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INVERTEBRATE MICROBIOLOGY

Identifying the Role of Elevation, Geography, and Species Identity in Structuring Turtle Ant (*Cephalotes* Latreille, 1802) Bacterial Communities

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Abstract

Bacterial communities in animals are often necessary for hosts to survive, particularly for hosts with nutrient-limited diets. The composition, abundance, and richness of these bacterial communities may be shaped by host identity and external ecological factors. The turtle ants (genus *Cephalotes*) are predominantly herbivorous and known to rely on bacterial communities to enrich their diet. *Cephalotes* have a broad Neotropical distribution, with high diversity in the South American Cerrado, a geologically and biologically diverse savanna. Using 16S rRNA amplicon sequencing, we examined the bacterial communities of forty-one *Cephalotes* samples of sixteen different species collected from multiple locations across two sites in the Cerrado (MG, Brazil) and compared the bacterial communities according to elevation, locality, species, and species group, defined by host phylogeny. Beta diversity of bacterial communities differed with respect to all categories but particularly strongly when compared by geographic location, species, and species group. Differences seen in species and species groups can be partially explained by the high abundance of *Mesorhizobium* in *Cephalotes pusillus* and *Cephalotes depressus* species groups, when compared to other clades via the Analysis of Composition of Microbiome (ANCOM). Though the *Cephalotes* bacterial community is highly conserved, results from this study indicate that multiple external factors can affect and change bacterial community composition and abundance.

Keywords NGS · Cerrado · Neotropical savanna · Formicidae · Cephalotini · Microbiota

Introduction

Symbiotic bacterial communities in insects are remarkably influential in shaping the diets, niches, and overall lives of their hosts [26, 38]. In many insects, these bacterial communities are essential, having codiversified with their hosts and shaped their evolution irrevocably [17, 18, 32]. In addition to codiversification, there are other factors that may influence the bacterial community within a host and include host phylogenetic history [50, 55, 56], the environment the host is living in [31, 53], and the developmental stages of the host [20, 51, 52].

The Neotropical ant genus *Cephalotes* Latreille, 1802 has been shown to have a stable core bacterial community [16, 19–21, 56]. *Cephalotes* have a mainly herbivorous, nitrogen-limited diet; species in the genus consume primarily nectar, pollen, fungi, bird droppings, and mammal urine [14, 16, 43]. The gut bacterial community of *Cephalotes* obtains necessary nutrients not provided in their diet by recycling nitrogen [21]. The association between *Cephalotes* and its symbiotic bacterial community is crucial for the host and has been largely stable for over 50 million years [16]. However, even in hosts with highly conserved bacterial communities, external factors can cause variation in the composition and abundance of these bacterial communities [20].

Cephalotes ants are found across the Neotropics and have the highest diversity in the Amazonian and Chacoan regions. Evidence from time-calibrated phylogenies indicates a likely recent adaptive radiation of *Cephalotes* in the Chacoan region;

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this region has the highest rate of diversification of any region *Cephalotes* is known to inhabit [46, 47]. The Cerrado, a vast Neotropical savanna, is the central in the Chacoan region, or "dry diagonal," of South America. It is extremely biodiverse and considered by many to be the most biologically rich savanna on Earth [9, 54]. Cerrado hosts an estimated 160,000 species of plants, fungi, and animals—including fifty-four *Cephalotes* species [5, 41]. The terrain is also compositionally diverse; most of the Cerrado is on sedimentary or crystalline plateaus which are broken up by a network of lower elevation depressions. The lower elevation regions, at about 100 to 500m, are a heterogeneous mosaic of plant communities, including broad gallery forest, tropical dry forests, marshlands, and grasslands [58]. The higher elevation regions of the Cerrado, ranging in elevation from 500 to 1700m, feature predominantly grassland vegetation. Much of this land is considered rupestrian grassland, a discontinuous ecoregion that is within the Cerrado, but differs in geography and vegetation and is known for its exceptionally nutrient-poor soil [39]. Still, rupestrian grassland is exceptionally biodiverse, more than the other regions of the Cerrado, and features more than 6000 plant species, with 90% of these plant families being endemic to the region [15].

The variety of elevations in the Cerrado make this system ideal for studying the effects of elevational difference on the bacterial communities of turtle ants. Changes in elevation can affect soil pH and vegetation types, which have been demonstrated to change soil bacterial community composition [57]. It is also well-known that elevation can impact the composition and richness of species of plants, vertebrates, and invertebrates [29]. Still, the symbiotic bacterial communities in these hosts have largely been overlooked. Most existing studies on elevation and host-associated bacterial communities focus on humans and other vertebrates; these studies have shown that differences in elevation can significantly influence composition and abundance of bacteria (Puerto Rican frogs, [22]; human skin, [27]; mesquite lizard, [36]). Comparably fewer studies have examined the impact that elevation could have on the bacterial communities of insects. In a recent study by Brown et al. [6], elevation had a small but significant effect on the bacterial communities of *Drosophila* pupae from an Australian rainforest; the authors suggest that these effects are due to differences in temperature and geographic location [6]. A study on *Apis cerana*, the Indian honey bee, compared bee gut bacterial communities between different elevations using 16S rDNA sequencing [60], and a study on *Lasioglossum* bees collected from the slopes of Mt. Kilimanjaro used 16S DNA metabarcoding to examine bacterial communities [33], and both studies identified bacterial taxa that were consistently more abundant at higher altitudes.

Additionally, multiple studies have shown that geographic location can impact the microbial communities of various insects, including ants [12, 31, 53, 61]. In *Cephalotes*, specifically, a few studies have examined the influence of geography on bacterial symbionts: one study found that the diversity of the endosymbiont *Wolbachia* in different populations of *Cephalotes atratus* was significantly influenced by geography [24]. Another study on the bacterial communities of different castes and life stages of thirteen *Cephalotes* species found that geographic distance explained some of the bacterial community dissimilarity [19]. Because the Cerrado has such varied terrain, environmental effects on the *Cephalotes* bacterial community may be different across locations in the Cerrado.

The species diversity of *Cephalotes* in the Cerrado also enables the study of the effects of relatedness and species identity on the bacterial community. The genus *Cephalotes* is further divided into species groups, defined initially by morphology [14], and later tested for monophyly using molecular data and revised [41, 45, 47]. Sanders et al. [56] found that *Cephalotes* samples from the same species group grouped together in principal coordinate analyses of beta diversity dissimilarities [56]. Flynn et al. [16] further corroborates this result, adding that host phylogeny influences bacterial communities specific to certain compartments of the *Cephalotes* gut [16]. A relatively rapid diversification of dominant lineages of Cerrado plants (4 million years ago or less; [59]) may also have influenced the evolution of *Cephalotes* in the Cerrado [47], which makes the Cerrado a particularly interesting place to examine the effects of species and relatedness on *Cephalotes* bacterial communities.

Because of the conserved nature of the overall *Cephalotes* bacterial community and the geographic, elevational, and species diversity of *Cephalotes* samples available in the Cerrado, we decided to investigate the impacts of external environment on *Cephalotes* bacterial community by comparing the bacterial communities of various *Cephalotes* ants. We used 16S rRNA amplicon sequencing and bioinformatics to examine the bacterial communities of *Cephalotes* specimens collected across multiple sites around two distinct Cerrado locations in the Brazilian state of Minas Gerais, each with varying ecoregions and vegetation types. The "Southwest" (SW) region, near the city of Uberlândia, is primarily mid-elevation (750–850m) Cerrado savanna, with little geographic or elevational variation within the collection areas. The "Southeast" (SE) region near Belo Horizonte varies more in elevation, with some samples collected from mid-elevation Cerrado, but most samples sourced from higher elevation rupestrian grasslands (>900m). This work explores the potential effects external factors like geography and location have on structuring

Cephalotes bacterial communities, as well as the differences in bacterial community that may be found between different *Cephalotes* species and species groups.

Methods

Obtaining Samples

The forty-one *Cephalotes* specimens in this study were collected from multiple sites in Brazil between 2011 and 2018 (Fig. 1). The samples were identified to species using the most recent species descriptions and keys [5, 14, 41]. Fourteen species matched existing species descriptions, while two distinct morphospecies did not and likely represent undescribed species. This gave a total of sixteen species of *Cephalotes* recognized in the present study. After collection in the field, specimens were immediately stored in 95% ethanol and kept at −20 °C until DNA extraction. Elevation data was recorded when available, and additional elevation data was supplemented by using known geographic coordinates on Google Earth (earth.google.com) to determine approximate elevation at collection locations. Specimen data including species and collection information is shown in Supplementary Material 1.

DNA Extraction and 16S rRNA Library Prep

DNA was extracted from the gaster in all ant samples. Following the protocol of Rubin et al. (2014), the extraction was performed with the DNeasy PowerSoil Kit (Qiagen, USA) with an added bead-beating step. All extraction steps were performed following the manufacturer recommended protocol and using methods described in Moreau [37] to best avoid contamination. Four negative controls were included in the extraction steps and later included in 16S rRNA amplification and library sequencing. Our library targeted the amplification of the V4 region of 16S rRNA (primers: 515F "Parada" forward primer, barcoded 5′-AATGATACGGCG ACCACCGAGATCTACACGCT XXXXXXXXXXXX TAT GGTAATT GT GTGYCAGCMGCCGCGGTAA [42]/806R "Apprill" reverse primer 5′ - CAAGCAGAAGACGGC ATACGAGAT AGTCAGCCAG CC GGACTACNVGGG TWTCTAAT [2]) and was performed following methods described in Caporaso et al. (2010) and the Earth Microbiome Project (EMP) protocol (http://www.earthmicrobiome.org/protocols-and-standards/). Three PCR reactions were

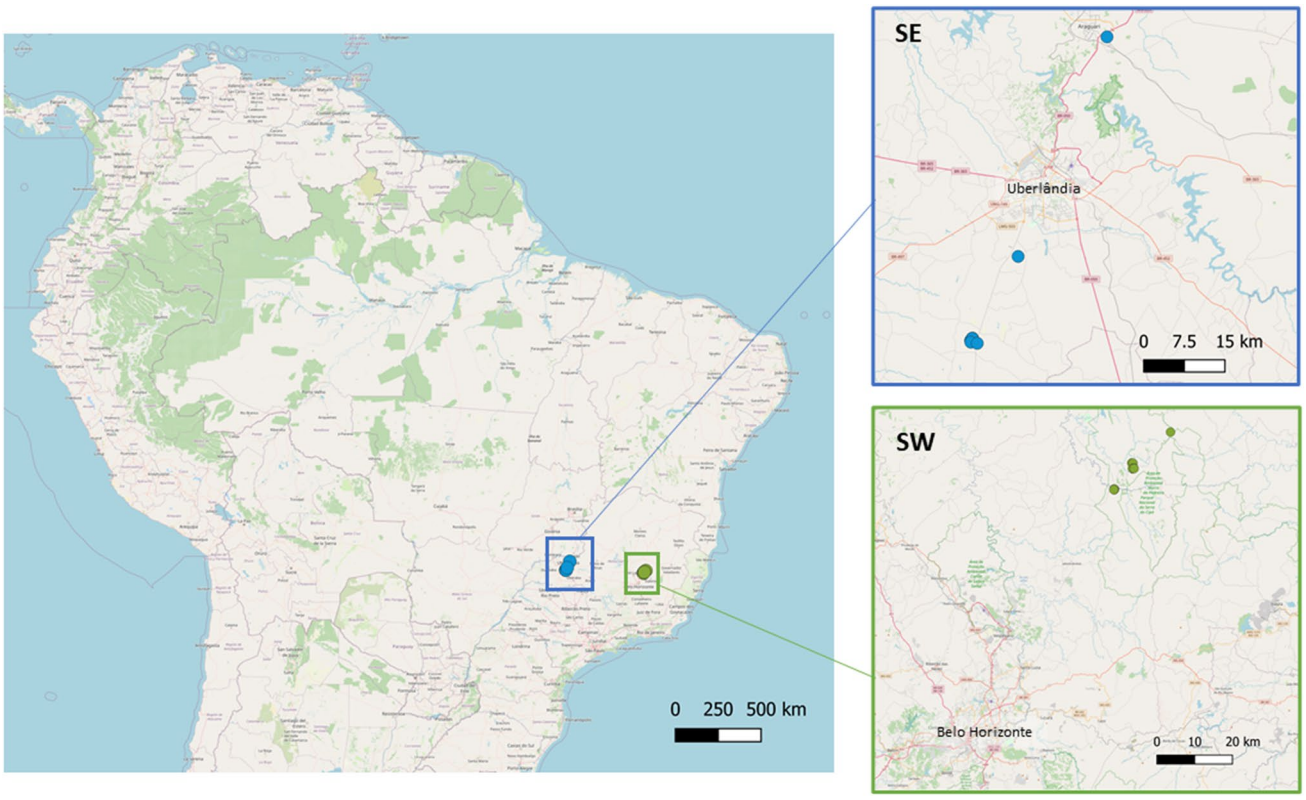

Fig. 1 Map of *Cephalotes* sampling locations within Minas Gerais, Brazil, with zoomed-in maps of the Southeast (SE) and Southwest (SW) collection regions

performed per sample; each 25 µl PCR reaction contained 12µl of certified DNA-free PCR water, 10µl of 5 PRIME HotMasterMix (1×) (5 PRIME, Gaithersburg, USA), 1 µl of forward primer (5 mM concentration, 200 final pM), 1 µl of reverse primer (5 mM concentration, 200 final pM), and 1 µl of template DNA (>0.20 ng/µl). The reaction was performed in a thermocycler under the following conditions: 94 °C for 3 min, 35 cycles at 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s, with a final cycle of 10 min at 72 °C. DNA amplification efficiency was confirmed using gel electrophoresis (1%).

Bacterial Quantification

To verify the quantity of bacteria present in each sample, qPCR was performed via real-time CFX Connect equipment (Bio-Rad, Hercules, USA), using the SYBRAdvanced 2X (Bio-Rad) SYBR Green SuperMix and 2µL of DNA. The 16S rRNA gene was amplified again using the universal primers 515 f (5′-GTGCCAGCMGCCGCGGTAA) and 806r (5′-GGACTACHVGGGTWTCTAAT) (http://earthmicrobiome.org/emp-standard-protocols/16s/). Following the parameters of Rubin et al (2014), standard curves were created from linearized plasmid-containing inserts of the *E. coli* 16S rRNA gene. All samples, including negative control samples, were analyzed in triplicate.

Sample Pooling, Cleaning, and Sequencing

All samples were pooled after quantification and cleaned using the QIAquick PCR Purification Kit (Qiagen, USA), following the protocols recommended by the manufacturers. The DNA pool was then diluted to 4 nM, denatured, and diluted to a final concentration of 6.75pM for sequencing at Argonne National Laboratory (Lemont, Illinois, USA). Sequencing was performed with the MiSeq Illumina V3 Reagent Kit 600 Cycles (2 × 300) using procedures and custom sequencing primers described in the supplementary methods in Caporaso et al. [8].

Bioinformatic Analysis

Demultiplexed sequence data were analyzed using the QIIME2 software package version 2020.8 [4] with the demux plugin (https://github.com/qiime2/q2-demux). Feature table construction and sequence quality control were performed via the DADA2 plugin [7]. Paired-end sequence reads were trimmed for better results, and the SILVA_132_QIIME database with 99% similarity was used to access ASVs (amplicon sequence variants) to assign taxonomic information from the data sequenced [49]. The classifier was implemented using the "feature-classifier fit-classifier-naïve-bayes" command. After the classifier had been obtained, the reads were classified by taxon using the "feature-classifier classify-sklearn command."

After construction of the feature table, data were imported into the R software (R Core Team 2020) using the QIIME2R package (https://github.com/jbisanz/qiime2R). Negative controls were filtered out of the feature table using the Decontam package [13]. The prevalence method of the Decontam package was used because it removed the largest number of contaminants and best filtered our data. Decontaminated data were then filtered on QIIME2 to remove mitochondria, chloroplast, and Hymenoptera-identified ASVs from the feature tables.

The "feature-table" and "taxonomy" objects were imported back to QIIME2, where "feature-table summarize" was used to create a visual summary of the 16S rRNA data. SEPP, implemented with the "q2-fragment-insertion" plugin, was used to estimate the bacterial phylogeny [35]. Alpha and beta diversity analyses were performed by using the "qiime feature-table core-features" command. The sampling depth for this command was set at 2000 reads per sample in an effort to keep features with low reads from skewing analyses. Significant differences in alpha diversity by categorical metadata column (elevation, geographic location, species identity, and species group identity) were analyzed with Kruskal-Wallis tests using the "alpha-group-significance" plugin [25] for Shannon's diversity index and Faith's Phylogenetic Diversity. Pairwise Kruskal-Wallis results (and pairwise PERMANOVA results in the case of beta diversity tests) were obtained by specifying "--p-pairwise" in the command line.

To test for significant differences in bacterial communities between categorical samples, we used the "qiime diversity beta-group-significance" plugin [1] using the default PERMANOVA method for two diversity metrics: Bray-Curtis dissimilarity and Jaccard index. Variables tested for influence on *Cephalotes* microbial communities were (1) altitude at which samples were collected, (2) geographic location of where samples were collected, (3) species identity of each sample, and (4) species group identity of each sample. Samples were divided into two groups based on relative altitude, with the "low" group defined as sea level to 1000 m above sea level and the "high" group defined as anything above 1000 m above sea level. The elevation groups reflect the range of elevations in our dataset and were defined in this way to highlight ecological differences between the mid-elevation "Cerrado" (750–900 m) and rupestrian grasslands (1000–1300 m). A smaller experiment, designed to more closely examine the effects of elevation, utilized only *Cephalotes pusillus* samples collected in 2017. Three *C. pusillus* colonies were collected from each of three different sites on the same mountain (9 colonies total): a low-elevation savanna, a mid-elevation transitional site featuring savanna and rupestrian grassland, and a high-elevation rupestrian

grassland site. These nine samples were analyzed separately (in addition to the analysis featuring all samples) and were assigned to the "low," "mid," and "high" groups based on collection site.

"Geographic location" here is assigned to two relative cardinal points of location based on longitude and latitude: Southeast (SE) and Southwest (SW). Geographic location was assigned independently of altitude or species identity. The correlation of the geographic distance, the differences between longitude and latitude of collection sites of each sample, and the bacterial community dissimilarity, represented by to the Bray-Curtis distance matrix produced via the "qiime diversity beta-group-significance" plugin, was tested using a Mantel test using the R package vegan [40]. Analysis of *Cephalotes* species groups followed species groupings originally characterized by de Andrade and Baroni Urbani (de Andrade and Baroni Urbani 1999). Species groups for more recently described species were confirmed using Oliveira and colleagues [41] and determined based on close species affinities and grouping synapomorphies for the two distinct morphospecies included in the study. These taxonomic groupings have also been upheld by phylogenetic analysis for the taxa included in the present study [45, 47].

To examine the "core" bacterial community (bacteria found in >50% of the samples), we input the same filtered and decontaminated table used in previous analysis and then used the "qiime feature-table core-features" command. The resulting "core" community was analyzed for alpha and beta group significance.

To test if there are any differently abundant taxa leading to the statistical distances inferred by Jaccard and Bray-Curtis, an Analysis of Composition of Microbiome (ANCOM) was used through the QIIME2 ANCOM plugin with the "qiime composition ancom" command [30]. An ANCOM produces the numbers of ASVs ultimately designated as a particular bacterial taxon, as well as a *W* statistic from a Wilcoxon signed-rank test on the centered log ratios. The test assumes that most taxa are not changing in abundance between host communities, and those that have a consistently large change in abundance rank between all communities have correspondingly large *W* statistic values. Three ANCOM analyses were performed to investigate significant differences shown among elevation, locality, and species group. The "core" community was also analyzed with ANCOM, with the same categories examined.

Maps in figures were created using QGIS 3.22.3 software (QGIS 2022). Barplot figures were made in Microsoft Excel. PCoA and NMDS plots in figures were created using the phyloseq R package [34].

Results

16S rRNA amplicon sequencing of 41 host turtle ant specimens (after Decontam, mitochondria and chloroplast filtering, and cutting off sampling at a depth of 2000 reads) produced 119 unique bacterial ASVs. The most prevalent ASVs found in each host sample's bacterial community are listed in Fig. 2. Names of ASVs and relative abundance in each *Cephalotes* sample's bacterial community are available in Supplementary Material 3. The most abundant ASV had 158,062 reads, and the least abundant sample had 1620 reads. Taxonomic assignments and relative abundance of the "core" bacterial community in each sample are presented in Supplementary Material 4.

Alpha Diversity and the Influence of Elevation, Geographic Location, Species, and Species Group

We found no differences ($p > 0.05$) in Shannon's diversity index and Faith's Phylogenetic Diversity Kruskal-Wallis tests for all metadata categories (Table 1 lists values of Kruskal-Wallis "all groups" comparison when examining the entire and "core" bacterial communities; see Supplementary Materials 5 and 6 for full lists of pairwise values).

The "core" bacterial community, however, did show significant differences in our alpha diversity analyses in a Faith's Phylogenetic Diversity Kruskal-Wallis test (p-value = 0.045, H = 12.85) and a Shannon's diversity index Kruskal-Wallis test (p-value = 0.277, H = 14.17) for the species group category. All other categories tested with Kruskal-Wallis did not show significant differences in alpha diversity.

Beta Diversity and Elevation

All p-values and pseudo-F values from the beta diversity analyses of elevation, geographic location, species, and species group are listed in Table 2, and full pairwise PERMANOVA results are listed in Supplementary Material 6 and 7. A PCoA figure of Bray-Curtis distance between samples labeled by "high" and "low" elevation category is shown in Fig. 3A.

The results of the beta diversity analysis suggest that the bacterial community of *Cephalotes* differs between different elevations. PERMANOVA comparison of the "high" and "low" elevation groups using the Bray-Curtis dissimilarity, which measures community richness and composition, showed significant results (p-value = 0.027, pseudo-F = 1.959), as did the Jaccard dissimilarity, which is the presence/absence measure of community

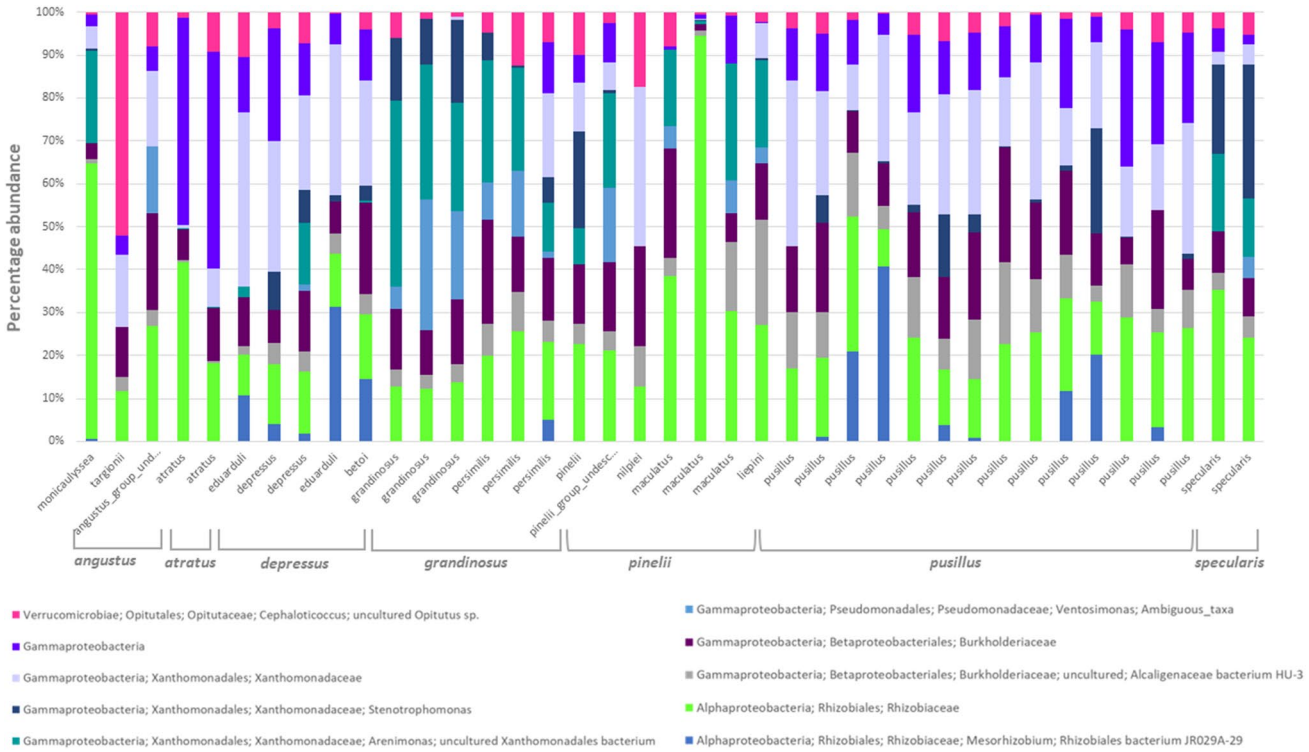

Fig. 2 Ten most prevalent taxon-assigned ASVs found in the bacterial community of each *Cephalotes* sample, shown here as percentage abundance of each taxon in each sample. Samples are labeled on the *x*-axis by species grouped into denoted species groups below the species labels. Each of the ten assigned ASVs includes the lowest level of taxonomic information available and corresponds to one of eight colors in the figure legend. Other taxon-assigned ASVs that were not in the top ten are listed in Supplement 3

richness (p-value = 0.005, pseudo-F = 2.412). These results suggest that the environmental differences between the "high" and "low" elevation groups are affecting community composition and abundance. The analysis of the "core" bacterial community showed no significance in the Bray-Curtis metric (p-value = 0.430, pseudo-F = 1.003) but was significant with the Jaccard metric (p-value = 0.043, pseudo-F = 2.529). Finally, the results of the smaller experiment with *C. pusillus* samples collected in 2017 showed no significant differences in beta diversity metrics between the "low," "mid," and "high" groups.

Table 1 Alpha diversity (Faith's phylogenetic diversity and Shannon index) of all groups compared with Kruskal-Wallis tests. Pairwise Kruskal-Wallis test results of alpha diversity metrics of both the complete and "core" bacterial communities are available in Supplementary Materials 5 and 6, respectively

	Faith's phylogenetic diversity		Shannon diversity	
	H	*p*-value	*H*	*p*-value
Total bacterial community				
Species	15.96266799	0.251151641	15.43072839	0.281240044
Species group	6.320524307	0.388258795	2.615297826	0.855347414
Elevation	3.691468254	0.054691452	2.786706349	0.095049405
Geographic location	2.962667994	0.227334223	3.4646016	0.176876983
Core (50%) bacterial community				
Species	16.35519956	0.230480025	18.53276921	0.138317863
Species group	**12.85180295**	**0.045450629**	**14.17507881**	**0.027740422**
Elevation	0.89379142	0.344451933	0.223214286	0.636601633
Geographic location	2.23172363	0.327632798	1.645365088	0.439251761

Boldface *p*-values indicate significance

Table 2 Beta diversity (Bray-Curtis dissimilarity and Jaccard index) of all groups compared with PERMANOVA. Pairwise PERMANOVA results are available in the Supplementary Materials

	Bray-Curtis		Jaccard	
	p-value	Pseudo-*F*	*p*-value	Pseudo-*F*
Total bacterial community				
Species group	**0.001**	**4.574191**	**0.001**	**3.813063**
Elevation	**0.027**	**1.9591**	**0.005**	**2.412073**
Geographic location	**0.023**	**1.704713**	**0.002**	**1.947414**
Core (50%) bacterial community				
Species group	**0.001**	**2.450132**	**0.001**	**5.412761**
Elevation	0.43	1.00312	**0.043**	**2.528923**
Geographic location	0.571	0.837833	0.423	1.016147

Boldface *p*-values indicate significance

Beta Diversity and the Influence of Geographic Location

PERMANOVA results between the two different geographic regions SW and SE were significant when compared with Bray-Curtis distance (p-value = 0.023, pseudo-F = 1.705), and Jaccard dissimilarity (p-value = 0.002, pseudo-F = 1.947) (Table 2). However, the "core" bacterial communities were not significantly different between the different collection locations in both the Bray-Curtis dissimilarity (p-value = 0.571, pseudo-F = 0.837) and Jaccard dissimilarity (p-value = 0.423, pseudo-F = 1.016). Figure 3B shows a PCoA figure of the Bray-Curtis distance between samples, labeled by the SW and SE geographic regions.

Mantel tests were performed to test for correlations between geographic distance and bacterial community dissimilarity. The tests used a distance matrix constructed using latitude and longitude values and distance matrices

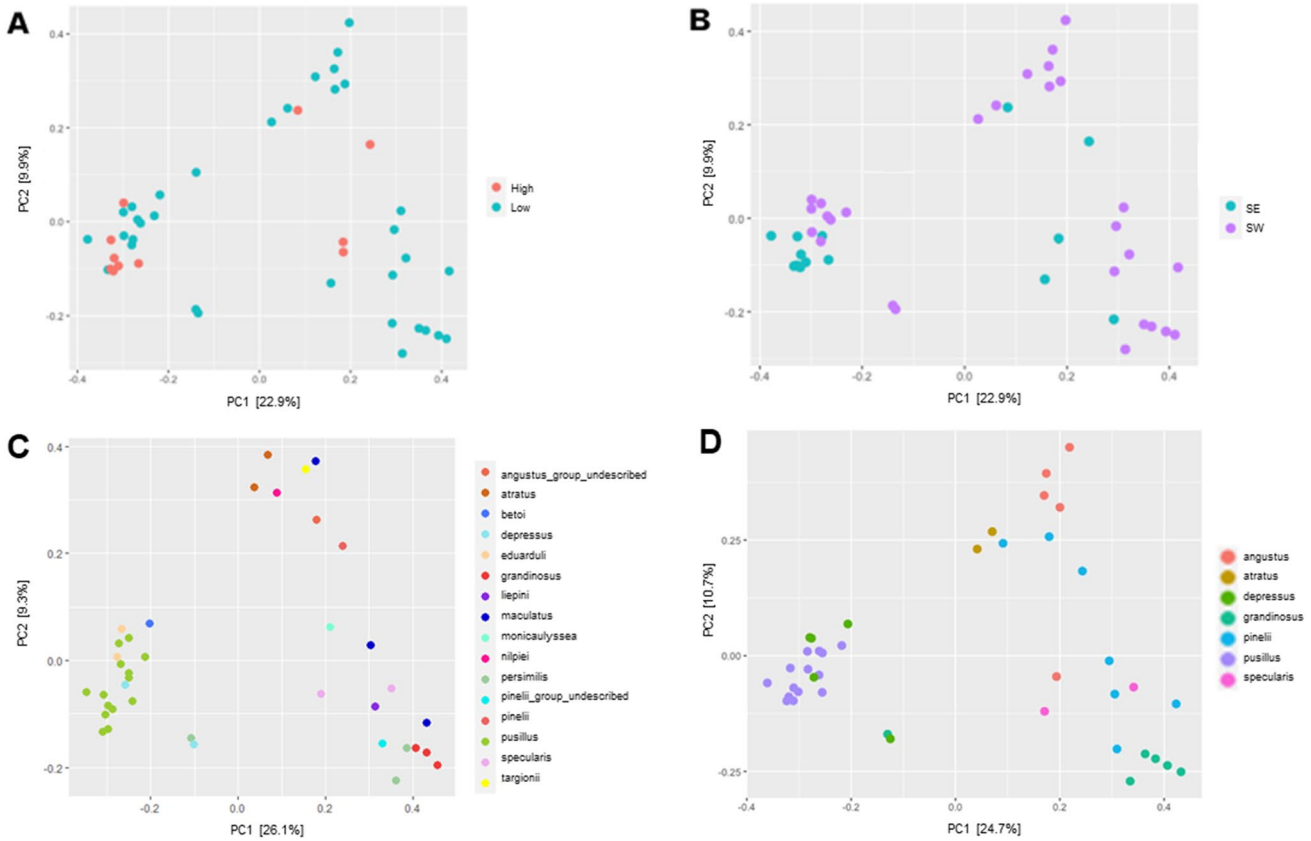

Fig. 3 Principal Coordinate Analysis (PCoA) plots of *Cephalotes* total bacterial communities at 2000 sampling read depth. **A** Bray-Curtis distance metric of samples collected at "high" altitudes (>1000m) and "low" altitudes (<1000m). Different colors indicate to which altitude group samples belong. **B** Bray-Curtis distance metric of samples collected from different locations in the Brazilian Cerrado. Two different colors indicate which of the two locations ("SE" and "SW") samples were collected. **C** Bray-Curtis distance metric of *Cephalotes* species included in the analysis. Sixteen total species are indicated by sixteen different colors. **D** Bray-Curtis distance metric of *Cephalotes* species groups. Seven different species groups are indicated by seven different colors. Significance was obtained by PERMANOVA

generated with Bray-Curtis and Jaccard. The Mantel test performed with the Jaccard distance matrix produced significant values (p-value = 0.0057, Mantel statistic = 0.151), but the test performed with the Bray-Curtis distance matrix did not (p-value = 0.714, Mantel statistic = 0.065).

Beta Diversity and the Influence of Species Group

To investigate the impact on bacterial communities of phylogenetically closely related species, samples were grouped together in taxonomically and phylogenetically defined species groups. Beta diversity PERMANOVA (pairwise and all-groups) requires more than one sample per category, so using species group allowed us to examine the influence of relatedness even though we had only one sample for some species (although species and species group are both represented in Bray-Curtis PCoA figures in Figs. 3C and D, respectively). Species group significantly predicts bacterial diversity when assessed with Bray-Curtis and Jaccard PERMANOVA (Bray-Curtis, p-value = 0.001, pseudo-F = 4.53603; Jaccard, p-value = 0.001, pseudo-F = 3.813). Results from analyses of the "core" bacterial community show similar significance to that of the total community (Bray-Curtis, p-value = 0.001, pseudo-F = 2.450; Jaccard, p-value = 0.001, pseudo-F = 5.412).

The *Cephalotes pusillus* species group (here representing both a species and a species group) differed significantly from all other groups in PERMANOVA comparisons; Figure 3B shows differences within *C. pusillus* between populations in the "SW" and "SE" geographic locations. Beta diversity analyses were performed on only *C. pusillus* samples using both geographic location and elevation as variables. Comparisons between geographic locations produced significant values (Bray-Curtis, p-value = 0.002, pseudo-F = 3.326; Jaccard, p-value = 0.001, pseudo-F = 4.512), as did comparisons between "high" and "low" elevations (Bray-Curtis, p-value = 0.009, pseudo-F = 2.592; Jaccard, p-value = 0.009, pseudo-F = 2.423). These results are illustrated in an NMDS plot in Fig. 4.

Results of Analysis of Composition of Microbiome

The Analysis of Composition of Microbiome (ANCOM) was performed to determine if certain bacterial taxa were influencing differences between elevations, geographic locations, species, and species group identity. No differently abundant bacterial taxa were found with significant differences between elevations, geographic locations, and species, but significant differences in abundance were found between *Cephalotes* species groups. Ten taxa were found to have significant differences in abundance between species groups: an undetermined Betaproteobacteriales genus (W = 118), *Mesorhizobium* (W = 118), *Arenimonas* (W = 117), *Ventosimonas* (W = 117), an undetermined Bacteroidia genus (W = 115), an undetermined Xanthomonadaceae genus (W = 113), an undetermined Gammaproteobacteria genus (W = 113), *Lysobacter* (W = 112), *Parviterribacter* (W = 110), and *Leucobacter* (W = 107). ANCOM was also performed on only the *Cephalotes pusillus* samples to examine bacterial taxa that may be contributing to bacterial community differences within that species/species group, but no significant taxa were found.

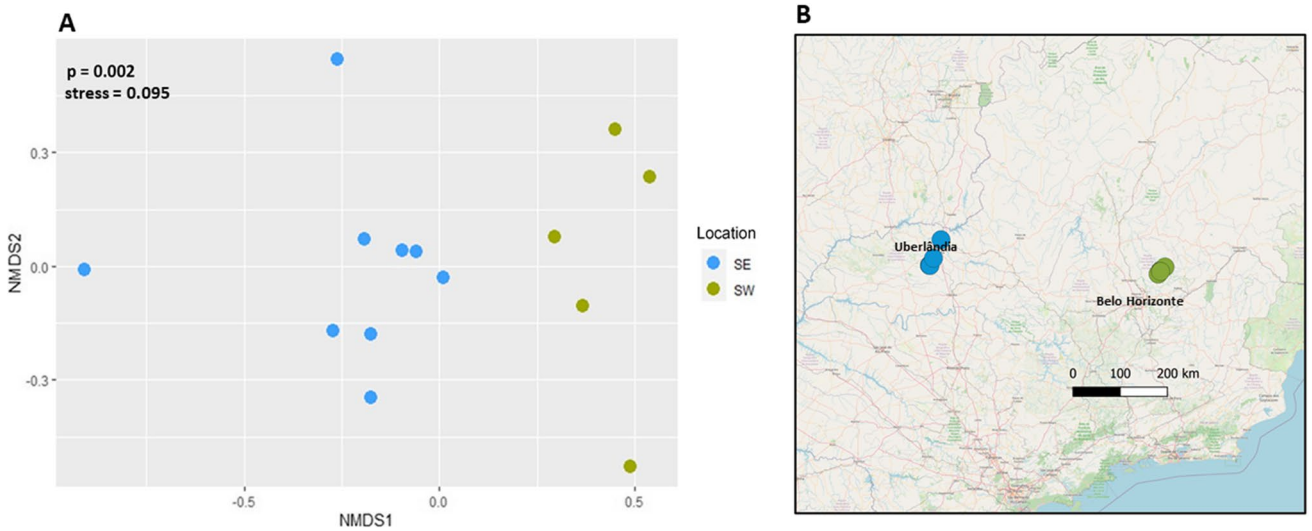

Fig. 4 Bray-Curtis NMDS plot of **A** *Cephalotes pusillus* samples including **B** a map of the collection sites, with Southwest (SW) and Southeast (SE) location groups labeled by color. Significance (p-value) was obtained by a PERMANOVA, and the stress value was found using the "ordinate" function from the R package phyloseq

Discussion

In our study, we used 16S rRNA amplicon sequencing to examine the bacterial communities of *Cephalotes* ants. We determined, using alpha and beta diversity metrics, that elevation, geographic location, species identity, and species group membership all significantly impacted the *Cephalotes* bacterial community to varying degrees. Ours is the first study to investigate the effects of elevation on the composition and abundance of ant bacterial communities and is among only few to investigate elevation as a factor impacting diversity in insect bacterial communities, generally.

Elevation Had a Small Effect on *Cephalotes* Bacterial Communities

When complete and "core" bacterial communities were compared by differences in elevation—in this case, pairwise between samples collected at 1000 m elevation and above and samples collected below 1000 m elevation—the complete bacterial community showed significant differences in composition and abundance, and the core bacterial community showed significant differences in composition. This finding suggests that low-elevation areas of the Cerrado may have different environmental effects on *Cephalotes* bacterial communities than high-elevation areas of the Cerrado.

Few published studies have explored the effects of elevation on insect bacterial communities, but those that have found similar results. Elevation had a small but significant effect on the bacterial communities of *Drosophila* pupae from an Australian rainforest, leading the authors to propose that variations in temperature associated with differences in elevation, and the changes in flora that these microclimates bring about may influence the bacterial communities of *Drosophila* and other insects [6]. Note that the collection altitudes in [6] (max, 880m; min, 70m) are lower in overall elevation but are 810m apart at their most different, not unlike the difference of 756m (max, 1293m; min, 547m) of our study. In contrast, the study of *Apis cerana* microbial communities [60] had collection elevations of 1 to 2268m, a nearly three-fold increase in range of elevation compared to study of Australian *Drosophila*. Though Sudhagar et al. [60] used a method of culturing gut bacteria and amplifying and sequencing 16S rDNA sequences and did not perform beta diversity tests like we did in this study, the authors found notable differences in composition between the high (>1000m), moderate (1000–500m), and low (100–500m) populations. Similarly, in a study of *Lasioglossum*, bees specimens from elevations varying from 830 to 3780m found that alpha diversity of ASVs in the gut bacterial communities of *Lasioglossum* bees increased with elevation, and that certain families of bacteria were more or less prevalent at higher elevations [33].

The influence of elevation in this study is particularly difficult to parse out because the highest elevation samples were largely from the same locality; if geographic location is influencing differences in bacterial communities, it may overshadow differences seen by just changes in elevation alone. Most samples were collected below 1000 m elevation (practically, around 800 meters elevation); these samples as a group showed a large variation in bacterial community composition and differences. Future studies may benefit from a higher diversity of collection locations and elevations within the Cerrado; a higher resolution of collection sites could reveal further variation within *Cephalotes* bacterial communities.

Species Group and Geographic Location Both Determine Bacterial Community Diversity

Overall, host species and species groups predicted the most significant differences in beta diversity and alpha diversity (among "core" bacterial communities) in *Cephalotes* bacterial communities in the Cerrado. This result is consistent with other studies exploring the effects that species and phylogenetic relatedness have on the *Cephalotes* bacterial community [16, 56]. In particular, the *Cephalotes pusillus* species and species group (as that species was the only included member of the *pusillus* species group), as well as the *Cephalotes depressus* species group to a slightly smaller extent, showed distinctive bacterial communities compared to the other species and species groups (Fig. 3D). These highly similar clusters have smaller sub-clusters based on geographic location and, specifically, the dissimilarity between the Southwest and Southeast location groups (the Northeast location group was represented by only one sample, so its effects are likely shadowed by the other two groups). This SW-SE dissimilarity is apparent in Fig. 3B in the highly similar cluster of *C. pusillus* and *C. depressus* samples within coordinates (−0.4, 0.1) and (−0.2, −0.1) and in the split among only *Cephalotes pusillus* in Fig. 4. Though species group has a large effect on the *Cephalotes* bacterial community diversity, geographic location has a notable effect on this community diversity as well and may suggest that some parts of the bacterial community are being acquired from the local environment.

The SW group, close to the western border of Minas Gerais (Brazil), is exclusively mid-elevation Cerrado savanna. The SE group is more central in the state and features samples collected mainly from hilly, rocky rupestrian grassland at higher altitudes. Soil bacteria in these regions differ significantly, with lower to mid-elevation Cerrado featuring more Proteobacteria and Actinobacteria and rockier, higher-elevation parts of the Cerrado featuring more Acidobacteria

and Firmicutes [48]. If bacteria are being environmentally acquired, these trends in soil bacteria may explain some of the differences in bacterial community composition. Results of ANCOM on all samples and on only *C. pusillus* samples, however, showed no significantly different taxa between the SW and SE locations, and all populations are very high in Proteobacteria (Fig. 5).

Ten Bacterial Taxa Are Driving Diversity Among *Cephalotes* Species Groups

The results of the ANCOM comparing *Cephalotes* species groups help explain the differences in community shown via Bray-Curtis PERMANOVA. While ten ASVs were identified as having significantly different abundance between species groups, only five taxa could be categorized to genus, and many of these have already been identified as part of the *Cephalotes* conserved core microbiome. Results of ANCOM corroborate that *Cephalotes pusillus* differs the most from other species groups in composition—*Cephalotes pusillus* completely lacks five taxa prevalent in other species groups (Fig. 6).

The PCoA results in Fig. 3D show that the *C. pusillus* and *C. depressus* species groups bacterial communities are the most similar to each other. However, results of ANCOM show variation in the bacterial communities of the *C. pusillus* and *C. depressus* species groups. The *Cephalotes depressus* species group only lacks one taxon that *C. pusillus* lacks, an undetermined Bacteroidetes that is found in relatively low abundance in all other groups. This similarity between the two species groups may be explained instead by one prevalent taxon, *Mesorhizobium*, of which both groups have high levels and are absent or found in very low levels in all other species groups. *Mesorhizobium* is a soil bacterium commonly associated with legume root nodules but has been found in ant bacterial communities. Birer et al. [3] identified the genus in cuticular bacterial communities of two ants that nest together inside root systems of epiphytic plants. Ishak et al. [23], when examining both external and internal bacterial communities in *Solenopsis* ants, found the genus in moderate abundance in *Solenopsis geminata* alates [23]. *Mesorhizobium* has not been identified in microbial surveys of Cerrado soil, but other nitrogen-fixing bacterial genera *Bradyrhizobium* and *Rhizobium* have been found in areas of the Cerrado with increased agriculture, particularly areas with soybean cultivation and pastureland [48]. It is possible that increased agricultural use of the Cerrado, and subsequent environmental acquisition of these agriculturally impacted soil bacterial communities, may explain the prevalence of *Mesorhizobium* in *Cephalotes* bacterial communities.

Additionally, both the *C. pusillus* and *C. atratus* groups lack *Ventosimonas* (Gammaproteobacteria: Pseudomonadales: Ventosimonadaceae), a novel bacterial genus isolated in *Cephalotes varians* in 2016 [28]. *Ventosimonas* is considered part of the "core" microbiome

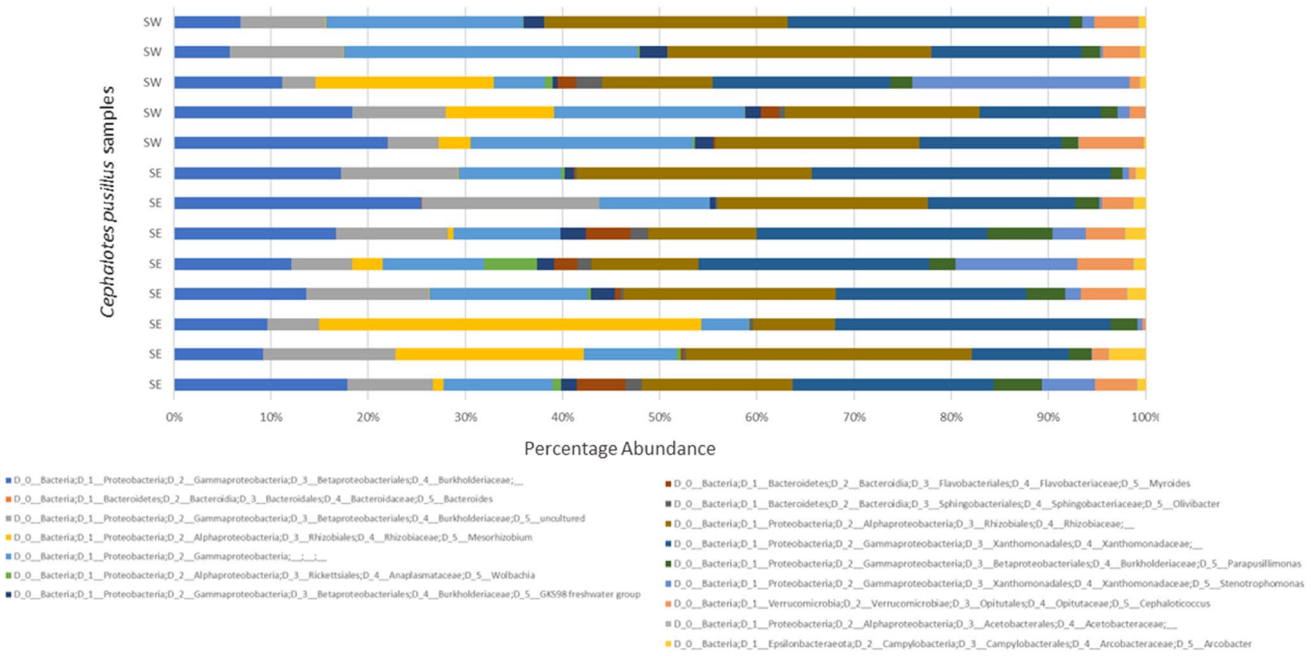

Fig. 5 Taxon-assigned ASVs found in the *Cephalotes pusillus* species groups from the Southwest and Southeast location groups. The most abundant thirteen taxa (representing >98% of community diversity) are included

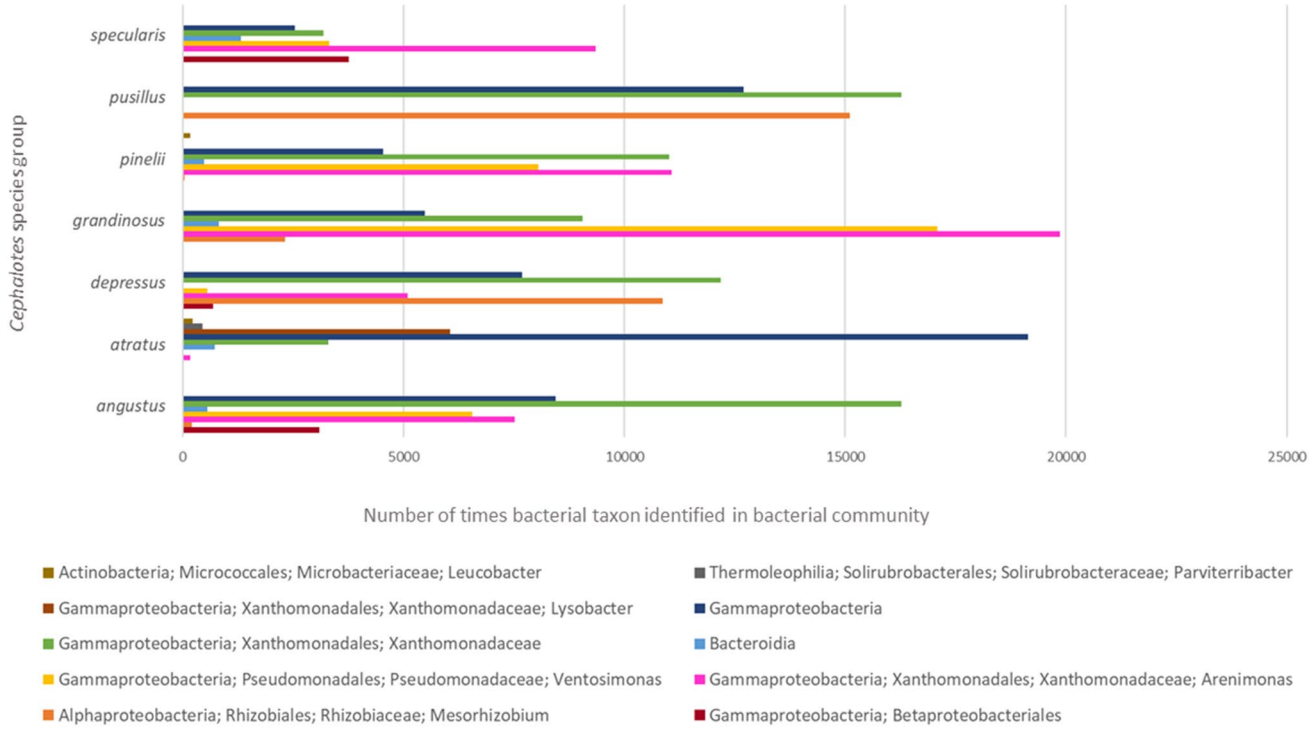

Fig. 6 Results of ANCOM. Bacterial taxa that show significant differences in abundance between each species groups bacterial community are shown, along with the number of times each taxon occurs in each species group bacterial community. Bacterial taxa are identified to genus if possible, and if not, to the lowest taxonomic rank able to be determined

of *Cephalotes*, so it is somewhat surprising that we did not detect it in *C. pusillus* and *C. atratus* [10]. Little else is known about Ventosimonas, as the genus has not been identified outside of the *Cephalotes* gut bacterial community. Additionally, little to no Pseudomonadales, the order in which *Ventosimonas* is included, was found in the *C. pusillus* and *C. atratus* bacterial communities, further setting these two species groups apart from other *Cephalotes* bacterial communities. Compared to other *Cephalotes* species featured in this analysis, *C. pusillus* and C. *atratus* are by far the most generalized in nesting ecology [43, 44] and diet ([11]; Powell pers. obs.), and this significant difference in bacterial community composition may be reflective of this high level of variability in the use of shelter and food resources relative to other species.

Conclusion

In this study, we examined the bacterial communities of forty-one different *Cephalotes* samples from the Brazilian Cerrado. The samples consisted of sixteen species from four locations in the Cerrado that varied in elevation. Though *Cephalotes* ants have highly conserved bacterial communities, significant differences in diversity were observed between groups based on elevation, locality, species, and species group. Although our results suggest that different altitudes may impact the bacterial communities associated with turtle ants, the most significant differences in bacterial composition and abundance were driven by geographic location, species, and species group. Specifically, differences between the Southwest and Southeast geographical location showed significant impacts on the bacterial community. The *Cephalotes pusillus* species group was the most different from all other species groups, but all species groups showed variation in bacterial community composition.

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Author Contribution All authors contributed to developing the overarching project ideas, while LCG and MOR developed the hypotheses tested in the study and led the research. MOR performed DNA extractions and 16S rRNA library prep. LCG did analyses, wrote the

manuscript, and made all figures, tables, and supplementary materials. SP collected and provided ant specimens. All authors discussed and contributed to the final manuscript.

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Data Availability All raw sequence data are publicly available in the NCBI SRA accession number PRJNA859790 and BioSample SUB11807550

Declarations

Competing Interests The authors declare no competing interests.

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