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Intracellular Symbiotic Bacteria of *Camponotus textor*, Forel (Hymenoptera, Formicidae)

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Abstract This study focuses on the weaver ant, *Camponotus textor*, Forel which occurs in some areas of the Brazilian Cerrado and Atlantic Forest, and its symbionts: *Blochmannia*, an obligate symbiont of *Camponotus*, and *Wolbachia*, known for causing reproductive alterations in their hosts. The main goal of this study was to investigate the presence, frequency of occurrence, and diversity of *Wolbachia* and *Blochmannia* strains in *C. textor* colonies. We found high infection rates (100%) and the occurrence of at least two distinct strains of *Blochmannia* (H_1 or H_7) in the same species. The observed haplotype variation within a single species may result from the high mutation rate of the symbiont. Similarly, the *Wolbachia* was found in all colonies with different rates of infections and a new strain (supergroup A) was deposited in the MLST database. The diversity found in the present study shows that there is still much to explore to understand about these symbiotic interactions.

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Introduction

Symbiotic relationships between insects and bacteria occur in virtually all orders, including Hymenoptera, and may be divided into primary (obligate) and secondary (facultative) interactions, both types have been identified in Formicidae [1, 2]. Obligate endosymbionts are the result of an ancient association with the host; they usually live inside specialized cells called bacteriocytes and contribute to ant nutrition. As a result of this association, the bacterial genome, which is vertically transmitted, may shrink [3]. *Blochmannia* is an example of an obligate symbiont that is commonly found in *Camponotus* species in the Northern Hemisphere [4].

In contrast, facultative symbionts are characterized by a more recent association and may be transmitted vertically or horizontally [5]. *Wolbachia*, for example, stands out for interfering in the reproduction of its hosts, but its role in the worker caste of ants is unknown. According to recent estimates, between 20 and 35% of arthropods are infected with *Wolbachia* [6]. *Wolbachia* has a large diversity of strains, which are divided into supergroups (A–F); the strains found in insects belong exclusively to supergroups A and B. Traditionally, *Wolbachia* infections were detected with the *wsp* gene [7]; however, due to its high rate of recombination [8] and strong selection for diversification [9] a multilocus sequence typing (MLST) approach has gained popularity [10].

In studies of intracellular bacteria, the term cospeciation is often used, and these associations between insects (host) and bacteria are well documented in the literature.

Examples include aphids, the tsetse fly, cockroaches, and their respective symbionts [11–13]. In ants, this type of interaction also results in a high degree of congruence between host and symbiont phylogenetic trees, indicating the occurrence of parallel diversification and of maternal transmission of the infection. However, the geographic distribution may only partially reflect the congruence between host and symbiont phylogenetic trees [14].

One of the best known genera of ants for having symbiotic relationships with bacteria is *Camponotus* Mayr, 1861 [15, 16] and in a recent study by Bronw and Wernergreen [17] found that 95–98% of bacteria found in *Camponotus chromaiodes* were *Blochmannia* and *Wolbachia*, but most studies of the microbiota and host are restricted to the Northern Hemisphere.

Camponotus textor, Forel as well as 14 other species of ants distributed around the world, is known as a weaver ant because it uses silk to construct its nests [18]. Although the existing literature suggests that this species is common in the forests of Central and South America, its precise distribution is not fully known [19]. In general, published works describe only behavioral traits, mostly related to nest construction using the silk produced by their larvae [20].

The taxonomy of the species is complicated. *C. textor* is often mistaken for *C. senex* (Smith) due to their morphological similarities. Recently, however, Ramalho et al. [21] distinguished the two species based on molecular data and ecological traits of specimens of both species collected in the Neotropics. Their results corroborate with Longino [22], who considers them as two separate species. Although *C. textor* has elaborate behaviors, little is known about its feeding habits, biology, ecology, and interactions with other organisms [20, 23, 24].

Other few studies involving ants and endosymbionts from the Neotropical region have already shown how diverse these associations are [25, 26]. Since the descriptions of primary and secondary symbionts in *Camponotus* are based on species from the Northern Hemisphere, the goal of this study was to investigate the presence, frequency of occurrence, and strain diversity of *Wolbachia* and *Blochmannia* in *C. textor* colonies, which are ants exclusively Neotropical, and evaluate the diversity of these endosymbionts.

Materials and Methods

Collection, Identification, and Total DNA Extraction

Camponotus textor workers were collected from eight localities in different regions of Brazil with either typical Cerrado or Atlantic Forest vegetation: Rio Claro, SP (22°23′42″ S, 47°32′33″ W), Araraquara, SP (21°43′29″ S, 48°1′7″ W), Ribeirão Preto, SP (21°12′42″ S, 47°48′24″ W), Santa Rita do Passa Quatro, SP (21°42′4″ S, 47°29′23″ W), São João da Boa Vista, SP (21°58′10″ S, 46°47′56″ W), Uberlândia, MG (two colonies: 18°53′10″ S, 48°15′39″ W, and 18°53′1″ S, 48°15′34″ W), and Ilhéus, BA (14°18′45″ S, 39°53′13″ W). The collected material was preserved in 80% ethanol and kept at −20 °C until DNA extraction. Specimens were identified by Dr. Jacques Delabie and deposited in the collection of CEPLAC, Ilhéus, BA (accession number 5692).

Total DNA was extracted from eight individual workers of each colony and preserved in 80% ethanol [19, 27]. We used primers Bloch16S-462F and Bloch16S-1299R [16] to screen for *Blochmannia*. We used primers wsp81f and wsp691r for the initial detection of *Wolbachia* [7, 28] and EF1α-532f and EF1α-610r [29] as positive controls. The amplification was performed using Taq DNA Polymerase, Recombinant (Invitrogen), following the protocol of the manufacturer. We used the thermal cycler parameters recommended by Baldo et al. [30] and Wernegreen et al. [16] to identify *Wolbachia* and *Blochmannia*, respectively.

Purification of the PCR product was performed using the GFX PCR DNA and Gel Band Purification kit (GE Healthcare). Samples were quantified in the Thermo Scientific NanoDrop 2000 (Uniscience) and sequenced using the BigDye Terminator v3.1 reagent kit (Applied Biosystems). Sequence reading was carried out in a 3130 Genetic Analyzer automated sequencer (Applied Biosystems).

Analyses: Blochmannia

After the sequences were edited, multiple *Blochmannia* infections were detected. As a result, cloning with the pGEM-T Vector System I (Promega) was required to isolate each sequence; we followed the protocol provided by the manufacturer. Miniprep followed Zhou et al. [31], and the sequencing reactions were prepared as described before. Samples that have succeeded in sequencing were deposited in GenBank (accession codes KX212263–KX212309, Ilhéus, Rio Claro, São João da Boa Vista and Santa Rita do Passa Quatro colonies). A haplotype network was constructed using sequences with highest similarity found in Genbank (E-values of 0.0 and 98% similarity with "*Candidatus* Blochmannia ulcerosus" AY334375.1, "*Candidatus* Blochmannia laevigatus" AY334370.1, and "*Candidatus* Blochmannia herculeanus" AJ250715.1) with the median-joining method in Network 4.5.1.0 [32].

To test whether there is geographic correlation of the colony with the several strains of *Blochmannia*, we use the Mantel test available in "vegan" package [33] of R software [34]. The geographical coordinates of the colonies were transformed to metric UTM using the "rgdal" package [35], and the genetic distance of each *Blochmannia* sequence

was calculated using the Kimura 2-parameter model [36] in PAUP 4.0 [37].

Analyses: Wolbachia MLST

The sequences generated were edited and aligned using BioEdit sequence alignment editor [38] and ClustalW [39]. The sequences obtained with the *wsp* primers allowed us to determine if the *Wolbachia* infections were single or multiple. If a single infection was confirmed, the *Wolbachia* MLST approach was initiated following the single infection protocol available at the MLST website (http://pubmlst.org/wolbachia/). All the analyses were run in triplicate (three different workers per colony). Double-infected colonies were excluded from the analyses. The alleles of each gene were compared one at a time with those deposited on the MLST database, and the sequence types (ST) were later confirmed through the concatenated analysis. The sequences from the *wsp* primers were compared with others in the same database [30].

The dataset was partitioned into the different genes, and an appropriate model of sequence evolution was chosen using the Akaike Information Criterion in ModelTest v3.06 [40]. The models selected were GTR_I_G for *gatB* and *wsp*, and GTR_G for *coxA, hcpA, ftsZ*, and *fbpA*. Phylogenetic reconstruction based on Bayesian analysis was carried out in MrBayes 3.1.2 [41] by running a 1,000,000-generation Markov chain and sampling every 1000 generations. The first 25% of the trees were discarded as burnin, and the probability values were calculated using the remaining trees. All the Formicidae STs in the MLST database were used for the comparative analysis. Since there is only one Formicidae B strain, other insects were added to compare and confirm the supergroups. The D strain was used as the outgroup.

Results

Blochmannia

All *Camponotus* specimens analyzed were positive for *Blochmannia*, with a total of 47 clones distributed in 22 different haplotypes, with 508-bp of the 16S rRNA region (Fig. 1). The network analysis revealed that *Blochmannia* haplotypes found in *C. textor* are distant (46 different nucleotides) from other *Blochmannia* sequences in *Camponotus* previously deposited in the database. As these *Blochmannia* from other *Camponotus* were selected to possess the highest similarity with the present study, it emphasizes how different the *Blochmannia* found in *C. textor* is.

In addition, there was more than one *Blochmannia* strain per worker, and these strains were not exclusive to a given geographic location. For example, haplotype 1 (H_1) was found in all locations and haplotype 7 (H_7) was only absent from São João da Boa Vista. There was no correlation between the *Blochmannia* genetic distances and the geographic locations of the colonies using the Mantel test ($r=0.023$, $P=0.3$). In addition, haplotype 1 (H_1) was the most frequent, followed by haplotype 7 (H_7), the two differed by a single nucleotide. The H_1 haplotype differs by only one nucleotide of the following haplotypes: H_3, H_22, H_5, H_4, H_21, H_14, H_13, by two nucleotides: H_2, H_18, H_17, H_15, H_19, H_20; by three nucleotides: H_12, H_16; and finally H_6 differ by five nucleotides. The H_7 haplotype differs by only one nucleotide of the following haplotypes: H_8, H_11 and H_9, and by two nucleotides H_10. All these mutations happened in different loci.

The haplotype network suggests that there are two distinct *Blochmannia* lineages (H_1 and H_7) in the ant population, and that the other haplotypes derive from H_1 and H_7 (Fig. 1). This could explain the co-occurrence of H_7 with H_8, H_9, and H_10, and the co-occurrence of H_1 with H_2 and H_3. Except the haplotype H_22 being co-occurring with H_7, all other haplotypes follow the pattern of the co-occurrence happens between haplotypes deriving from. For many others haplotypes found, we could not exclude the possibility of sequencing artifacts. Therefore, we decided to focus our discussions in these haplotypes that were repeatedly detected in different ant specimens (H_1 and H_7).

Wolbachia

Wolbachia was detected in all colonies analyzed with the *wsp* gene, but its rates of infection varied: 75% in Rio Claro, 37.5% in Santa Rita do Passa Quatro, 87.5 and 80% in Uberlândia I and II, and 100% in Araraquara, Ribeirão Preto, Ilhéus, and São João da Boa Vista. The electropherogram revealed both single and double infections. The only colonies with single infections were in Santa Rita do Passa Quatro (SP) and São João da Boa Vista (SP).

The triplicate MLST sequences from the single-infected colonies were compared with the sequences deposited in the *Wolbachia* MLST database. There was no variation within the species; in other words, all alleles for all individuals were identical. However, we found a new allele, *coxA* allele 185, and a novel ST, ST347, was deposited in the MLST database as a consequence. The sequences generated by *wsp* were included in an additional analysis, which revealed hypervariable regions (HVR1: allele 37, HVR2: allele 38, HVR3: allele 41, and HVR4: allele 37) and 100% similarity with *wsp* allele 58.

The Bayesian inference analysis (using Bayesian posterior probabilities, BPP) of the 42 concatenated sequences

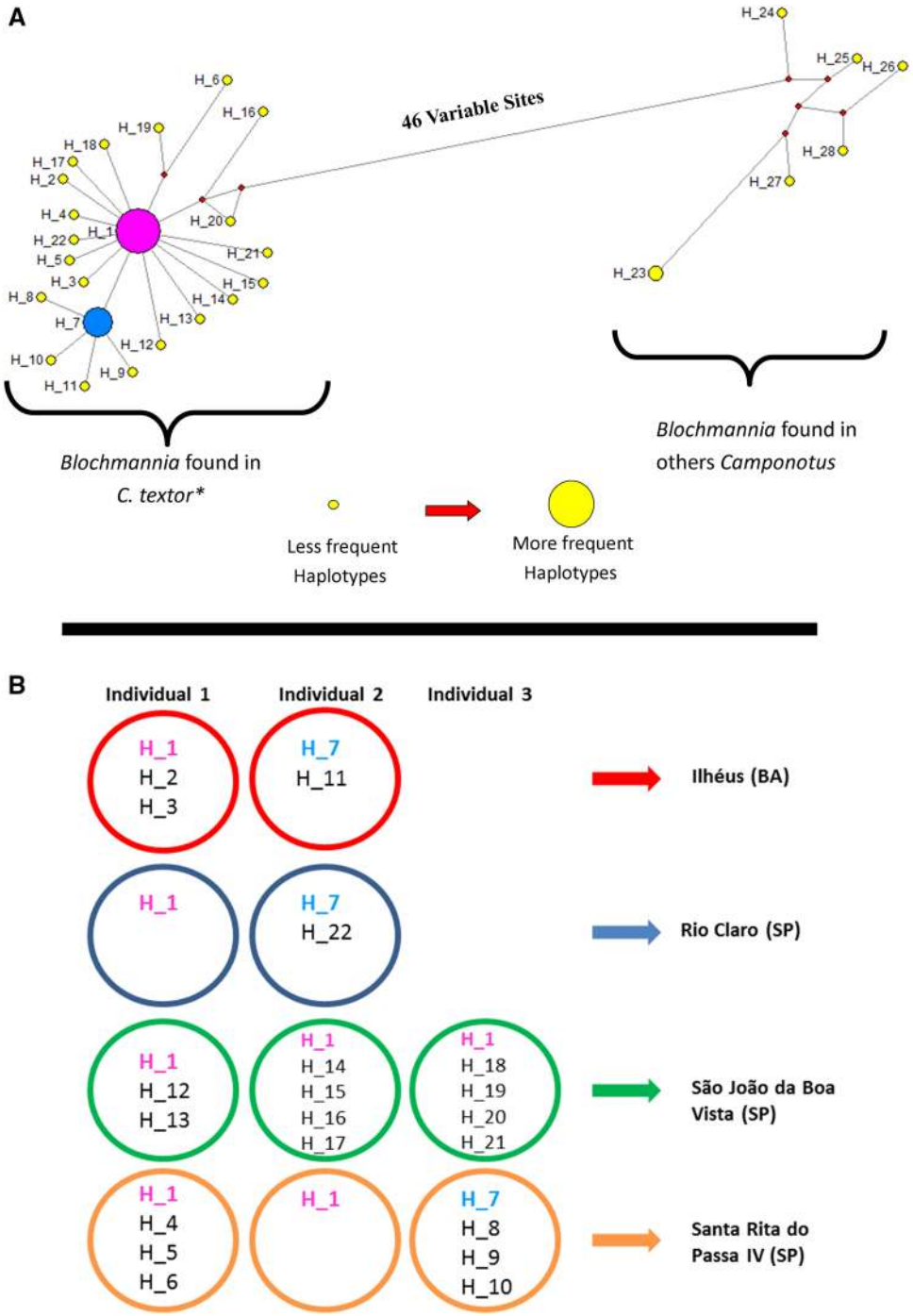

Fig. 1 *Blochmannia* haplotypes in *Camponotus textor*. **a** Haplotype network showing the higher frequency of H_1, followed by H_7. **b** Distribution of the different haplotypes from each analyzed individual. The haplotype size represents the frequency found, and the point in *red* was added by the software as hypothetical haplotype. **C. textor* from this study. (Color figure online)

in different supergroups revealed that ST347 was more closely related to supergroup A STs, thus characterizing this new strain (Fig. 2). However, the strain found in the present study is separated from the strains from North America and the Old World. Combined with ST45, this new strain forms a strongly supported clade (BPP = 90) that is separated from other supergroup A strains. Supergroup A and supergroup B were separated by 100 bp.

Discussion

Blochmannia

Corroborating the findings of Sameshima et al. [15] and Wernegreen et al. [16], all analyzed individuals had *Blochmannia* suggesting that the bacteria are fixed within populations of species in the ant genus *Camponotus*, as

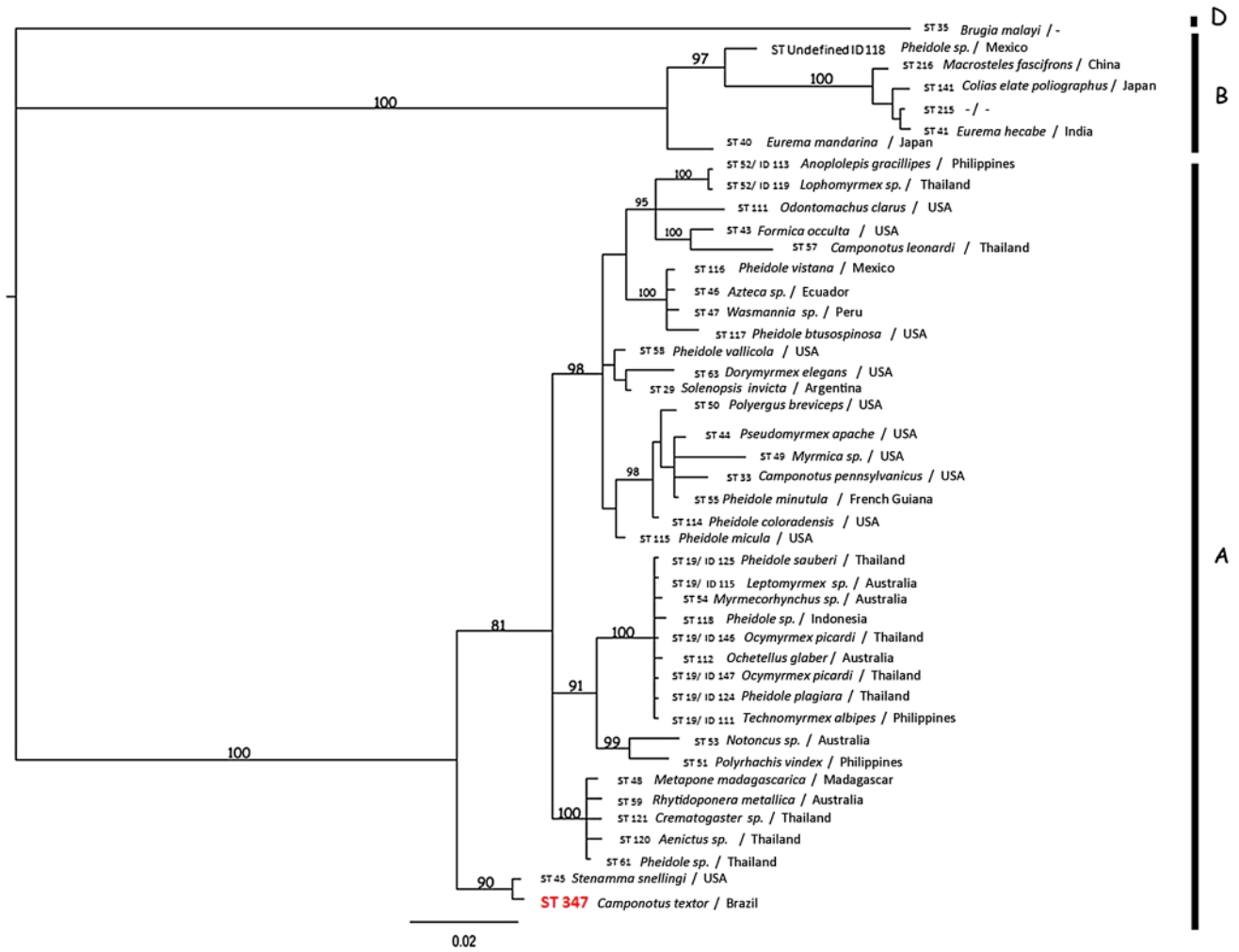

Fig. 2 Bayesian inference analysis of the sequences in the *Wolbachia* with the host/location available in MLST database. The *Wolbachia* strain found in *Camponotus textor* and identified through this work is highlighted (ST347), and belongs to the supergroup A clade. The symbol "-" means that the information was unavailable

observed in *Formica truncorum* and its obligate endosymbiont *Wolbachia* [42]. Additionally, *Blochmannia* strains found in the present work were very different to "Candidatus Blochmannia ulcerosus" (AY334375.1, USA), "Candidatus Blochmannia laevigatus" (AY334370.1, USA), and "Candidatus Blochmannia herculeanus" (AJ250715.1, USA), which confirms the high interspecific diversity. We believe that this huge difference could be because (I) there are few studies in South America that analyzed the diversity of this endosymbiont, and (II) this bacterium has a high rate of mutation [43]. Besides that, there was no correlation between the geographical location and strain similarity based on the intraspecific haplotype network of the *Blochmannia* sequences found in *C. textor*, i.e., shared strains occurred in different geographical regions (Fig. 3).

The intracellular endosymbionts that live in bacteriocytes are vertically transmitted [44]. The fact that the bacteria are located inside a specialized organ associated with female reproductive tissues suggests that the speciation processes of the host and its endosymbiont are interconnected [45, 46]. Phylogenetic congruence suggests the absence of horizontal transfer [43], contrasted to the recurrent recombination among strains of free-living bacteria [47]. The present study analyzed different colonies from different locations and showed the same pattern of diversity of *Blochmannia* (H_1 and H_7, in different locations). Therefore, there is no evidence of horizontal transmission of *Blochmannia* among these populations of *C. textor*, suggesting that the long-distance migration of the ants happened in the past and that the common ancestor of this ants has been carrying these strains ever since [15, 16, 44].

The high intraspecific diversity of *Blochmannia* haplotypes in this species should also be noted. Based on the frequencies observed, we cannot exclude the possibility of

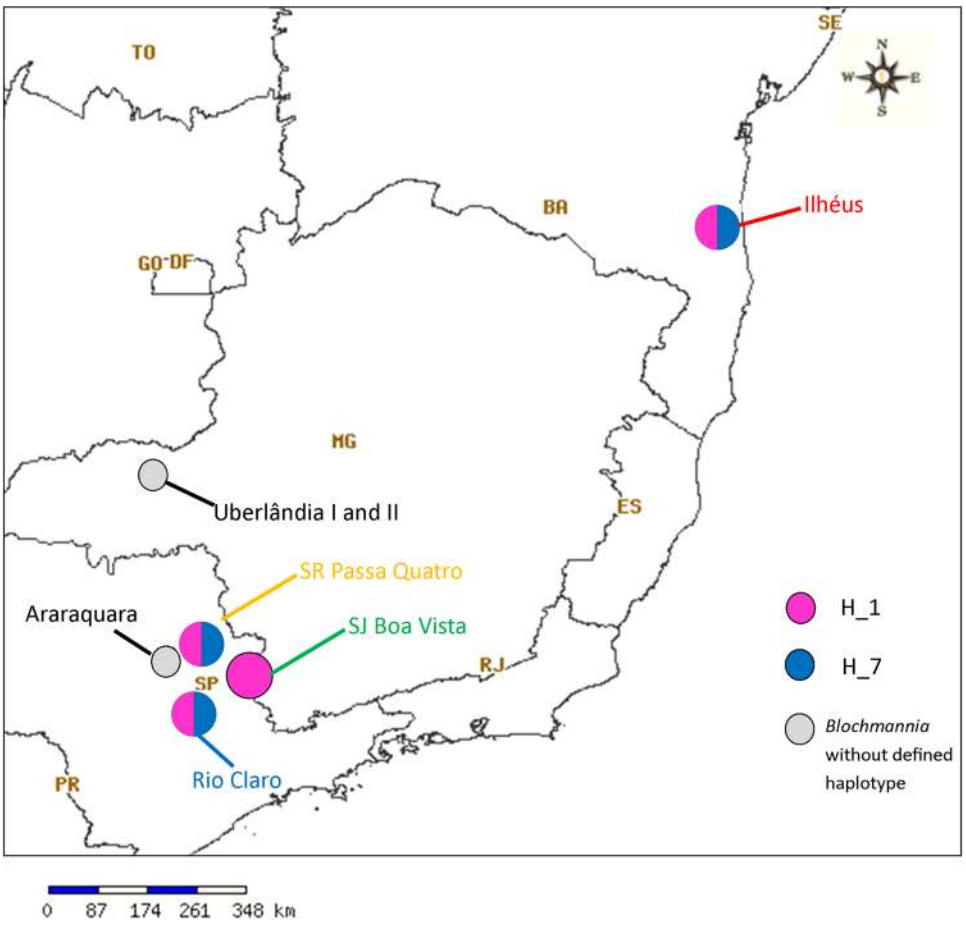

Fig. 3 Distribution map of *Blochmannia* haplotypes found in *Camponotus textor*. Haplotype 1 (H_1) was the most common and it is highlighted in *pink*. Haplotype 7 (H_7) is highlighted in *blue*. Note that there are colonies with the presence of both haplotypes. Locations where we confirmed the presence of *Blochmannia*, but we cannot define the haplotype by the technique of cloning, are highlighted in *gray*. (Color figure online)

at least two distinct haplotypes coexisting in the same host species. Degnan et al. [43] observed a high mutation rate in *Blochmannia*, which might explain the occurrence of haplotype variation within a single species. Nevertheless, we should consider three hypotheses: (1) this diversity is a sequencing artifact; (2) there are multiple copies of 16S in the *Blochmannia* genome; and (3) there are multiple strains of *Blochmannia* in *C. textor*. Given the high frequency of haplotypes identified in this study, the possibility of a sequencing artifact may be rejected, at least for H_1 and H_7, which were frequently found in different colonies. The second hypothesis merits further investigation. Because we did not analyze the whole genome of *Blochmannia*, we do not know if there are multiple copies of the 16S genome. However, we checked whether there is precedence in the publicly available genome sequence of the *Blochmannia* endosymbiont from *C. obliquus* strain 757, GenBank accession # NZ_CP010049, and it was found in only one copy of the 16S gene [48]. Therefore, we also believe that this hypothesis does not apply to *C. textor*. The third hypothesis is the most plausible; although we sampled different colonies from different locations, we observed the same two haplotypes in each one of them (either H_1 or H_7). Due to the high mutation rate of *Blochmannia*, it is possible that these diversified strains of *Blochmannia* had already been present in the ancestor of *C. textor*, and that they spread along with their host species as the geographic range of *C. textor* expanded.

In our study, a pattern emerged where every worker analyzed harbored either haplotype H_1 or H_7 (Fig. 1b). Interestingly, however, these two dominant haplotypes never co-occurred, that is, with our sampling protocol we were unable to find haplotypes H_1 and H_7 in the same individual. More studies are needed to address the possibility of incompatibility or competition between these haplotypes. It is important to note that little is known of the general biology of this host ant, and in particular if the colonies are polygynous or monogynous. If this species is monogynous, the single queen may harbor both haplotypes, excluding the possibility of incompatibility or competition. However, an additional question may be raised: why would these multiple strains not be quickly lost due to genetic drift? Genetic drift does not prevent the co-occurrence of multiple strains of *Wolbachia* within the same individual [25, 26, 49, 50], and we believe the same could be the case for *Blochmannia*, explaining its diversity. If functional

divergence has occurred, akin to the recent diversification of symbionts in cicadas reported by Campbell et al. [51], more studies will be needed to understand the diversity of *Blochmannia* in *Camponotus* sp.

Wolbachia

Camponotus textor had a high rate of *Wolbachia* infection compared to *Solenopsis* spp. from the same region [25], suggesting that the bacteria may be at or near fixation, as suggested by Wenseleers et al. [42] in *F. truncorum*, Fabricius. However, the ST and the *wsp* gene did not vary among different colonies, suggesting that the *Wolbachia* infection may have occurred a long time ago in the common ancestor of the populations. According to Watanabe et al. [52], there are three possible explanations for the presence of similar strains of *Wolbachia* in related species: vertical transmission by a common ancestor [53], horizontal transmission [54], and introgressive hybridization between the hosts [55, 56].

Introgressive hybridization may be discarded because we are reporting similar strains of *Wolbachia* within a species *C. textor*. Therefore, the observed distribution of *Wolbachia* may be caused by(a) vertical transmission by a common ancestor, maintained despite the geographic separation of the colonies, or (b) horizontal transmission induced by similar host–parasitoid or predator–prey interactions [52]. These two hypotheses are also supported by the results of Salunke et al. [57], who used MLST to study *Wolbachia* in butterflies.

Phylogenetically, several strains of *Wolbachia* have been detected in different species of ants using the MLST methodology, and the great majority belongs to supergroup A and the ST347 strain. The distribution of the supergroups (A, B, and D) in the haplotype network and the reconstructed phylogeny confirm that the supergroups are completely separated. Haplotype 31 and haplotype 10 (which are equivalent to ST347 and ST45, respectively) were closely related, both in the network and in the phylogenetic tree, but they are distant from the other strains in supergroup A. The similarity among the variants in family Formicidae was also confirmed through MLST, indicating that there are differences between the strains found in ants and those from other insects, and between the variants in the New World and the Old World [6].

Conclusion

Research into symbiotic interactions of ants of the genus *Camponotus* often focuses on *Blochmannia*, but the actual diversity of the bacterial community of this genus is still unknown. In the general context, we were able to find at least two strains of *Blochmannia* present in the same species of *C. textor*, an ant occurring only in the Neotropical region. One possible explanation for the occurrence of these strains could be the high mutation rate of *Blochmannia*. In the same species, the high infection rate was also observed for *Wolbachia*, and a new strain was deposited in the MLST database. However, these new ST and wsp genes were the same for all *C. textor* colonies analyzed, suggesting that the *Wolbachia* infection occurred in the past in the common ancestor of these populations, before the colonies split. New studies with *C. textor* using next-generation sequencing technologies are needed to obtain more data on the role of symbiotic relationships and their implication for the biology of the host.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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