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"Cytochrome c oxidase I DNA sequence of *Camponotus* ants with different nesting strategies is a tool for distinguishing between morphologically similar species"

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Abstract The great diversity of *Camponotus*, high levels of geographic, intraspecific and morphological variation common to most species of this genus make the determination of the interspecific limits of *Camponotus* a complex task. The Cytochrome c oxidase 1 (COI) gene was sequenced in this study to serve as an auxiliary tool in the identification of two taxa of *Camponotus* thought to be morphologically similar. Additionally, characteristics related to nesting were described. Five to fifteen workers from twenty-one colonies were analyzed, collected from twigs scattered in the leaf litter and from trees located in different regions of Brazil. Phylogenetic reconstructions, haplotype network, and nesting strategies confirmed the existence of two species and that they correspond to *Camponotus senex* and *Camponotus textor*. Our results emphasize that the COI can be used as an additional tool for the identification of morphologically similar *Camponotus* species.

Keywords Atlantic forest · Brazilian savannah · Leaf litter · Tree vegetation · Twigs

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Introduction

Camponotus Mayr 1861 is cosmopolitan, and it is currently the most diverse genus of ants with more than 1000 described species, subspecies and varieties (Bolton 2015). The genus is not monophyletic (Brady et al. 2000), and the high degree of variation in morphological characters makes taxonomy extremely complex and challenging (Garcia et al. 2013).

Camponotus ants have a preferentially nocturnal habit and generalist diet (Yamamoto and Del-Claro 2008). They may exhibit mutualism with cochineals, aphids and leafhoppers and feed on extrafloral nectaries (Hölldobler and Wilson 1990). Some species prey on herbivorous insects (Del-Claro et al. 1996) and honeybees (Akre and Hansen 1990), and they may destroy wooden frameworks and electrical installations in urban environments (Bueno and Campos-Farinha 1999).

Camponotus species live in a variety of habitats and microhabitats; colonies may be polygynous, and nests are built in interstices or on the ground, in twigs or rotten twigs (Fernandes et al. 2012; Souza et al. 2012; Matta et al. 2013). They can nest in trees using silk produced by the larvae to weave nests (Santos and Del-Claro 2009). Many species build secondary or satellite nests in different environments (Bueno and Campos-Farinha 1999; Matta et al. 2013). This feature is another challenge to the biological characterization of the species because these nests are related to different functions (Lanan et al. 2011).

In Brazil, the genus is recorded in a wide variety of native vegetation, including the Cerrado (Brazilian savannah) (Oliveira and Brandão 1991; Del-Claro and Oliveira 2000), the Brazilian Atlantic Domain (Delabie et al. 2000; Mentone et al. 2011; Suguituru et al. 2013), the Amazon Rainforest (Vasconcelos and Delabie 2000) and the Caatinga (tropical dry forest) (Ulysséa and Brandão 2013), as well as in agricultural areas (Marinho et al. 2002). However, the great morphological similarity among many species of the genus immensely complicates their accurate identification (Ronque et al. 2015); thus, many of the studies which refer to species of *Camponotus* are limited to listing morphotypes or reasonably distinguishable forms.

C. senex (Smith 1858) and *C. textor* (Forel 1899) are examples of morphologically similar species. Smith (1858), when describing *C. senex* from material from Brazil, did not mention the use of silk for nest building. Forel (1879) studied specimens derived from the Cordoba, Mexico, collection and noted the morphological similarity with *C. senex* and the presence of silk in the nest. In 1899, Forel described specimens from Costa Rica, including *C. senex textor*, and mentioned the presence of silk. However, in 1905, after analyzing specimens from Brazil, the author concluded that they corresponded to *C. senex* and reported that the nests were built with silk. Wheeler (1915) perpetuated the association between *C. senex* and nests that included silk and was followed by Wheeler and Wheeler (1953), Schremmer (1979) and Hölldobler and Wilson (1983, 1990). Longino (2006) analyzed the morphology of specimens from Costa Rica and separated *C. senex textor* into *C. senex* and *C. textor*. This author reported that only *C. textor* has larvae that produce silk and describes some ecological aspects and nesting strategies specific to *C. senex* and *C. textor*. Nevertheless, doubts persisted because Robson et al. (2015) recently referred to *C. senex* as having silk-producing larvae when comparing the behavior of this species with that of *Dendromyrmex* during nest building.

Studies have demonstrated the efficiency of the COI method for identifying species (Silva-Brandão et al. 2009), especially those considered morphologically similar (Ronque et al. 2015). The method is based on the fact that, throughout evolutionary history, DNA sequences have accumulated mutations that make them unique to each species (Hebert et al. 2003).

"A high quality faunal inventory and a refined taxonomy are essential prerequisites for understanding the ecological processes of ecosystems" (Silva et al. 2015). Therefore, in this study, we present the results of molecular and phylogenetic analysis of a segment of the mitochondrial gene cytochrome oxidase 1 (COI) as well as a description of biological aspects related to the nesting of two morphologically similar species of *Camponotus*.

Materials and methods

Characterization of collection areas and nests

Nests were collected from trees and twigs scattered on the leaf litter (Fig. 1; Table 1) of fragments of the Atlantic Forest and the Brazilian savannah vegetation (Colombo and Joly 2010) (Fig. 2). The twigs with colonies of *Camponotus* were measured (length and diameter), and the adult and immature (egg + larvae) individuals were counted. The colonies on twigs scattered on the leaf litter were named Group I (GenBank accession code: KT364225–KT364233), and the colonies on canopy trees, Group II (GenBank accession code: KM37004–KM371011).

### DNA extraction and genetic characterization of COI

The worker ants collected were stored in a freezer at −20 °C for DNA extraction. Five workers were analyzed from each colony found on twigs scattered in the leaf litter. For those in trees, fifteen workers were analyzed per colony. The difference in the number of workers subjected to molecular analysis is related to the size of the populations of the colonies on twigs and trees.

Total DNA was extracted after macerating the tissues in a lysis solution (100 mM Tris, pH 9.1, 100 mM NaCl, 50 mM EDTA, 0.5 % SDS). Subsequently, proteinase K was added to the samples, which were then incubated at 55 °C for 3 h. The protein residue was precipitated with 5 M NaCl, and the supernatant was washed with 100 and 70 % alcohol for DNA precipitation. The material was dried for 10 min in a Thermo Savant DNA 120 SpeedVac®, and the DNA was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8).

The mitochondrial gene Cytochrome c oxidase 1 (COI) was amplified using the GoTaq® Flexi DNA Polymerase Kit (Promega, Madison, WI, USA). The specific primers for *Camponotus*, CampR and CampF (Ramalho et al. 2016) were used for COI amplification, since the universal primers did not work. The PCR occurred under the following conditions: 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 48 °C for 1.5 min and 68 °C for 2.5 min, with a final extension at 65 °C for 7 min. The samples were purified with the kits GFX PCR and Gel Band Purification (GE Healthcare) and quantified in a NanoDrop 2000 spectrophotometer (Thermo Scientific). Sequencing reactions used the BigDye Terminator kit (V.3.1) (Applied Biosystems). Amplicons were sequenced in both directions in a 3130 Genetic Analyzer automated sequencer (Applied Biosystems).

The consensus sequences obtained in this work for each colony were edited manually using the program BioEdit

Fig. 1 Arboreal nest (**a**) with workers and larvae on the external (**b**) and internal parts (**c**); *Camponotus* worker leaving a hole on twigs scattered in the leaf litter (**d**)
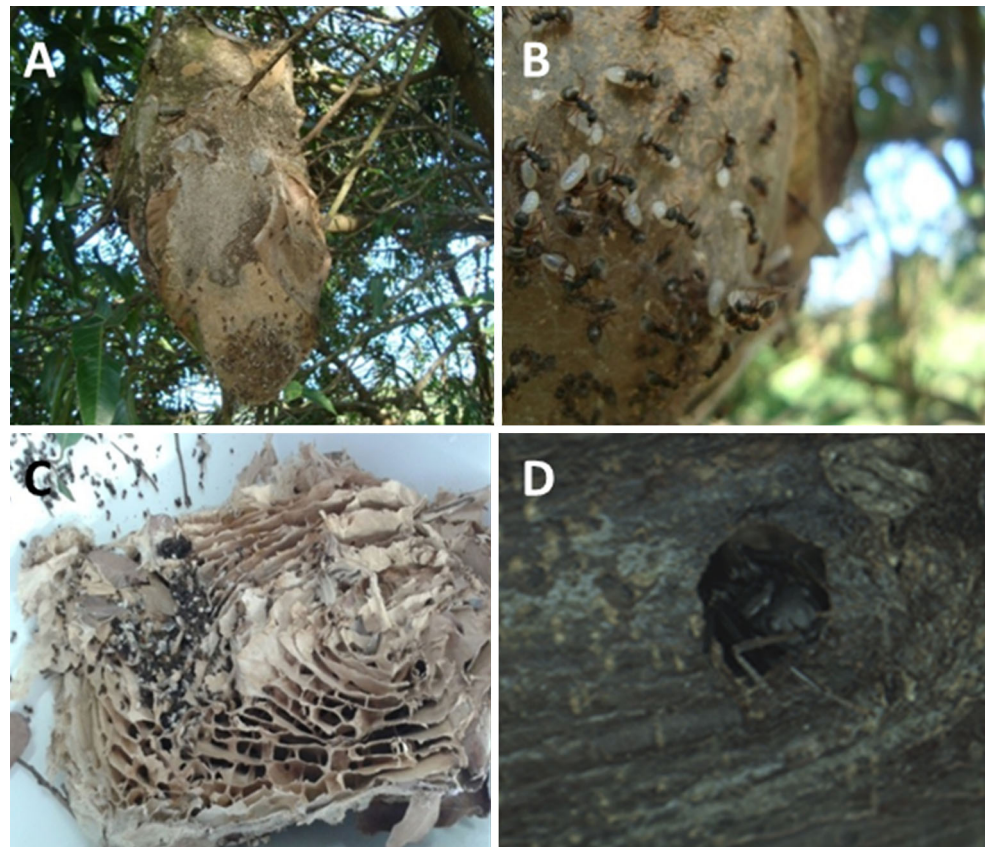
Table 1 Structure and total number of nests according to the collection localities

Nest		City/State
Structure	Total number	
In hollow galleries of twigs scattered in the leaf litter. (Group I)	1	São Paulo/São Paulo
	4	Igaratá/São Paulo
	2	Itaquaquecetuba/São Paulo
	2	Suzano/São Paulo
In trees. The nest is made up of leaves intertwined with silk. (Group II)	1	Araraquara/São Paulo
	1	Ilhéus/Bahia
	1	Ribeirão Preto/São Paulo
	1	Rio Claro/São Paulo
	1	São João da Boa Vista/São Paulo
	1	Santa Rita do Passa Quatro/São Paulo
	2	Uberlândia/Minas gerais

Sequence Alignment Editor (Hall 1999) and aligned with the Clustal W tool (Thompson et al. 1994). Consensus sequences were confirmed as coding sequences using the ORF Finder (Open Reading Frame Finder) of the NCBI (http://www.ncbi.nlm.nih.gov/) and compared with sequences deposited in GenBank using BLASTn. Taxa with ≥85 % similarity to our consensus sequences were selected as outgroups for phylogenetic reconstructions.

Phylogenetic analyses of maximum parsimony were performed using the software PAUP 4.0 (Swofford 1998) with heuristic search parameters and 1000 bootstrap replicates. The TN93 + L sequence evolution model was chosen as the best model for maximum likelihood analysis under the Akaike Information Criterion. The analysis was performed in MEGA 6.6 (Tamura et al. 2013) using nearest-neighbor interchange (NNI) and 1000 bootstrap
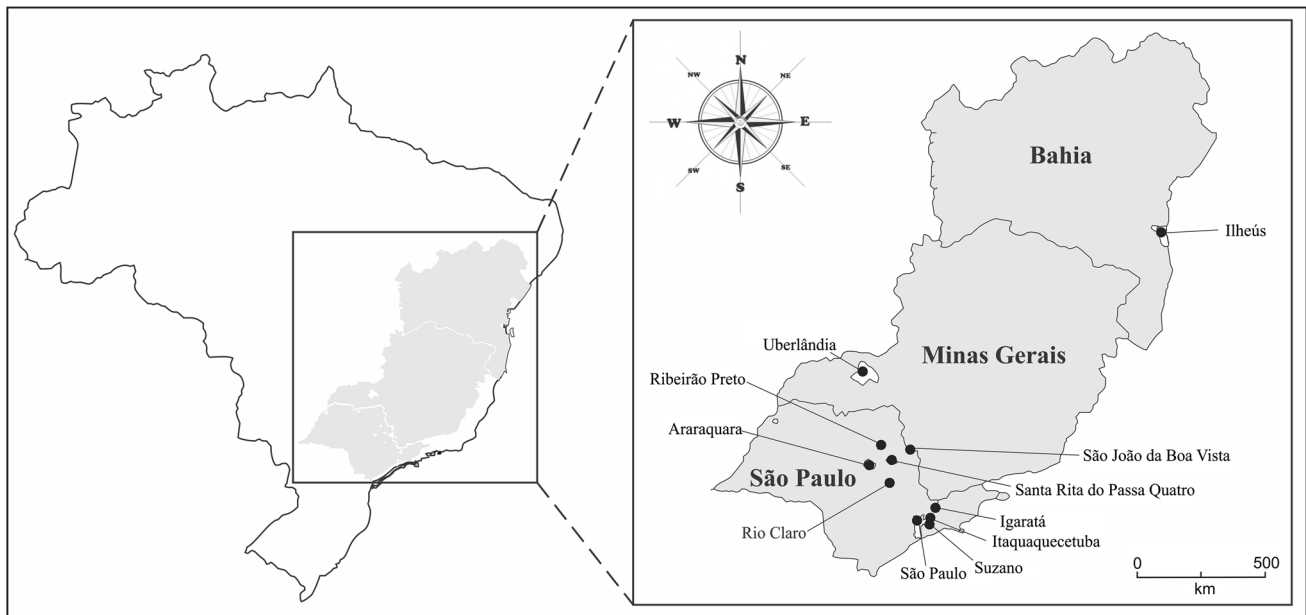
Fig. 2 Geographical location of the collection areas of *Camponotus* nests in three Brazilian states

replicates. The genetic distance of worker ants from *Camponotus* colonies was calculated using the APE package (Paradis et al. 2004) under the Kimura 2-parameter (K2P) (Kimura 1980) and the rgdal package (Bivand et al. 2013) of the software R (R Core Team 2015). The haplotype network was generated with the median-joining method using Network 4510 (Bandelt et al. 1999).

The all specimens used (belonging to the group I and group II; Table 1) were identified according to Longino (2006), and confirmed by Dr. Rodrigo Machado Feitosa (Federal University of Paraná) and Dr. Jacques Hubert Charles Delabie (State University of Santa Cruz and Cocoa Research Center). The specimens are deposited in the reference collection at the University of Mogi das Cruzes (see Suguituru et al. 2015) and the Executive Committee of the Cocoa Farming Plan (Comissão Executiva do Plano da Lavoura Cacaueira-CEPLAC) collection.
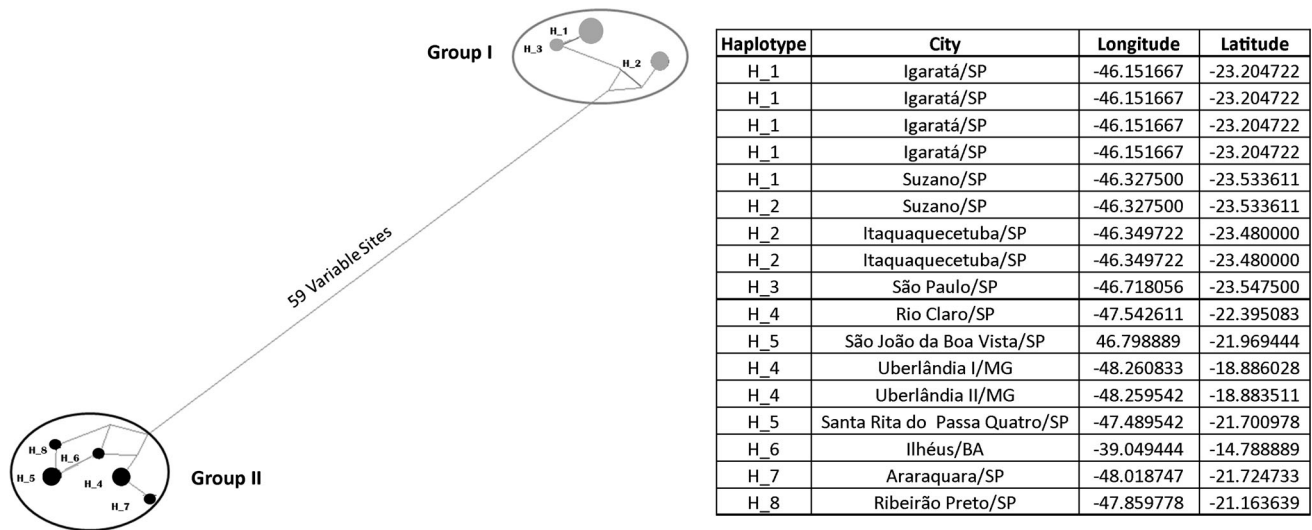
Results

The consensus sequences in Group I were found in twigs within fragments of native Atlantic Forest vegetation present in urban parks (Table 1; Fig. 1d). The twigs measured from 11.05 ± 0.45 to 35.99 ± 4.77 mm in diameter and 35 to 22.3 cm in length. The colonies had 12 to 138 workers and 3 to 73 immature individuals. Nests from Group II were suspended in trees (Table 1; Fig. 1a) located at the edge of native Atlantic Forest or savannah vegetation fragments. The number of workers and/or immature individuals

exceeded 100 (Fig. 1b, c). These our results add new information to the literature on nest characteristics.

Consensus sequences fragments were obtained for 464 bp of the COI gene. Three different haplotypes were present in Group I and five in Group II; there were 59 polymorphic sites between the both groups (12.71 %), yielding the haplotype network (Fig. 3). The haplotype diversity (*h*) was 0.639 and 0.857 for Group I and Group II, respectively. In Group I, the haplotype H_1 grouped workers from the cities of Suzano and Igaratá, H_2 grouped workers coming from the municipalities of Itaquaquecetuba and Suzano and H_3 had workers from the city of São Paulo (Table 2). In Group II, we found five distinct haplotypes. The haplotype H_4 grouped consensus sequences of workers from Rio Claro and Uberlândia; the H_5 haplotype grouped those from São João da Boa Vista and Santa Rita do Passa Quatro; and haplotypes H_6, H_7 and H_8 represented the workers from Ilhéus, Araraquara and Ribeirão Preto, respectively (Table 2).

For phylogenetic reconstruction, the following GenBank sequences were selected to form the outgroup: *C. castaneus* (Latreille 1802); *C. americanus* (Mayr 1862); and *C. quadrinotatus* (Forel 1886). *Oecophylla smaragdina* (Fabricius 1775) was added because it also belongs to the subfamily Formicinae and shows weaving behavior. *Odontomachus laticeps* (Roger 1861) has plesiomorphic characteristics compared with the other ants (see Moreau et al. 2006). The phylogenetic hypotheses obtained (Maximum Likelihood and Maximum Parsimony, Fig. 4a, b, respectively) show, with high support values, both groups

Haplotype	City	Longitude	Latitude
H_1	Igaratá/SP	-46.151667	-23.204722
H_1	Igaratá/SP	-46.151667	-23.204722
H_1	Igaratá/SP	-46.151667	-23.204722
H_1	Igaratá/SP	-46.151667	-23.204722
H_1	Suzano/SP	-46.327500	-23.533611
H_2	Suzano/SP	-46.327500	-23.533611
H_2	Itaquaquecetuba/SP	-46.349722	-23.480000
H_2	Itaquaquecetuba/SP	-46.349722	-23.480000
H_3	São Paulo/SP	-46.718056	-23.547500
H_4	Rio Claro/SP	-47.542611	-22.395083
H_5	São João da Boa Vista/SP	46.798889	-21.969444
H_4	Uberlândia I/MG	-48.260833	-18.886028
H_4	Uberlândia II/MG	-48.259542	-18.883511
H_5	Santa Rita do Passa Quatro/SP	-47.489542	-21.700978
H_6	Ilhéus/BA	-39.049444	-14.788889
H_7	Araraquara/SP	-48.018747	-21.724733
H_8	Ribeirão Preto/SP	-47.859778	-21.163639

Fig. 3 Haplotype network analysis of workers that nest on twigs (Group I-*gray* circle) and trees (Group II-*black* circle). (Color figure online)

Table 2 Pairwise comparison of p-distances between haplotypes of *Camponotus* with different nesting habits. H_1 to H_3 of *Camponotus* recorded in twigs scattered in the leaf litter, and H_4 to H_8 are haplotypes of *Camponotus* recorded in arboreal nests

	H_1	H_2	H_3	H_4	H_5	H_6	H_7	H_8	
H_1	/////								
H_2	0.010897181	/////							
H_3	0.00216456	0.008699375	/////						
H_4	0.157508047	0.151496404	0.154634476	/////					
H_5	0.160398229	0.154345415	0.157508047	0.006521832	/////				
H_6	0.157508047	0.151496404	0.154634476	0.004338422	0.002164506	/////			
H_7	0.160398229	0.154345415	0.157508047	0.002164506	0.008714818	0.006521832	/////		
H_8	0.157508047	0.151496404	0.154634476	0.008714818	0.002164506	0.004338422	0.010917464	/////	

Legend:
- Intraspecific nucleotide variation of Group I
- Intraspecific nucleotide variation of Group II
- Interspecific nucleotide variation of different nests

as monophyletic and the individuals from Group II most closely related to *C. quadrinotatus*. *C. americanus* and *C. castaneus*, chosen for the outgroup, were also sister groups and thus, despite the relatively low support for the major relationships between these groups of species, we can demonstrate that the colonies of Group I and Group II consist of distinct and monophyletic taxonomic units.

Discussion

The phylogenetic hypothesis that we obtained, associated with the genetic distances and haplotype diversity data, allows us to conclude that the *Camponotus* workers belong to two distinct species. These results, together with observations of the nesting habits of the workers in Groups I and II and the morphological characteristics proposed by Longino (2006), show that the colonies located in twigs scattered in the leaf litter (Group I) belong to *C. senex*. The colonies hanging from trees in nests formed by leaves interspersed with silk (Group II) belong to *C. textor*. These

observations corroborate the description and identification based on morphological characters described by Longino (2006). But, they do not support the suggestion that the two species should be merged into a single one (Mackay 2004). It is important to clarify that this work has not performed extensive phylogenetic study of *Camponotus*, however mitotypes were still grouped in two a monophyletic clade with strong support: Group I (*C. senex*) and Group II (*C. textor*). A large study involving all subfamilies of *Camponotus* and several genes could help reveal the phylogenetic relationships of this diverse group.

Nests of *C. senex* are common in moist environments, from mature forest to anthropogenically disturbed sites (Longino 2006). In this study, we found that *C. senex* nested only in urban parks composed of native forest. Extensive surveys were conducted of twigs scattered in the leaf litter of the Atlantic Forest (Fernandes et al. 2012) and in a *Eucalyptus* forest with developed understory (Souza et al. 2012), and no *C. senex* nests were observed. Nests of *C. textor* were observed in the canopy of tropical forests (Longino 2006), and in our study, we found them in trees
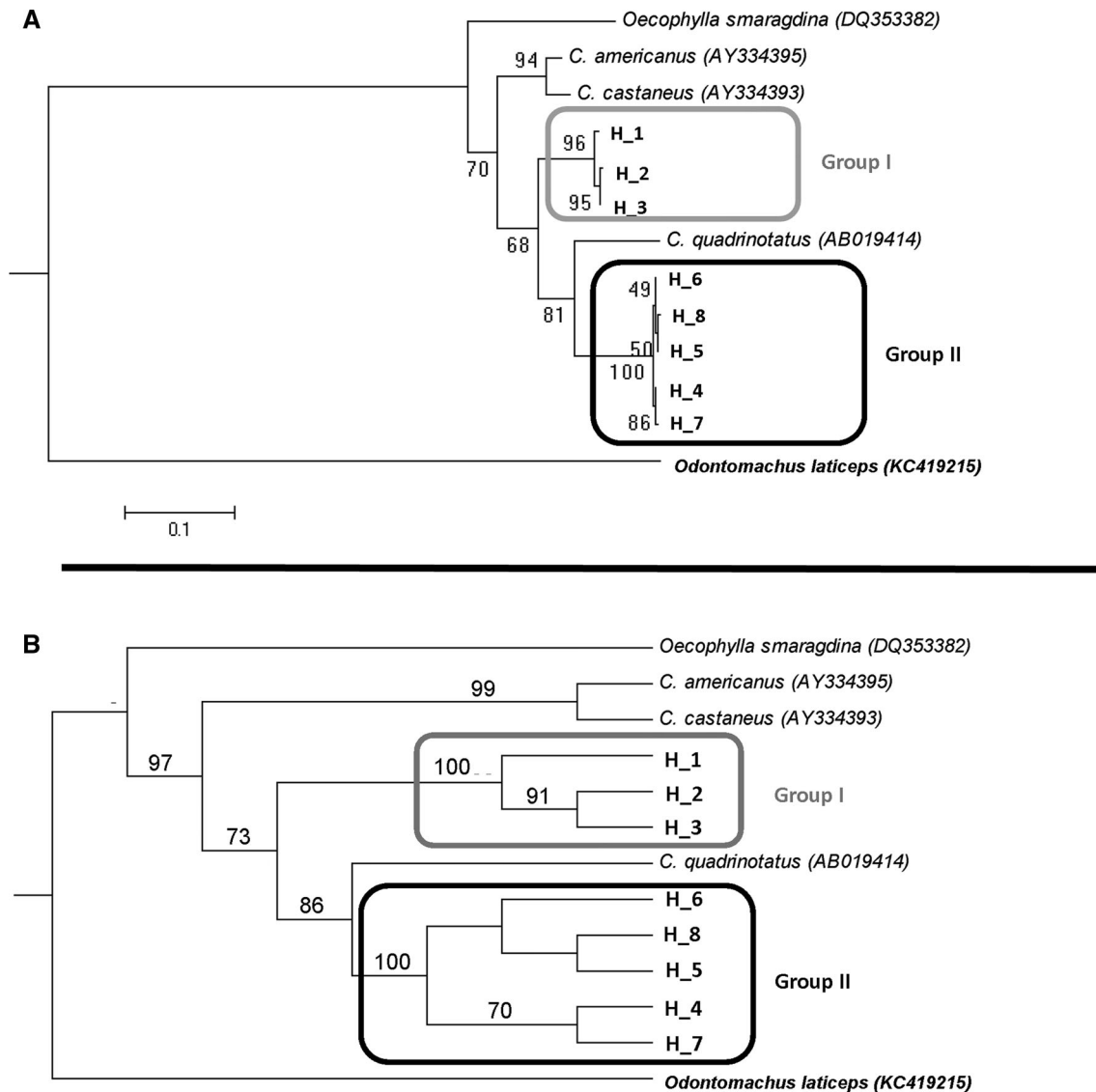
Fig. 4 Phylogenetic analysis with Maximum Likelihood and Maximum Parsimony (both showing boostrap support) of *Camponotus* that nest on twigs dispersed in the litter (Group I) and tree (Group II) obtained using mitochondrial DNA. **a** Single tree inferred under maximum likelihood search shown with branch lengths proportional to estimated divergence with a TN93 + G model of sequence evolution. **b** Cladogram tree with boostrap support values. Sequences obtained from GenBank were added in these analysis

of the Atlantic Forest and savannah vegetation, either anthropogenically disturbed or not. There is also no record of workers of these species foraging on the vegetation of areas located in the Atlantic Domain of the Brazilian Southeast region (Morini et al. 2006; Munhae et al. 2009; Suguituru et al. 2015). The few reports available indicate that there is no habitat overlap between *C. senex* and *C. textor* and this information was not previously described in the literature.

These arboreal species of *Camponotus* differ in the size of their nests; those of *C. senex* are much smaller than those of *C. textor*. The populations found inside twigs scattered in the leaf litter are relatively small because the

space is limited (Hölldobler and Wilson 1990). *C. senex* probably occupies twigs previously drilled by wood-boring insects (Longino 2006; Powell 2013), including *C. renggeri* (Ronque et al. 2015). *Myrmelachista* spp. (Nakano et al. 2012, 2013) and *Pseudomyrmex phyllophilus* (Ketterl et al. 2003) are also arboreal species whose colonies may be found on twigs on the ground of the study areas, but in these cases, the twigs probably housed the colony before falling into the leaf litter.

For many species of arboreal ants, fallen twigs in the leaf litter represent an ephemeral shelter or satellite nests containing workers and immature individuals, and this may be the case for *C. senex*. The existence of satellite nests

increases the chances for territory defense, protection of the host plant and survival of the colony itself, which, when concentrated in one place, is at increased risk of predation (Santos and Del-Claro 2009). This behavior is relatively common among ants (Debout et al. 2007) and may also be the case for *C. textor*. During our expeditions, we observed more than one nest per tree or nests in trees that were very close to each other.

By comparison, twigs scattered in the leaf litter may be considered structurally simpler nests and require low investment by ants to occupy them, and many species of *Camponotus* make use of this type of structure (Ronque et al. 2015; Jiménez-Soto and Philpott 2015). The species quickly occupy these nests (Jiménez-Soto and Philpott 2015) but also move more frequently looking for new places to nest (Byrne 1994; McGlynn 2012). However, this seems not to be the case for *C. textor*, which suspends the nests in trees and constructs them with leaves intertwined with silk, making them comparatively more stable structures.

These biological characteristics associated with the genetic divergence of 12.71 % between *C. senex* and *C. textor* allow us to conclude that they are actually two distinct species. For Hymenoptera, COI sequence divergence generally ranges from 8 to 16 % (Hebert et al. 2003). In addition to interspecific divergence, intraspecific differences were also detected for the two species. *C. textor* shows greater genetic variation than *C. senex*. However, the colonies of *C. senex* were collected from geographically close localities, allowing greater gene flow and reduced genetic variation (McGlynn 2012). Furthermore, these results may be interpreted as support for the molecular separation of the species because, despite the high genetic variation among *C. textor* workers, all the populations studied were securely associated with the same species. The intraspecific divergence was less than 3 %, which, for ants, has been considered insufficient variation for recognizing distinct species (Smith et al. 2005).

Although recent studies have demonstrated the importance of COI in species identification (Ojha et al. 2014; Paknia et al. 2015; Smith et al. 2015), the delimitation of a species is very complex for the information contained in only one gene to be considered (Green 1996). The use of this technique should be associated with environmental, behavioral and morphological characteristics, as demonstrated by Ronque et al. (2015), Darienko et al. (2015) and Gomes et al. (2015). That is, a COI should be used as an additional tool, especially in hyperdiverse groups, including *Camponotus* (Smith et al. 2005). Thus, our results reinforce the assumption that different approaches in the process of delimitation of a species are essential when analyzing in an integrated fashion. We hope that the results presented here will assist in the correct identification of these species of *Camponotus*, especially in biodiversity studies, and encourage new integrative studies in an attempt to delineate potentially problematic taxa.

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