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The Diversity and Distribution of *Wolbachia*, Rhizobiales, and *Ophiocordyceps* Within the Widespread Neotropical Turtle Ant, *Cephalotes atratus* (Hymenoptera: Formicidae)

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Abstract

Ants are an ecologically and evolutionarily diverse group, and they harbor a wide range of symbiotic microbial communities that often greatly affect their biology. Turtle ants (genus *Cephalotes*) engage in mutualistic relationships with gut bacteria and are exploited by microbial parasites. Studies have shown that associations among these microbial lineages and the turtle ant hosts vary geographically. However, these studies have been limited, and thorough within-species analyses of the variation and structure of these microbial communities have yet to be conducted. The giant turtle ant, *Cephalotes atratus* (Linnaeus 1758), is a geographically widespread, genetically diverse Neotropical species that has been sampled extensively across its geographic range, making it ideal for analysis of microbial associations. In this study, we verified the presence, genetic variation, and geographic patterns at the individual, colony, and population level of three microbial groups associated with the giant turtle ant: *Wolbachia*, a genus of facultative bacteria which are often parasitic, affecting host reproduction; Rhizobiales, a mutualistic order of bacteria hypothesized to be an obligate nutritional symbiont in turtle ants; and *Ophiocordyceps*, a genus of endoparasitic fungi infecting many arthropod species by manipulating their behavior for fungal reproduction. In this study, we found varying degrees of prevalence for two distantly related genotypes (haplogroups) of *Wolbachia* and high degree of prevalence of Rhizobiales across colonies with little genetic variation. In addition, we found low occurrence of *Ophiocordyceps*. This study highlights a key first step in understanding the diversity, distribution, and prevalence of the microbial community of *C. atratus*.

Introduction

Ants can be one of the players in several complex symbiotic relationships such as ant-plant, ant-animal, and ant-microbe interactions. It has been known for over a century that ants possess intracellular bacteria, as first recognized by Blochmann (1882) for *Camponotus* and *Formica* (subfamily: Formicinae). It has since been shown that ants are also a common host to a variety of symbionts, with parasitic and mutualistic interactions (Feldhaar *et al* 2007; Stoll *et al* 2007; Pinto-Tomas *et al* 2009; Frost *et al* 2010; Evans et al. 2011; Russell *et al* 2012; Martins *et al* 2012; Vieira *et al* 2017). Some ant groups at least partially owe their ecological success to internal mutualists. This idea is well-studied in carpenter ants (Formicinae: *Camponotus*), whose gut bacteria (*Blochmannia*) provide amino acids and nitrogen

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recycling for the host (Feldhaar *et al* 2007), and fungus-growing ants (Formicinae: Attini), which possess antibiotic-producing bacteria (*Streptomyces*) that inhibit the growth of a parasitic fungus in the ants' fungus gardens (Currie *et al* 1999). There are many other ant-microbe interactions that are not as well-studied, despite the growing evidence for the importance of symbionts in the ecology and evolution of their hosts. In order to glean information on a microbe's life history, or the coevolutionary history between host and microbe, a critical first step is to analyze the presence and diversity of host-associated microbes.

The turtle ants are a widespread, arboreal, mainly Neotropical ant group (Price *et al* 2014). Hu *et al* (2013) surveyed the gut microbiota of the turtle ant species *Cephalotes varians* (subfamily: Myrmicinae Smith 1876), revealing varying microbial abundance and genotypes between colonies and across the geographic range of this species. Little is known of the variation within other *Cephalotes* species, however. Likely, the most common turtle ant species *Cephalotes atratus* (Linnaeus 1758) occupies an unusually large range for an insect, one that spans the entirety of South America's longitude (Kempf 1951, de Andrade & Urbani 1999, see Fig 1-A). This broad range lends to the diversity of habitats that *C. atratus* occupies, which predominantly includes lowland wet forest but also savanna and xeric scrublands (de de Andrade & Urbani 1999). The species' larger body size and enormous colonies also allow collection of large samples throughout its geographic range (Corn 1976).

Wolbachia is a commonly encountered reproductive parasite that targets invertebrates, with a list of effects such as cytoplasmic incompatibility, parthenogenesis, and male killing or feminization (reviewed in Werren *et al* 2008). Approximately 65% of insect species are host to the parasite, highlighting its abundance (Hilgenboecker *et al* 2008). These bacteria are incredibly well-documented across a wide range of ant genera, showcasing the commonness of the microbe (Frost *et al* 2010, Frost *et al* 2014, Russell *et al* 2012, de Souza *et al* 2009, Souza *et al* 2014, Kautz *et al* 2013, Martins *et al* 2012, Ramalho *et al* 2017a, Ramalho *et al* 2017b, Ramalho *et al* 2018), although it is still unknown how and if this bacterium affects ant hosts, particularly in the case of sterile ant workers that are unable to reproduce. In ants, estimates range from 29% to 50% prevalence in colonies (Russell *et al* 2009b, Wenseleers *et al* 1998). Kautz *et al* (2013) found high colony prevalence of *Wolbachia* within four of seven examined *Cephalotes* species, including *C. atratus*, suggesting *Wolbachia* parasitizes *C. atratus*. Despite its commonness, the specific methods through which *Wolbachia* parasitizes ants are unclear. Recent evidence rejects cytoplasmic incompatibility and parthenogenesis as viable methods, leaving male killing and feminization as possible candidates, at least in *Monomorium pharaonis* (subfamily: Myrmicinae; Linnaeus 1758) (Pontieri *et al* 2017). *Wolbachia* transmits itself vertically from mother to offspring (Wernegreen *et al* 2009, Ramalho *et al* 2018) and can also be horizontally transmitted between species as they interact in their environment (Stouthamer *et al* 1999). The reproductive cycle of ants, in which a single queen produces the entirety of a colony, should therefore predict the same *Wolbachia* morph across a colony, or even a population, although horizontal transmission makes strain variation possible (Ahmed *et al* 2015).

As *Cephalotes* is a mainly herbivorous group, the diet of many of the species is poor in some key nutrients such as nitrogen (Russell *et al* 2009a). It has been shown that their herbivory is facilitated by symbiotic bacteria specific to the gut (Russell *et al* 2009a; Hu *et al* 2018) as in other insect groups (Engel & Moran 2013). Bacteria of the order Rhizobiales are noteworthy symbionts in root nodules of plants but are also found in lichens and herbivorous ants (Erlacher *et al* 2015; Russell *et al* 2009a). While Rhizobiales is nitrogen-fixing in plant-microbe symbiosis, it is unclear if the same process is mirrored in the turtle ant Rhizobiales strains, although it has been hypothesized due to the nitrogen-poor diet of herbivorous ants (Russell *et al* 2009a; Hu 2015). However, genes for a urea-recycling complex were found in Rhizobiales found in the digestive tracts of several *Cephalotes* species (Hu *et al* 2018).

Lastly, *C. atratus* makes an ideal system for studying interactions with another symbiont: the tropical, parasitic fungi genus *Ophiocordyceps* (previously *Cordyceps*, see Sung *et al* 2007). Famously referred to as "zombie-ant fungi," *Ophiocordyceps* is able to infect a variety of arthropod hosts with differing life history traits. Several species of *Ophiocordyceps* rely on ants for propagation, with spore-infected individuals dying and the eventual germination of *Ophiocordyceps* fruiting bodies continuing infection near and within colonies (Araújo *et al* 2015). *Cephalotes atratus* was a notable host to *Ophiocordyceps* in early observations of the fungus, with workers extracting infected individuals from the colony and dropping them from the canopy to the forest floor (Evans 1982).

We analyze the presence, genetic variation, and geographic patterns of three microbes, *Wolbachia*, Rhizobiales, and *Ophiocordyceps*, of a widespread and extensively collected turtle ant species, *C. atratus*. We survey these microbes across the geographic range of *C. atratus* at the individual, colony, and population level. We then examine whether there is distinct genetic diversification within each symbiont. This survey and analysis provide an important first step in understanding the interactions and coevolutionary history between this host and its microbes.

Materials and Methods

Collection and extraction

Ants were collected across the geographic range of *C. atratus*, including north and south Peru, northwestern

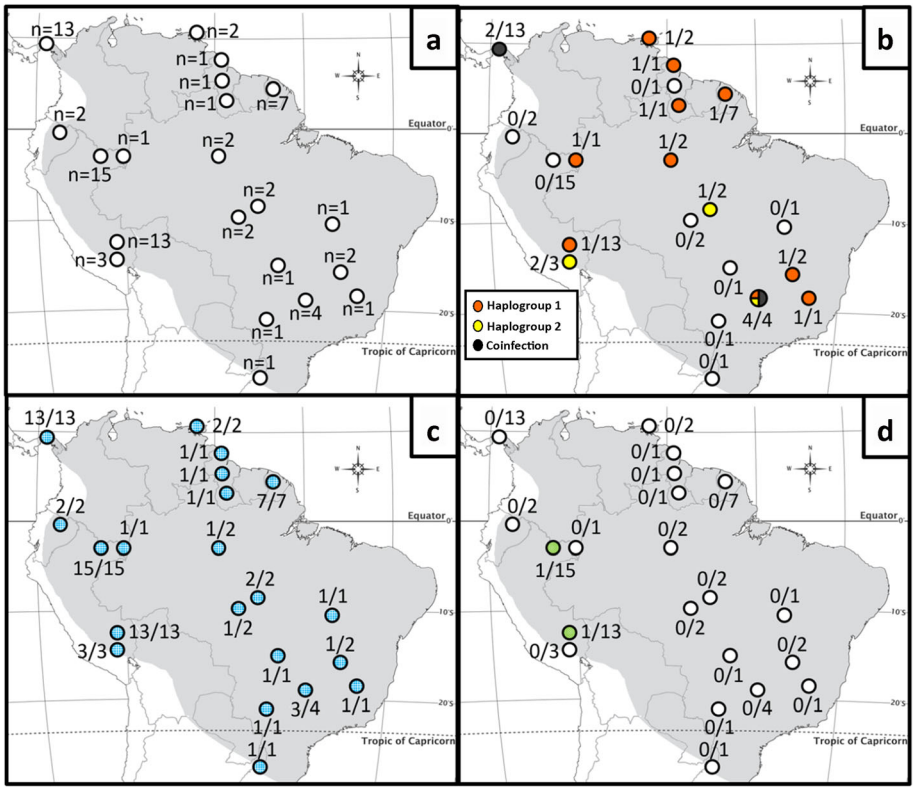

Fig 1 *Cephalotes atratus* and symbiont collections**.** Gray area denotes *Cephalotes atratus* geographic range. A**.** Distribution of *Cephalotes atratus* samples included in this study. *n* indicates number of colonies sampled at the location. Individuals per colony vary based on the success of the collection. B. Presence of *Wolbachia* within sampled colonies. Two different haplogroups were recovered (haplogroup 1 = orange; haplogroup 2 = yellow). Four colonies had coinfection with both haplogroups 1 and 2 (coinfected = black). No geographic structuring was apparent. C. Presence of Rhizobiales within sampled colonies. D. Presence of *Ophiocordyceps* in sampled colonies. The fungus was only detected in Peru (green = presence; white = screened, but not detected).

and southeastern Brazil, French Guyana, and Panama, from a total of 76 colonies (see Fig 1-A and Additional File 1). Adult *C. atratus* workers were stored in 95% ethanol and rinsed with sterile deionized water before extraction. DNA extraction was performed on entire ants using a bead-beating method and the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) following the manufacturer's protocol.

Screening and primer development

Primers were chosen or developed based on deposited gene regions in GenBank, as screening methods include comparison between known and new microbe sequences: *Wolbachia* wsp 81F (5'-TGGTCCAATAAGTGATGAAGAAAC) and 691R (5'-AAAAATTAAACGCTACTCCA) (Zhou *et al* 1998); Rhizobiales (16S rRNA) Ceph_Rh_392F (5'-TCTTTCAC CGGAGAAGATAA) and Ceph_Rh_814R (5'-CGGAATGT TTAATGCGTTAG) (present study); and *Ophiocordyceps*-specific fungal elongation factor-1α (TEF) TEF9F (5'-GACGTCCA ACTTCATCAAGA) and TEF361R (5'-ACTTGACTTCAGTG GTGAC) (present study)).

Wolbachia wsp primers 81F and 681R, as described in Zhou *et al* (1998), were selected for this study , and the surface coding protein gene wsp is known to be highly variable and is considered a fast-evolving gene region that is useful for typing different genotypes (and haplogroups) of *Wolbachia*. These bacteria were screened at both the individual and colony level. In total, 301 individuals from 76 colonies were screened, with an average of 4 individuals screened per colony.

Rhizobiales primers were developed using Primer3 v2.3.4 in Geneious v9.1.6 (Kearse *et al* 2012) and were based on 16S rRNA sequences for *Cephalotes* and its sister group *Procryptocerus* (Russell *et al* 2009a). Rhizobiales was screened at both the individual and colony level, with 190 individuals from 76 colonies screened. Ten individuals were initially screened per colony, but the number was reduced to three per colony as initial screening revealed no sequence variation at the colony level.

Primers were developed for *Ophiocordyceps* based on *C. atratus*-specific sequences from Sanjuan *et al* (2015) using Primer3. *Ophiocordyceps* was screened across 76 colonies, with an average of 9 individuals per colony. *Ophiocordyceps* was screened at the colony level, meaning all nine individuals from the same colony were pooled together after DNA extraction.

PCR amplification

Polymerase chain reaction (PCR) was performed using GE Healthcare Illustra Pure taq Ready-to-go PCR beads and protocol and took place in a BIO-RAD C1000 touch thermal cycler. PCR cycle conditions for *Wolbachia* consisted of initial denaturation for 5 min at 95°C; 30 annealing cycles of 95°C for 30 s, 52°C for 45 s, and 72°C for 90 s; and final extension at 72°C for 7 min. PCR cycle conditions for Rhizobiales consisted of initial denaturation for 5 min at 95°C; 30 annealing cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 90 s; and final extension at 72°C for 7 min. *Ophiocordyceps* followed the same amplification parameters as *Wolbachia*. Negative and positive controls were included for all PCRs, but the possibility of low infection that was not detectable by the traditional PCR technique and sequencing by the Sanger method cannot be excluded. Gel electrophoresis was conducted to test the success of the PCR process. Positive PCR products were purified with ExoSAP-IT PCR Product Cleanup Reagent (Applied Biosystems Inc., Carlsbad, CA). Clean PCR products were cycle sequenced with 25 cycles of the following thermal cycler conditions: initial denaturation for 1 min at 96°C, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Cycle sequence reactions were subsequently cleaned using an ethanol/EDTA precipitation procedure, following the recommendations of Moreau (2014). Gene regions were Sanger sequenced using an ABI 3730 automated sequencer with Big Dye Terminator chemistry (Applied Biosystems Inc.) in the Pritzker DNA Lab at the Field Museum of Natural History, Chicago, IL, USA.

Sequence analysis

Successful sequences were edited in Geneious, aligned using the Geneious alignment algorithm, and subsequently visually inspected and compared. Sequence identity was confirmed using BLAST searches. Successfully sequenced samples were deposited in GenBank (accession codes MK967806, MK977729–MK977742, MN003028, and MN003029). To test whether there is specificity of these recovered bacteria, a phylogeny for the ant-associated *Wolbachia* wsp, 16S rRNA Rhizobiales, and *Ophiocordyceps* sequences recovered from this study was inferred using ant-associated sequences available from GenBank with high degree of similarity. Phylogenies were inferred using maximum likelihood with 10,000 bootstrap generations with the IQ-TREE software (Trifinopoulos *et al* 2016) with the best model of substitution implemented (TMV + F + G4 for *Wolbachia*, TIM + F + G4 for Rhizobiales, and TN + F + G4 for *Ophiocordyceps*). A haplotype network was constructed with the median joining method in Network 4.5.1.0 (Bandelt *et al* 1999).

The genetic distance of all *Wolbachia* sequences recovered in this study was calculated using the R software (R Development Core Team 2018) and ape package (Paradis *et al* 2004). Geographical distances of samples were transformed in UTM distance metric through the R rgdal package (Bivand *et al.* 2013). The correlation of genetic and geographical distances was tested to verify influence of geographical location through Mantel test with the R vegan package (Oksanen *et al.* 2016).

Results

This study recovered a total of 92 positive infections: 18/76 for *Wolbachia*, 72/76 for Rhizobiales, and 2/76 for *Ophiocordyceps*. In total, 162 individuals were infected: 64 for *Wolbachia* and 98 for Rhizobiales. *Wolbachia* was present in 21.3% of total individuals and 23.68% of successfully sequenced colonies (Fig 1-B). The prevalence of *Wolbachia* varied between several populations with 11 colonies entirely infected, whereas 3 only included a few infected individuals (all of these single-infected), and 4 presented coinfections by *Wolbachia*. Coinfection is common and has been reported previously in the literature (Russell *et al* 2009b; Ramalho *et al* 2017a), which we detected when more than two sequences for *Wolbachia* were recovered from a single sample. Therefore, these samples are not included in the consequent analyses, although they were recorded as having with *Wolbachia* infection. Therefore, we found that variation is present at the colony-level and two distinct *Wolbachia* clades were recovered from *C. atratus* (Fig 2A and 2B). These clades clearly separate the two haplogroups found by network analyses (see Fig 2B), and furthermore the wsp phylogeny reveals that the two haplogroups are not sister clades (Fig 2A).

The haplotype network of *Wolbachia* sequences present in *C. atratus* recovered from the wsp gene resulted in four different sequences (Fig 2B). But it should be noted that H_1 and H_2 have only one different nucleotide between them, so for our analyses, we grouped these two into major haplogroup 1, same for H_ 3 and H_4, which belong to major haplogroup 2. However, there is a difference of 71 nucleotides (13%) between the two major haplogroups, and these are colored according to the clades present in the phylogenetic analysis (Fig 2). Ten colonies were infected with *Wolbachia* haplogroup 1, and four were infected with haplogroup 2. The HVRs regions of the wsp gene also recovered the same patterns found in network analysis (Table 1). Our results from the wsp gene also showed that there is no correlation between the genetic distance of the *Wolbachia* and the geographic distances of the hosts, and this was confirmed with the Mantel test ($r = -0.016$, $P = 0.467$).

Rhizobiales was found in 51.6% of total individuals screened and was present in 94.7% of screened colonies

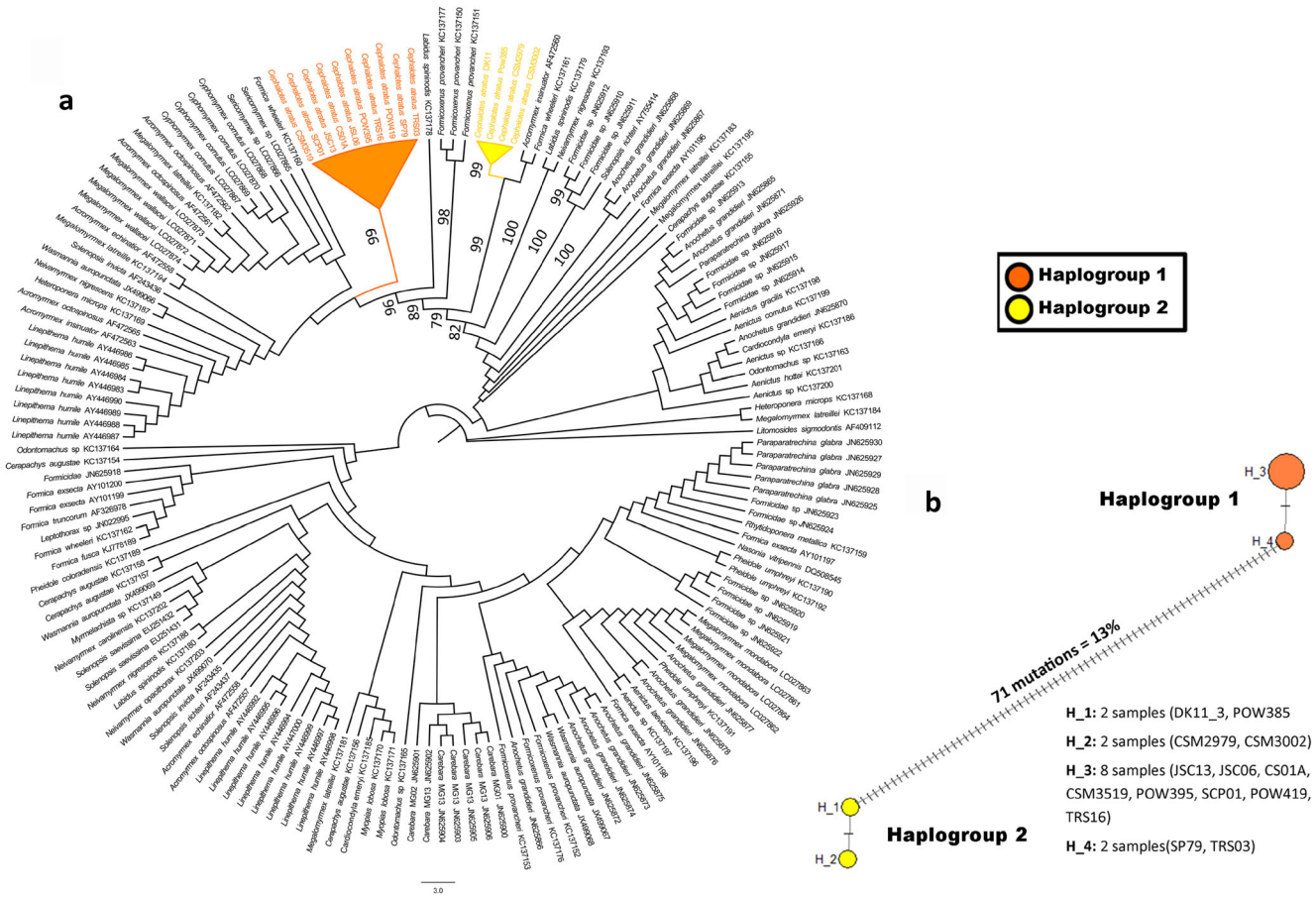

Fig 2 *Wolbachia* found in Formicidae through sequencing of the wsp gene. A. ML-tree of recovered *Wolbachia* haplogroups (haplogroup1 = orange; haplogroup2 = yellow). The haplogroups are not sister groups. B. Haplotype network of *Wolbachia* from *Cephalotes atratus* samples.

(Fig 1-C). There was no variation for the 16S rRNA region in sequenced individuals from different colonies and different geographic locations. This also reveals that this region of the gene has been highly conserved for this bacterium and across the geographic range of the host. 16S rRNA phylogenetic analysis reveals that this Rhizobiales strain from *C. atratus* belongs to a specific clade within other *Cephalotes* and *Procryptocerus* (the sister group to *Cephalotes*). However, some strains of Rhizobiales belonging to other closely related *Cephalotes* species are outside the clade (see Fig 3A).

Our results reveal that although not as common as for *Wolbachia* and Rhizobiales, infection by *Ophiocordyceps* was found in 2.63% of successfully sequenced colonies. Of the two positive colonies (SP36 and SP70), both from Peru, one included visibly infected individuals, whereas the other colony was not known to be infected (see Fig 1-D). Phylogenetic analysis of the two different recovered strains of *Ophiocordyceps* from *C. atratus* shows that they are from different clades. *Ophiocordyceps* from SP36 colony are nested with other *Ophiocordyceps* from *C. atratus* (KC610740), but there are other *Ophiocordyceps* of *C. atratus* in a distinct clade of fungi recovered from this study (Fig 3B).

Discussion

The nature of symbiont-host interactions has consequences for the patterns of the evolution and ecology of both symbionts and hosts. Transmission mode is reflected in the life-history traits of a symbiont, as some modes require symbionts to survive outside of a host more so than others, and the stability of host-symbiont interactions can often be predicted by its transmission mode (Brown & Wernegreen 2016). The geographic distribution and diversity of a host may predict the geographic or evolutionary variation of symbionts, or vice versa (Yun *et al* 2011, Ramalho *et al* 2017a). Additionally, the presence of a host-associated symbiont may be a leading factor in the diversification of a host, as in the case of herbivorous ants (Russell *et al* 2009a, Hu 2015). These and other factors can influence the genome of both the host and symbiont. Our study provides an important first step in understanding the interactions and coevolutionary

Table 1 *Wolbachia* found in *Cephalotes atratus* through sequencing of the wsp gene with the four HVR regions highlighted.

Locus	Allele	Length	Contig	Start position	End position
wsp	729	480	JSC13	50	529
HVR1	21	33	JSC13	50	148
HVR2	21	48	JSC13	149	292
HVR3	291	51	JSC13	293	445
HVR4	21	28	JSC13	446	529
wsp	729	480	JSL06	50	529
HVR1	21	33	JSL06	50	148
HVR2	21	48	JSL06	149	292
HVR3	291	51	JSL06	293	445
HVR4	21	28	JSL06	446	529
wsp	729	480	CS01A	50	529
HVR1	21	33	CS01A	50	148
HVR2	21	48	CS01A	149	292
HVR3	291	51	CS01A	293	445
HVR4	21	28	CS01A	446	529
wsp	729	480	CSM3519	50	529
HVR1	21	33	CSM3519	50	148
HVR2	21	48	CSM3519	149	292
HVR3	291	51	CSM3519	293	445
HVR4	21	28	CSM3519	446	529
wsp	729	480	POW395	50	529
HVR1	21	33	POW395	50	148
HVR2	21	48	POW395	149	292
HVR3	291	51	POW395	293	445
HVR4	21	28	POW395	446	529
wsp	729	480	SCP01	50	529
HVR1	21	33	SCP01	50	148
HVR2	21	48	SCP01	149	292
HVR3	291	51	SCP01	293	445
HVR4	21	28	SCP01	446	529
wsp	729	480	POW419	50	529
HVR1	21	33	POW419	50	148
HVR2	21	48	POW419	149	292
HVR3	291	51	POW419	293	445
HVR4	21	28	POW419	446	529
wsp	729	480	TRS16	50	529
HVR1	21	33	TRS16	50	148
HVR2	21	48	TRS16	149	292
HVR3	291	51	TRS16	293	445
HVR4	21	28	TRS16	446	529

Locus	Allele	Length	Contig	Start position	End position
wsp	28	480	TRS03	50	529
HVR1	21	33	TRS03	50	148
HVR2	21	48	TRS03	149	292
HVR3	25	51	TRS03	293	445
HVR4	21	28	TRS03	446	529
wsp	28	480	SP79	50	529
HVR1	21	33	SP79	50	148
HVR2	21	48	SP79	149	292
HVR3	25	51	SP79	293	445
HVR4	21	28	SP79	446	529
wsp	730	471	DK11_3	50	520
HVR1	260	33	DK11_3	50	148
HVR2	40	45	DK11_3	149	283
HVR3	42	52	DK11_3	284	439
HVR4	39	27	DK11_3	440	520
wsp	730	471	CSM2979	50	520
HVR1	260	33	CSM2979	50	148
HVR2	40	45	CSM2979	149	283
HVR3	42	52	CSM2979	284	439
HVR4	39	27	CSM2979	440	520
wsp	730	471	CSM3002	50	520
HVR1	260	33	CSM3002	50	148
HVR2	40	45	CSM3002	149	283
HVR3	42	52	CSM3002	284	439
HVR4	39	27	CSM3002	440	520
wsp	730	471	Pow385	50	520
HVR1	260	33	Pow385	50	148
HVR2	40	45	Pow385	149	283
HVR3	42	52	Pow385	284	439
HVR4	39	27	Pow385	440	520

Haplogroup 1

Haplogroup 2

history between a widespread turtle ant species *C. atratus* and its *Wolbachia*, Rhizobiales, and *Ophiocordyceps*.

The vertical maternal transmission of *Wolbachia* predicts the infection of an entire colony through a single infected queen. This prediction is consistent with the results, as a majority of colonies were infected in their entirety. Some colonies were only partially infected, which may be explained as *Wolbachia* has been shown to be lost in some workers over the course of development as in the case of leaf-cutter ants (Van Borm *et al* 2001), but further analysis is needed to confirm this for other species. Many studies have reported the presence of multiple *Wolbachia* infections in the same individual including this one, but most of them do not go very deeply into characterizing it (Baldo *et al* 2006, Russell *et al*. 2009), and it is possible that this is a source of diversity related to *Wolbachia* by recombination of the strains (Stahlhut *et al* 2010). Arai *et al* (2019) in a Lepidoptera study argue that for the host, such multiple infections may be beneficial by decreasing offspring mortality compared to a single infection by decreasing the chance of cytoplasmic incompatibility (CI). From the point of view of this symbiont, these multiple infections are more stable, and this high density of infection guarantees the passage from *Wolbachia* to the descents. Bailly-Bechet *et al* (2017) also suggest that these

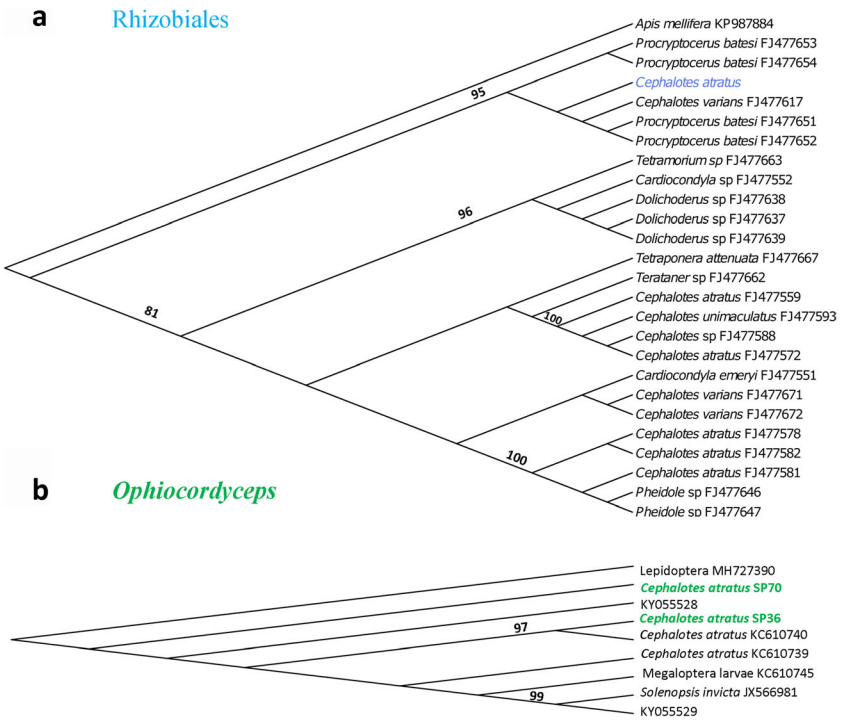

Fig 3 A. Maximum likelihood tree of Rhizobiales 16S rRNA gene region. The recovered strain of *Cephalotes atratus* Rhizobiales (highlighted in blue) is within a clade including other *Cephalotes* and *Procryptocerus* (the sister group to *Cephalotes*) strains. B. Maximum likelihood tree of *Ophiocordyceps* elongation factor-1α (TEF) gene region. The two recovered strains of *Ophiocordyceps* (highlighted in green) from *Cephalotes atratus*.

multiple infections may be more susceptible to horizontal transmission compared to simple infection. As horizontal transmission of *Wolbachia* in ants is not common, only few studies confirm that occasionally it can happen in related hosts (Tolley *et al* 2019, Russell *et al* 2009). More studies that take into account multiple *Wolbachia* infections in ants need to be conducted to understand their impact on this symbiotic relationship.

Although our data are robust and no geographic pattern was discerned for the two *Wolbachia* haplogroups, the analysis was retrieved with only a single gene wsp, and Baldo *et al* (2006) has reported that this gene has a high rate of recombination. Future studies addressing other techniques such as MLST may reveal more about the evolutionary history of this symbiosis. Subsequent analyses of *C. atratus* colonies could tease apart any potential patterns related to the biogeography of host and symbiont.

The high degree of prevalence of Rhizobiales in *C. atratus* is consistent with the idea of its obligate nutritional role for the host. While no variation was found in this gene region, other regions may be subject to higher rates of change. Previous work on *C. varians* detected two Rhizobiales strains across its range in the Florida Keys (Hu *et al* 2013); however, that range was decidedly smaller with ants distributed across multiple islands rather than a continuous continental region like that of *C. atratus*. Additional sequencing with other gene regions could reveal similar results; however, high conservation across the genome is possible with obligate symbionts. The lack of variation of Rhizobiales across all the *C. atratus* samples and this strain grouping with other *Cephalotes* and *Procryptocerus* (the sister group to *Cephalotes*) strains in the phylogenetic analysis may indicate that this bacterium is not being picked up from the environment but rather suggests coevolution of this obligatory symbiont and host.

Ophiocordyceps is likely more common in *C. atratus* than these results suggest. As this fungal parasite is ephemeral and only horizontally acquired from the environment by single workers, obtaining an infected individual through random sampling would be less likely than the other microbes we have screened, as Rhizobiales and *Wolbachia* are likely to be passed down from a queen to the entirety of its colony. Additionally, the ability for a colony to purge itself of infected individuals has been found (Evans 1982) suggesting the majority of sampled colonies would be mostly uninfected. These results do show a positive *Ophiocordyceps* sample from a colony that did not have visibly infected individuals, suggesting our methods are robust enough to be carried out with additional colonies that do not have *Ophiocordyceps* visibly germinating. Our data from the phylogenetic analysis of *Ophiocordyceps* also confirm the lack of specificity of the fungus with the host, since in spite of grouping with other *C. atratus*, other species of ants and other insects were also present in the clade recovered from *Ophiocordyceps* of this study; therefore, this also corroborates the idea of the ephemerality of this infection.

In conclusion, these screening results present new insights regarding three symbionts of the giant turtle ant, *C. atratus*. This study also highlights the need for further analysis regarding additional *C. atratus* symbionts, as well as the symbionts of other *Cephalotes* species and across other host genera. We hope to continue this work to further elucidate the interactions between *C. atratus* and its symbionts and potentially map out phylogeographic relationships between this widespread species and its microbial community.

Competing Interests The authors declare that they have no competing interests.

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