Hymenoptera: Formicidae)

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The closely related *Camponotus renggeri* and *Camponotus rufipes* (subgenus *Myrmothrix*) often live in sympatry in the Brazilian ‘cerrado’ savannah, and are distinguished by nuances in their blackish body colour and by the colour of the legs. Variation in morphological characters, however, makes species separation difficult and it has been suggested that the two species should be merged into one. As appropriate species identification is essential for studies in ecology and evolutionary biology, here we examine how natural history data (habitat preference, nesting biology) and molecular tools (nuclear and mitochondrial markers) perform in distinguishing sympatric populations of *C. renggeri* and *C. rufipes*. In our study area, *C. rufipes* was only seen in cerrado *sensu stricto* (scrub of shrubs and trees), whereas *C. renggeri* occurred in cerrado *sensu stricto* and cerradão (closed woodland). *Camponotus renggeri* nested underground or in fallen/erect dead trunks, whereas *C. rufipes* constructed distinctive nests of dry straw. Nest persistence through time was higher in *C. rufipes*, especially in the hot/rainy season. Nest distribution was random in *C. renggeri* and aggregated in *C. rufipes*. Molecular data consistently showed that, regardless of the source of genetic variation, the uppermost hierarchical level of divergence is observed between species, unambiguously differentiating the individuals identified as *C. renggeri* and *C. rufipes* as two independent evolutionary lineages. Mitochondrial data throughout the species’ geographical ranges further confirmed a consistent genetic divergence between *C. renggeri* and *C. rufipes* along their distribution in Brazil. Our integrated approach combining morphological traits with natural history and molecular data confirms that *C. renggeri* and *C. rufipes* are valid species that can be separated in our study area relatively well.

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INTRODUCTION

Classification of species into recognizable and distinguishable entities is a scientific tool that helps biologists observe and interpret the natural world. Although Carl Linnaeus' pioneering work using external morphology and binomial nomenclature to distinguish species is still widely employed today (with many changes), modern taxonomy is based more on evolutionary relationships and takes into account a considerable amount of information to assess both higher- and lower-level relationships (Schlick-Steiner *et al.*, 2010; Ward, 2010; Hamilton, 2013). Nowadays, the most promising method for appropriately identifying species, as well as demarcating between-species boundaries, is to incorporate many types of information into the same study. Indeed, besides morphology, the inclusion of other types of data from areas such as chemistry, behaviour, ecology, and molecular biology has greatly invigorated systematics research of different groups in the past few decades – ants are no exception (Lucas *et al.*, 2002; Ward, 2010; Seppä *et al.*, 2011). Studies on ant systematics have increasingly taken multidisciplinary approaches that combine chemical (Morrison & Witte, 2011; Touchard *et al.*, 2014), behavioural (Basibuyuk & Quicke, 1999; Fleury *et al.*, 2010; Trager, 2013), natural history/ecological (Longino, 1991; King & Trager, 2007; Schmidt & Shattuck, 2014), and molecular data (Bernasconi, Pamilo & Cherix, 2010; Jansen & Savolainen, 2010; Hosoishi & Ogata, 2014) to assist species identification in difficult groups where the use of traditional morphological characters is problematic.

The ant genus *Camponotus* Mayr is one of these difficult taxa, consisting of over 1000 described species worldwide (Bolton *et al.*, 2006). Approximately 650 taxa have been named in *Camponotus* for the New World, but an on-going revision will reduce the numbers to about 440 valid species, with nearly 140 new species (Mackay, 2004). The large size of the genus and the difficulty in defining reliable morphological characters (most vary within a species or species complex) make species identification challenging for *Camponotus* ants. Nevertheless, new species of *Camponotus* are still frequently described worldwide (e.g. Mackay & Barriga, 2012; Karaman & Aktaş, 2013).

In the Neotropical region, *Camponotus* species are widely distributed in a variety of environments and in Brazil these range from open semi-arid environments to densely covered rainforests (Kempf, 1972). The natural history and ecology of Brazilian *Camponotus*, however, are perhaps best documented in the so-called 'cerrado' savannah that occupies nearly 22% of the land surface of the country (Oliveira & Marquis, 2002). The cerrado biome is a biodiversity hotspot (Myers *et al.*, 2000) encompassing a mosaic of

vegetation physiognomies in the central plateau of Brazil, which ranges from open grassland to dense woodlands following gradients in soil fertility, groundwater regimes, and fire (Oliveira-Filho & Ratter, 2002). The difficulty in delimiting the numerous ground- and plant-foraging *Camponotus* species found in cerrado is revealed by the large number of ecological studies in which they could not be distinguished with certainty, or were simply referred to as morphospecies (e.g. Del-Claro & Oliveira, 1999; Ribas *et al.*, 2003; Schoederer *et al.*, 2010; Christianini, Mayhé-Nunes & Oliveira, 2012; Frizzo, Campos & Vasconcelos, 2012).

Camponotus renggeri (Emery) and *Camponotus rufipes* (Fabricius) belong to the New World subgenus *Myrmothrix*, which is characterized by numerous long, erect, brownish or golden hairs that are conspicuous on the antennal scapes and legs. The two species are usually distinguished by nuances in their blackish body colour (*C. renggeri* is shiny; *C. rufipes* is matte), number of erect hairs on different body parts, and colour of the legs (yellowish in *C. renggeri*; reddish in *C. rufipes*) (Fig. 1; Hashmi, 1973). However, variation in these

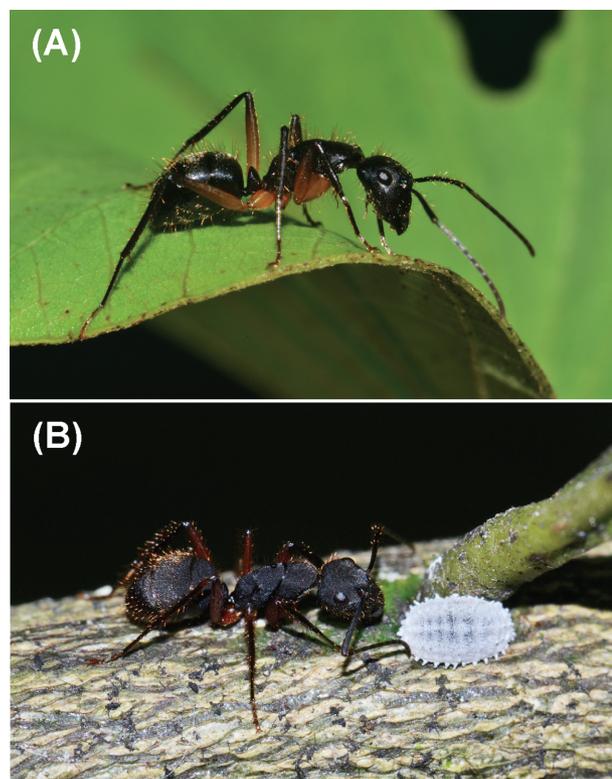


Figure 1. Workers of (A) *Camponotus renggeri* and (B) *Camponotus rufipes*. The two species are usually differentiated in the field by nuances in the integument colour (*C. renggeri* is shiny; *C. rufipes* is matte), and colour of the legs (yellowish in *C. renggeri*; reddish in *C. rufipes*). Photographs courtesy of L. Mota.

morphological characters, combined with the many similarities in their ecology in cerrado (Oliveira & Brandão, 1991; Del-Claro & Oliveira, 1999; Ribas *et al.*, 2003), frequently make *C. renggeri* and *C. rufipes* hard to differentiate in the field. Indeed, this taxonomic uncertainty has persisted since the species' descriptions. *Camponotus renggeri* was initially described as a subspecies of *C. rufipes* (Bruch, 1914), only acquiring specific status a few years later (Luederwaldt, 1918). This classification was then challenged by Wheeler (1923), but subsequent work confirmed *C. renggeri* and *C. rufipes* as differentiated biological entities (Emery, 1925; Kusnezov, 1952; Hashmi, 1973). More recently, Mackay (2004) suggested that colour variation in the body and legs justifies merging these two species into one highly variable species, *C. rufipes*.

The closely related *C. renggeri* and *C. rufipes* often live in sympatry in cerrado areas, where they are seen feeding on with fruits on the ground (Christianini *et al.*, 2012) and plant and insect exudates on foliage (Oliveira & Brandão, 1991; Del-Claro & Oliveira, 1999), as well as insect prey (Silvestre, Brandão & da Silva, 2003; Sendoya, Freitas & Oliveira, 2009). The two species have variable types of nests, which can be built in rotten wood, natural hollows of tree trunks, underground, or in above-ground straw mounds (Hashmi, 1973; Mackay, 2004; Weidenmüller *et al.*, 2009; Ronque, 2013). As appropriate species identification is essential for studies of ecology, behaviour, and evolutionary biology, the aim of the current paper was to provide reliable tools to allow delimitation of the closely related *C. rufipes* and *C. renggeri*, and at the same time assist future taxonomic studies on the genus. Our work was motivated by the ambiguous interpretations of the morphological traits utilized to delimit these species. In addition, we noted that change in leg colour of alcohol-preserved specimens further complicates the differentiation of these species in the laboratory (M. Azevedo-Silva, pers. observ.). We examined how behavioural and ecological traits (foraging substrates, nest types and distribution patterns, nest residence time, habitat type) and molecular biology (nuclear and mitochondrial markers) perform in distinguishing sympatric populations of *C. rufipes* and *C. renggeri* inhabiting an area of cerrado savannah in southeast Brazil.

MATERIAL AND METHODS

STUDY SITE

The cerrado biome of tropical South America covers about 2 000 000 km² and presents variable physiognomies throughout its distribution, ranging from open grassland to forest with a discontinuous herbaceous layer; the assortment of savannah formations covering the entire range is collectively referred to as the

cerrados (Oliveira & Marquis, 2002). Field work was carried out in a cerrado reserve in Mogi-Guaçu (22°18'S, 47°11'W), São Paulo state, southeast Brazil. Observations and collections were performed in two vegetation physiognomies that are typical of the cerrado biome: (1) the 'cerrado *sensu stricto*', which consists of a dense scrub of shrubs and trees of 3–8 m tall and a fair amount of herbaceous vegetation, and (2) the 'cerradão', which consists of a closed woodland with crown cover of 50–90% and trees of up to 14 m that cast considerable shade so that the ground layer is much reduced (Fig. 2; see Oliveira-Filho & Ratter, 2002). The climate of the region is characterized by a hot and rainy season (summer) from October to March, and cold and dry season (winter) from April to September. Average annual temperatures range from 20.5 to 22.5 °C; the average accumulated rainfall varies from 1100 to 1200 mm in the hot/rainy period, and 250 to 300 mm in the cold/dry period (data from 1961 to 1990 from the reserve's climatological station at the cerrado reserve).

NESTING HABITS AND FORAGING ECOLOGY OF ANTS

Nests of *C. renggeri* and *C. rufipes* were located in the field by following ant workers attracted to honey and tuna baits placed on the ground and on vegetation, in both cerrado *sensu stricto* (6000 m²) and cerradão (5000 m²). The two species were distinguished in the field *a priori* by the colour of their bodies (shiny or matte), and legs (yellow or red) (Fig. 1). Ant nests of both species were described according to their external structure, building material, and location. The following nest types were observed in the field: (1) underground; (2) fallen dead trunk; (3) erect dead trunk; (4) dry straw; (5) dry straw and trunk. The occurrence of different types of nests in colonies of each species was compared with a G-test.

Nests of *C. renggeri* ($N = 46$) and *C. rufipes* ($N = 40$) were tagged in December 2011, and their persistence during 9 months was determined by direct inspections of marked nests in April 2012 and August 2012. Nests were considered active if ants and fresh midden material were seen in the immediate vicinity. We recorded the proportion of nests of each species persisting during the hot/rainy season and during the cold/dry season, and nest persistence between species was compared with a G-test.

The spatial pattern of nest distribution in *C. renggeri* and *C. rufipes* was evaluated in April 2012 and August 2012, based on the nearest neighbour index. This index indicates a random distribution if $R = 1$, a maximum aggregate distribution if $R = 0$, and a uniform distribution if $R = 2.1491$ (Clark & Evans, 1954).

The foraging substrate (ground or vegetation) used by workers of each species was monitored for 130 h

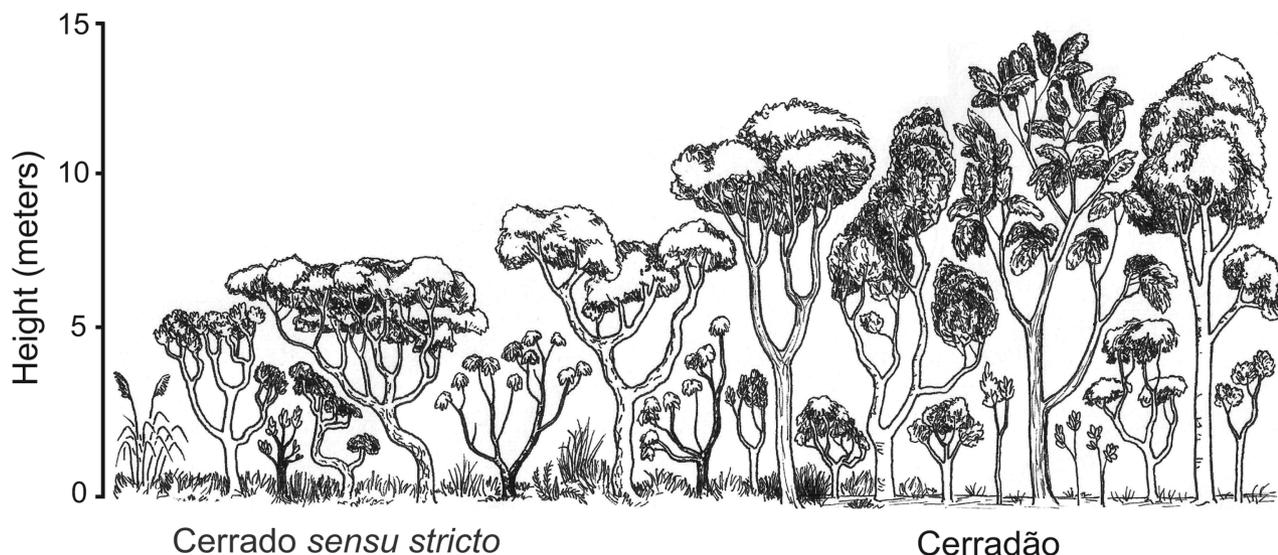


Figure 2. Main vegetation physiognomies of the cerrado reserve at Mogi-Guaçu, Brazil. *Cerrado sensu stricto* consists of a dense scrub of shrubs and trees and a fair amount of herbaceous vegetation, whereas the *cerradão* is a closed woodland with a reduced ground layer. Nests of *Camponotus renggeri* ($N = 46$) were found in *cerrado sensu stricto* (22%) and *cerradão* (78%), whereas *Camponotus rufipes* ($N = 40$) occurred only in *cerrado sensu stricto*. Drawing by L. Mota.

in hot/rainy season and 130 h in cold/dry season. Behavioural observations were carried out in four nests of *C. renggeri* and *C. rufipes* in each period. Workers were followed as they left the nest and the foraging substrate was tagged. The proportion of ants foraging on the ground or vegetation in each season was calculated for each focal colony of *C. renggeri* and *C. rufipes*, and compared using a Mann–Whitney U -test. To avoid variation in the availability of foraging substrate between different cerrado physiognomies, for the sake of comparison in this analysis we only considered the observations of *C. renggeri* and *C. rufipes* colonies occurring in *cerrado sensu stricto*. Four colonies of each species were collected for demographic data, mainly the number of workers and queens, between September and December 2012. Voucher specimens are deposited at the Museu de Zoologia da Universidade Estadual de Campinas (ZUEC, Campinas, Brazil; registration numbers 2465 to 2482).

DNA COLLECTION

Workers of *C. renggeri* and *C. rufipes* were collected for molecular analyses in September 2012 and February 2013. All individuals were preserved in 100% ethanol and stored at -20°C . Total genomic DNA was extracted from entire workers following a modified cetyltrimethyl ammonium bromide extraction protocol (Saghai-Marooof, Soliman & Jorgensen, 1984). We hypothesized that if *C. renggeri* and *C. rufipes* are different species, they would present a more similar genetic composition within species than between the two species.

We tested this prediction using microsatellites and cytochrome c oxidase subunit I (*COI*) sequences.

MICROSATELLITE AMPLIFICATION AND ANALYSES

According to Bernasconi *et al.* (2010), microsatellites are helpful for species identification and should be used primarily in local-scale studies to avoid geographical influences, especially when Bayesian clustering analyses are employed. We thus sampled a total of 94 *C. renggeri* and 104 *C. rufipes* workers at the cerrado reserve, from 30 different colonies of each species (one to five workers per colony; see Hale, Burg & Steeves, 2012). The ants of both species were genotyped at the same 28 polymorphic microsatellite loci developed by Azevedo-Silva *et al.* (2015), which were amplified using PCR following the protocols proposed by the authors. In each forward primer we added a M13 tail (5'-CACGACGTTGTAAAACGAC-3') at its 5' end (Schuelke, 2000), which enabled us to score the amplified microsatellite fragments on a Li-Cor 4300 DNA analyser (Li-Cor Biosciences, Lincoln, NE, USA). Allele lengths were determined using SagaTM software (Li-Cor Biosciences, Lincoln, NE, USA).

To better understand how the genetic diversity of each species is organized, and to examine the genetic composition of individuals within each species, we used a Bayesian approach implemented in STRUCTURE 2.3.3 (Pritchard, Stephens & Donnelly, 2000) without *a priori* assumptions of subdivisions in the populations. Under an admixture model and correlated allele frequencies, 60 independent Markov chain Monte Carlo

(MCMC) runs were carried out with 5.0×10^5 iterations following a burn-in period of 5.0×10^5 iterations for each value of the number of clusters (K) ranging from 1 to 10. We determined the most likely number of clusters (K) using the *ad hoc* ΔK statistic method proposed by Evanno, Regnaut & Goudet (2005) (see Fig. S2). To deal with label switching and multimodality issues in this analysis we used the software CLUMPP 1.1.2 (Jakobsson & Rosenberg, 2007). To evaluate population structure, we also conducted a principal coordinates analysis (PCoA), which was implemented in the ADE-4 package (Thioulouse *et al.*, 1997).

To further evaluate the evidence of putative hybrid individuals between *C. renggeri* and *C. rufipes*, we performed a different Bayesian approach implemented in the software NEWHYBRIDS (Anderson & Thompson, 2002). The six genotype classes investigated were pure parent A, pure parent B, F1 progeny, F2 progeny, backcrosses with parent A, and backcrosses with parent B. We carried out the analyses independently, each one with 5.0×10^5 iterations of MCMC chains after 5.0×10^5 burn-in steps considering Jeffrey-type and uniform distribution priors for every combination of the parameters π and θ .

COI AMPLIFICATION AND ANALYSES

For analyses of mitochondrial gene, we used a subset of the individuals collected in the cerrado at Mogi-Guaçu – one worker from six and seven different colonies of *C. rufipes* and *C. renggeri*, respectively. Furthermore, in order to evaluate if the patterns of divergence between *C. renggeri* and *C. rufipes* found in the field are consistent throughout the geographical distributions of both species, we also included specimens from eight other localities (see Table S1, Fig. S1). In such cases only one individual per locality was available, precluding microsatellite analyses based on allele frequencies (Hale *et al.*, 2012) for these individuals, which were only considered for the COI marker. For PCR amplifications, we used the universal primers LCO1490 (5'-GGTCAACAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACC AAAAATCA-3') (Folmer *et al.*, 1994) with the following thermocycling conditions: 94 °C for 5 min; 40× (94 °C for 30 s, 45 °C for 1 min, 72 °C for 1 min 30 s); and 72 °C for 1 min 30 s. Amplification products (672 bp) were purified and double-sequenced using the primers mentioned above with BigDye Terminator v. 3.1 (Life Technologies) in a ABI3500 automated sequencer (Applied Biosystems). We edited the sequences using the software ChromasPro (Technelysium Pty Ltd) and aligned them with MUSCLE (Edgar, 2004) in MEGA v. 6.0 (Tamura *et al.*, 2013). The sequences were deposited in GenBank (see accession numbers in Table S1).

We determined the pairwise genetic distances within and between *C. renggeri* and *C. rufipes* using the Kimura two-parameter (K2P) nucleotide substitution model (Kimura, 1980). The inferred divergences were used to reconstruct a neighbour-joining (NJ) tree with MEGA v. 6.0 (Tamura *et al.*, 2013), considering only unique haplotypes. We estimated branch supports by bootstrapping 10 000 replicates. To further comprehend the genetic relationship amongst individuals, we also performed a median-joining (MJ) network analysis (Bandelt, Forster & Röhl, 1999) using PopART 1.0 (<http://popart.otago.ac.nz/index.shtml>). The haplotype (*h*) and nucleotide diversity (π) were calculated using DNAsp 5.1 (Librado & Rozas, 2009).

RESULTS

NATURAL HISTORY AND ECOLOGY

In our study area, nests of *C. renggeri* were found both in cerrado *sensu stricto* (22% of the nests) and cerrado (78%), whereas *C. rufipes* was only observed in cerrado *sensu stricto*. The nesting habits of *C. renggeri* and *C. rufipes* colonies differed significantly with respect to the structure and building materials ($G = 37.62$, $P < 0.0001$; Fig. 3). Whereas *C. renggeri* colonies were found in three different categories of nests (underground, fallen dead trunk, and erect dead trunk), *C. rufipes* commonly constructed two additional, distinctive types of nests of dry straw (Fig. 3).

Nest persistence through time differed between the two species according to the season. In the hot/rainy period (from December to April), nests of *C. rufipes* were more persistent (71% remained in original site) than those of *C. renggeri* (41%) ($G = 9.16$, $P = 0.002$). In the cold/dry season (from April to August), however, nest residence time did not differ significantly between nests of *C. rufipes* (61%) and *C. renggeri* (39%) ($G = 3.04$, $P = 0.081$).

The two species also differed in the distribution pattern of their nests, irrespective of the period of the year. *Camponotus renggeri* nests were randomly distributed in December ($R = 0.79$; $P = 0.072$), April ($R = 0.79$; $P = 0.074$) and August ($R = 0.97$; $P = 0.9$). By contrast, nests of *C. rufipes* presented an aggregated distribution in December ($R = 0.42$; $P < 0.0001$), April ($R = 0.44$; $P < 0.0001$), and August ($R = 0.42$; $P < 0.0001$).

The percentage of ants foraging on the ground or vegetation was similar in *C. renggeri* and *C. rufipes*, regardless of the season. In the hot/rainy season, 70 to 90% of the workers per colony of both species foraged on the ground ($U = 5.5$; $P = 0.47$; $N = 4$), whereas in the cold/dry season this percentage ranged from 70 to 100% in both *C. renggeri* and *C. rufipes* ($U = 2.0$; $P = 0.08$; $N = 4$).

The number of individuals in excavated colonies ranged from 105 to 340 workers and one to seven

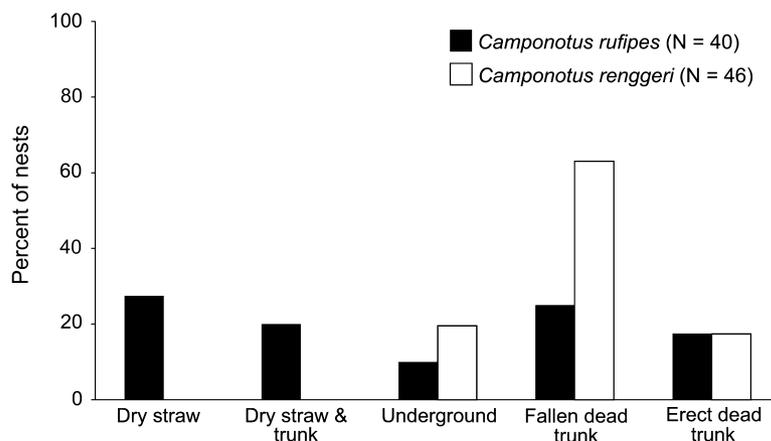


Figure 3. Frequency distribution of nest categories in *Camponotus renggeri* and *Camponotus rufipes* in the cerrado reserve at Mogi-Guaçu, Brazil. The species differed in the structure and building materials used for nesting.

dealted queens for *C. renggeri* (215.25 ± 96.58 , mean \pm SD, $N = 4$), and from 251 to 3654 workers and one to two dealted queens for *C. rufipes* (1410.75 ± 1524.22 , mean \pm SD, $N = 4$).

MICROSATELLITE ANALYSES

Regarding the STRUCTURE analysis, the most likely number of groups is $K = 2$; that is, yellow legs and shiny integument (*C. renggeri*) and red legs and matte integument (*C. rufipes*) clustered separately (Fig. 4A, see also Fig. S2). Similarly, the PCoA retained 40.13 and 19.83% of the total variance in the first and the second axes, respectively, consistently indicating that individuals are genetically more similar within the two putative species than between them (Fig. 4C). The PCoA also indicated a genetic structure within *C. rufipes*, whereas for *C. renggeri* the genetic diversity seemed to be more homogeneously distributed across the sampling site.

We did not detect any evidence of hybrids between *C. renggeri* and *C. rufipes*, and the NEWHYBRIDS analysis indicated a very similar pattern to that found using STRUCTURE (Fig. 4B). Thus, there is no evidence for hybridization or backcrossing, indicating that individuals are all 'pure' *C. renggeri* or *C. rufipes*.

COI ANALYSES

Within *C. renggeri* the average K2P distance was 0.27% (range: 0–0.6%), whereas within *C. rufipes* this average was 2.21% (range: 0–6.9%). The mean distance between *C. renggeri* and *C. rufipes* was 13.93% (range: 12.8–15.2%; Fig. 5). Therefore, the genetic distances between individuals from the two *Camponotus* species were consistently higher than between individuals within each species, regardless of the locality (Fig. 6, see also Table S1, Fig. S1). The NJ analysis considering these

divergence values resulted in two monophyletic clades with strong bootstrap support, corresponding with the differentiation of the individuals into two species (*C. renggeri*, bootstrap = 100 and *C. rufipes*, excluding the haplotype H8, bootstrap = 99, Fig. 6A).

In agreement with NJ, the haplotype network also unveiled a clear differentiation between the two species: amongst the 13 haplotypes identified, there is no haplotype shared by both species, which are separated by a minimum of 78 mutational steps (Fig. 6B). In *C. renggeri*, we did not find any haplotypes not sampled in our study, and the mutational steps amongst unique sequences did not exceed three steps. By contrast, haplotypes not sampled were found in *C. rufipes* and the divergence amongst the haplotypes was higher than in *C. renggeri*, indicating that *C. rufipes* has higher genetic diversity (Fig. 6B). This was confirmed by the lower values of h and π estimated for *C. renggeri* (0.803 and 0.00176, respectively) compared with those estimated for *C. rufipes* (0.848 and 0.02079). Thus, our COI results indicate that the genetic divergence pattern found in Mogi-Guaçu for the two species persists even with the inclusion of samples from eight other localities 66 to 1335 km distant from the study area (see Fig. S1).

DISCUSSION

This study combined behavioural, ecological, and molecular data to confirm that *C. renggeri* and *C. rufipes* are different species. The results complement the main morphological traits (colour of legs and brightness of integument; Fig. 1) designated by Hashmi (1973) to delimit these two species, and do not support the suggestion that they should be merged into a single species (Mackay, 2004). Although variation in body and leg colour admittedly makes the two species difficult to

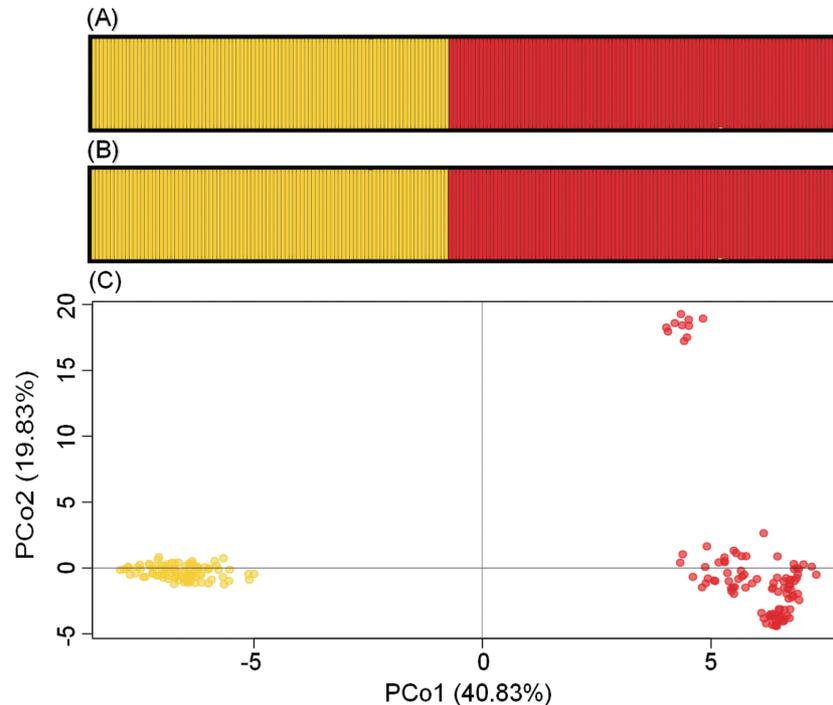


Figure 4. Genetic structure analyses of *Camponotus renggeri* (yellow) and *Camponotus rufipes* (red) workers from Mogi-Guaçu (Brazil), using microsatellites. A, model-based assignment of individuals to the most likely number of clusters ($K = 2$) using STRUCTURE software. B, model-based assignment of individuals to different classes of hybrids or ‘pure’ species. Each individual is represented by a vertical line and the colours indicate the probability of the individual being assigned to a group in (A), or a hybrid or ‘pure species’ class in (B). C, scatterplot of the model-free principal coordinates analysis considering the two first principal coordinates (PCo1 and 2).

differentiate in the field (worsened in alcohol-preserved specimens), our study using natural history and molecular data reinforces the importance of an integrative approach for the correct delimitation of species that closely resemble each other, corroborating a current trend in ant systematics (e.g. Bernasconi *et al.*, 2010; Schlick-Steiner *et al.*, 2010; Seppä *et al.*, 2011; Schmidt & Shattuck, 2014). According to Brown (2000: 70), a character as applied to biosystematics “is any trait of use in making a comparison”. As such, behavioural and ecological traits can be helpful in assisting ant ecologists in species-level taxonomy, especially in problematic genera (e.g. *Camponotus*, *Pheidole*, and *Crematogaster*), in which clear demarcation of species based on morphology alone can be difficult (see Ward, 2010). For instance, marked differences in nesting habits in closely related camponotine ants – e.g., *Camponotus* (current study) and *Polyrhachis* (Liefke *et al.*, 1998) – have proven to be important traits for species separation in the field. Ultimately, the ways in which ant populations exploit the available resources in their habitat, including where and how to construct a nest, are intrinsic species-specific traits that directly contribute to niche differentiation, and that can help us

understand species coexistence within ant communities (Blüthgen & Feldhaar, 2010).

NATURAL HISTORY DATA

Environmental conditions such as temperature, moisture, and vegetation structure can mediate choice of nesting site in ants (Blüthgen & Feldhaar, 2010; McGlynn, 2012, and included references). In our study area, all nests of *C. rufipes* were located in cerrado *sensu stricto* (open canopy) whereas *C. renggeri* nested more frequently in cerradão (dense canopy), indicating a possible differential habitat preference by the two species (Fig. 2). The types of nests built by *Camponotus* species are in general variable, and in cerrado savannah the occurrence of this genus can be affected by the availability of fallen trunks in the understory, plant density and richness, and the occurrence of plant and insect exudates (which are food sources) on foliage (Morais & Benson, 1988; Ribas *et al.*, 2003; Schoederer *et al.*, 2010). Although both *C. renggeri* and *C. rufipes* use trunks as nesting sites, the latter species commonly builds distinctive nests of dry straw (Fig. 3; see also Mackay, 2004). Animal-built structures can be

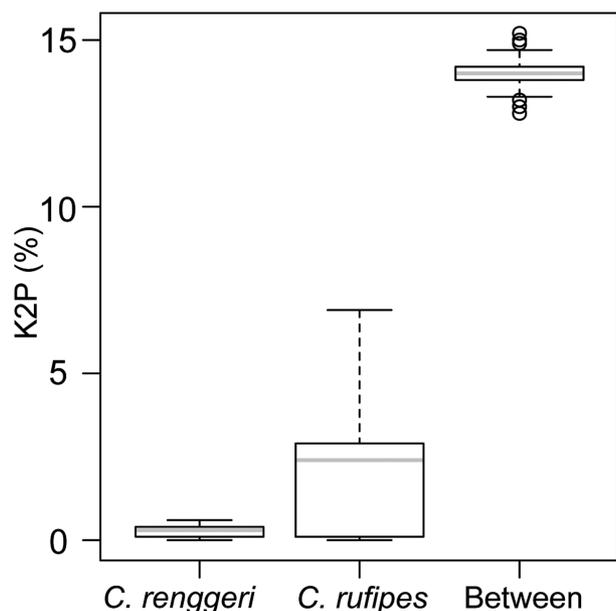


Figure 5. Box plots of Kimura two-parameter (K2P) distance of 672 bp cytochrome *c* oxidase subunit I sequences within and between *Camponotus renggeri* and *Camponotus rufipes*. Boxes indicate interquartile range (upper line, quartile 3; lower line, quartile 1). Horizontal lines with boxes indicate median and whiskers the minimum and the maximum values. Outliers are shown as individual circles.

regarded as extended phenotypes (Turner, 2002); nest construction and architecture in ants are important behavioural traits resulting from the innate cooperative behaviour of colony members, and represent species-typical extended phenotypes (Hölldobler & Wilson, 2008).

Residence time of ant colonies can be associated with biotic and abiotic factors such as competition, natural enemies, prey availability, environmental fluctuations, or even nest type (e.g. Leal & Oliveira, 1995; Blüthgen & Feldhaar, 2010; McGlynn, 2012; Moyano & Feener, 2014). Ant species that have simple or fragile nests, which require a low investment to construct, tend to relocate their colonies more frequently than species with more complex nests (Hölldobler & Wilson, 2008; McGlynn, 2012). In our study site, colonies of *C. renggeri* nested preferentially in hollowed-out, decaying fallen trunks (63% of the nests; Fig. 3), which probably accounted for the lower persistence of their nests during the hot/rainy season compared with the more stable nests of *C. rufipes*. Indeed, in a previous study we observed in the field that mechanical damage to decaying trunks containing *C. renggeri* colonies led to massive outflows of workers carrying broods (Ronque, 2013). It is thus possible that *C. renggeri* nests suffered destruction during summer storms, causing relocations or deaths of colonies in this period. Different nest distribution patterns in *C. renggeri* (random) and *C. rufipes*

(aggregated) further enhance the distinctiveness of these two species. The aggregated pattern in *C. rufipes* is apparently related to a polydomous habit, which is reinforced by field observations of ant traffic between neighbouring nests (see Matta, Morini & Hilsdorf, 2013; Ronque, 2013). Additional investigation is clearly needed to properly assess the factors mediating temporal and spatial patterns in the nesting biology of these two species in cerrado savannah.

Foragers of *C. renggeri* and *C. rufipes* were seen searching for food on soil and vegetation in the area of cerrado *sensu stricto*, with both species showing similar intensities of foraging activity in both substrates. Indeed, these ants commonly feed on plant and insect exudates on cerrado foliage (Sendoya & Oliveira, 2015) and are also seen on the ground feeding on arthropod prey and fallen fleshy fruits (Silvestre *et al.*, 2003; Christianini *et al.*, 2012). However, when tending honeydew-producing treehoppers, *C. rufipes* extends its activities throughout the day whereas *C. renggeri* remains mainly nocturnal (Del-Claro & Oliveira, 1999). Excavated colonies of *C. renggeri* and *C. rufipes* in the current study presented numbers of workers within the range reported for other species in the genus (Hölldobler & Wilson, 1990); *C. rufipes* apparently has larger colonies but more samples are needed for more accurate comparison with *C. renggeri*. The occurrence of more than one queen in some of the colonies in the present study suggests facultative polygyny in the two species, a trait associated with polydomous and ephemeral nests in this genus (Hölldobler & Wilson, 1990).

MOLECULAR AND GENETIC DATA

Our molecular data were concordant with the species' behavioural and ecological data in consistently showing that, regardless of the source of genetic variation (nuclear or mitochondrial), the uppermost hierarchical level of divergence is observed between species. The model-based and multivariate microsatellite analyses, and divergence based on the mitochondrial *COI* gene, in addition to the absence of interspecific hybrids, allowed us to differentiate unambiguously (in genetic terms) the individuals morphologically identified as *C. renggeri* and *C. rufipes* as two independent evolutionary lineages (Figs 4–6). The combination of microsatellites and partial *COI* sequences has also been used to delimit ant species from other groups. For instance, Bernasconi *et al.* (2010) validated *Formica lugubris* Zetterstedt and *Formica paralugubris* Seifert as two different species using different molecular markers. This differentiation, however, was not confirmed for *Formica fusca* Linnaeus and *Formica lemani* Bondroit, in which the mtDNA marker data satisfactorily separated these species whereas the allozyme

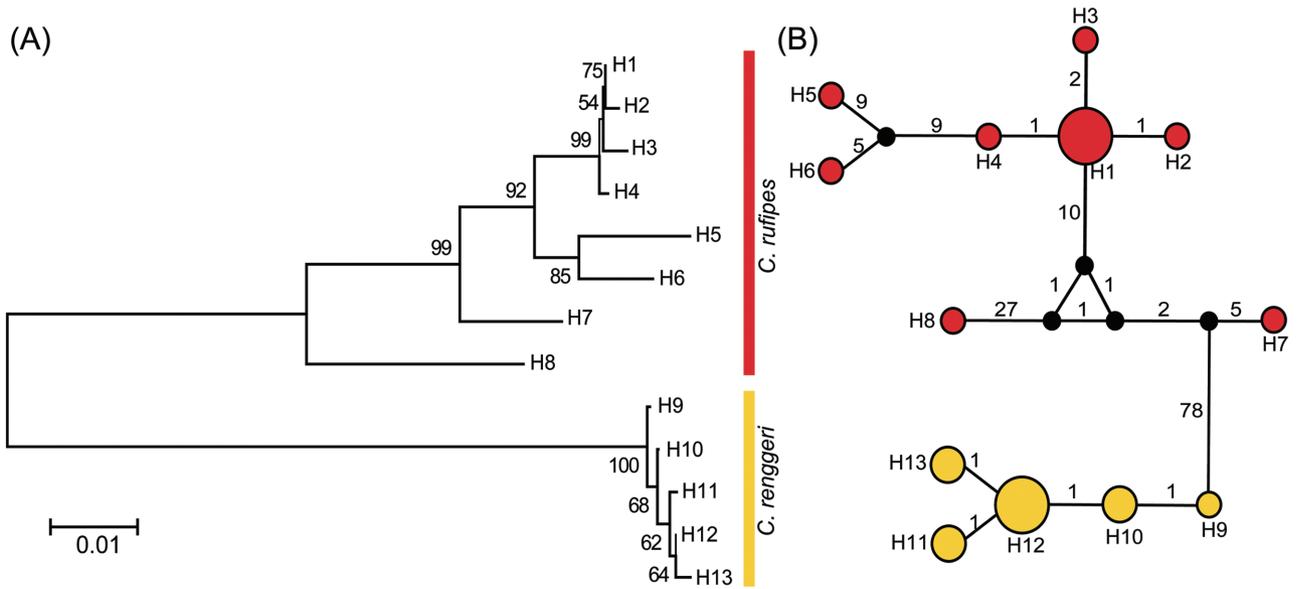


Figure 6. Analyses of the cytochrome *c* oxidase subunit I haplotypes of *Camponotus renggeri* (yellow) and *Camponotus rufipes* (red). A, neighbour-joining tree constructed with Kimura two-parameter distances between *C. renggeri* and *C. rufipes* with bootstrap support values based on 10 000 replications indicated on each branch. B, median-joining network amongst the obtained haplotypes. Values on the branches represent the numbers of mutational steps distinguishing the haplotypes, represented as circles whose areas are proportional to the number of individuals with that haplotype.

and microsatellite data did not, revealing possible male-based hybridization (Seppä *et al.*, 2011). By contrast, Steiner *et al.* (2006) refuted the hypothesis that the host ant *Myrmica rubra* (Linnaeus) and its parasite, *Myrmica microrubra* Seifert, are distinct species by confirming with combined molecular data the shared gene pool between the host and the parasitic ants.

Our analyses based on mtDNA, which also included samples 66 to 1335 km away from Mogi-Guaçu, revealed a strong differentiation of 13.93% between the *COI* sequences of *C. renggeri* and *C. rufipes*, as expected for Hymenoptera, in which the *COI* sequence divergence generally ranges from 8 to 16% amongst species (Hebert, Ratnasingham & Waard, 2003). Specifically for ants, Smith, Fisher & Hebert (2005) suggested that a threshold ranging from 2 to 3% of genetic divergence is appropriate for ant specimen identification or species discovery, considering *COI* as an animal barcode. We found the mean distance between *C. renggeri* and *C. rufipes* to be within this range. That is, all individuals identified as belonging to the same species had at least 97% similarity in their *COI* nucleotide composition, except for the haplotype H8 of *C. rufipes*, the discrepancy in which could be the result of phylogeographical factors and processes affecting the population sampled. Our results indicate that *C. rufipes* has higher genetic variation, which is also more conspicuously structured when compared

with *C. renggeri* (Figs 4C, 6). This difference may be interpreted as another important feature separating these species into different biological entities and is also in accordance with the species' natural history in terms of nest spatial distribution and persistence through time. In Mogi-Guaçu, nest relocation by *C. renggeri* occurred in the hot/rainy season, when nuptial flights occur (Ronque, 2013). This behaviour over generations could promote increased gene flow, minimizing genetic differentiation amongst colonies (McGlynn, 2012).

To summarize, we hope that the integrated approach employed in our study can also prove useful for researchers facing similar problems in delimiting ant species that closely resemble one another. By combining available morphological distinctive traits (Hashmi, 1973) with natural history (habitat preference, nesting biology) and molecular data (nuclear and mitochondrial markers), we were able to confirm that *C. renggeri* and *C. rufipes* are valid species that can be separated relatively well in our study area. Although *C. renggeri* and *C. rufipes* may also occur in other types of vegetation in Brazil (e.g. Amazon and Atlantic rain forests, Pantanal floodplains), our study with sympatric populations strongly suggests a differential habitat preference between the two species in the cerrado landscape (although ecological differences can also be subject to geographical variation). Data on mitochondrial markers from eight additional localities further confirmed that

the substantial genetic divergence between *C. renggeri* and *C. rufipes* is consistent along the species' distributions in Brazil (Table S1, Fig. S1). Our work was motivated by the difficulties experienced by ant ecologists in delimiting the many *Camponotus* species found in the ant-rich cerrado savannah. Given the relevance of the cerrados as a biodiversity hotspot (Myers *et al.*, 2000) and the diverse interspecific interactions involving ants in this ecosystem (Silvestre *et al.*, 2003; Oliveira & Freitas, 2004; Christianini *et al.*, 2012), we hope that our study can facilitate species delimitation between *C. renggeri* and *C. rufipes* by field biologists, and also stimulate further multidisciplinary ant research in this currently threatened biome.

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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:

Figure S1. Map of South America showing the distribution of cerrado savannah (shaded area) in Brazil. The study area, Mogi-Guaçu, is shown in green. Additional samples for cytochrome *c* oxidase subunit I analyses of *Camponotus renggeri* and *Camponotus rufipes* were collected in eight localities, indicated by the black dots. See also Table S1.

Figure S2. The most probable number of clusters (K) based on the *ad hoc* ΔK statistics method proposed by Evanno *et al.* (2005), following the admixture model in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). We analysed the genotypes of 94 *Camponotus renggeri* and 104 *Camponotus rufipes* workers from Mogi-Guaçu for 28 polymorphic microsatellite loci.

Table S1. Specimens of *Camponotus renggeri* and *Camponotus rufipes* sampled for cytochrome *c* oxidase subunit I analyses, with localities (and their geographical reference), corresponding haplotype, and GenBank accession number. Distance (km) of each locality from our study area at Mogi-Guaçu: Serra do Caparaó (584), Itirapina (66), Chapada dos Veadeiros (913), Jalapão (1335), Serra do Cipó (487), Serra do Japi (105), Campinas (70), Brasília (750). See also Figure S1.