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"Ecological and evolutional implications of ant-microbiome associations"

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P r e s e n t e:

Por medio de la presente hacemos constar que el trabajo de Tesis titulado **"Ecological and evolutional implications of ant-microbiome associations",** fue realizado por el candidato a Doctor en Ciencias, el ***MC Mario Josué Aguilar Méndez*** para obtener el grado de Doctor en Ciencias en Biología.

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Hacemos constar que el trabajo es original, y se realizó con la calidad y rigor científico requerido **y está concluido, por lo que, los abajo firmantes, directores de Tesis, autorizamos continuar con los trámites correspondientes para la obtención de grado.**

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To Estela for the past

To Dante for the future

ABSTRACT

Host-microbial gut interactions have been studied in a large variety of taxa. In almost all groups, a strong relationship between microbes and their hosts has been found but the evolutive and ecological mechanisms that determine those associations haven't been fully described. Ants (Hymenoptera: Formicidae) are an interesting model for exploring the ecological and evolutive implications in the microbiome stability. They are the dominant insect group in most terrestrial habitats and exhibit diverse life history strategies. They have a remarkable evolutionary strategy - eusociality, with overlapping generations within the colony, a cooperative brood care and a reproductive division of labor. The close relationship between individuals in the colony provides higher microbiome stability among the nestmates. The constant grooming in ants often prevents the growth of bacteria on the surface of individuals and influences the microbial communities inside the nest. Many ant species have glands that secrete a broad range of chemicals, usually with antimicrobial properties that enforce the aseptic conditions in the colony. We theorize that grooming, eusociality and gland products generate a highly selective pressure on the microbial communities associated with ants and could direct a highly specialized microbiome or core microbiome. Here we propose a comparison of the ants' metagenome from a range of species that differ in their trophic strategies, nesting preferences, habitat and phylogenetic relationship. We hypothesize that the trophic strategies could be the principal factor that leads the microbial community composition among the ant´s species, and the environmental gradient will be the main factor that influences the microbial community at the population level. Ants were collected from different habitats in México (deserts, tropical rain forest, cloud forests) and from all the main subfamilies. Gasters from seven individuals per nest have been dissected and DNA extracted using the power soil modified protocol, and the V4 region of the bacterial 16S SSU rRNA was amplified in an Illumina MiSeq sequencer. COI sequences from ants of the same colonies were also amplified. We retrieved 74 different ant species from 334 nests across 46 different habitats in México ranging from Cuatro Cienegas, Coahuila, to Bacalar, Chetumal. We report one new record in México and 19 new records (state-based) of 14 species. We describe the core metagenome of 243 colonies representing 7 ant subfamilies and we compare them at different taxonomic levels. We discuss the importance of the ant´s evolutive history and environment as factors that could give stability to the core microbiome and we analyze the core microbiome among different populations of species from the genera *Camponotus*, *Pogonomyrmex* and *Atta*. We propose that at higher taxonomic levels the microbiome of 5 of the 7 subfamilies could be the principal factor for microbiome stability. Finally, we discuss the possible implications of the high abundance of few sOTUs of *Wolbachia* among our samples.

Keywords:

FORMICIDAE, Holobiont, Metagenome, Microbiome, Molecular Microbial Ecology

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TABLE OF CONTENTS

INTRODUCTION 10
Ants as an evolutionary model 11
Sources of variability in ants 13
HYPOTHESIS 14
GENERAL OBJECTIVE 15
SPECIFIC OBJECTIVES 15
MATERIALS AND METHODS 16
Ant colonies collection and preservation 16
Ant identification 17
Metagenomic analysis 17
DNA extraction 17
Primary PCR (quality control) 18
Amplicon library preparation and sequencing 18
Bioinformatic analysis 20
Ant Phylogeny analysis 22
DNA Extraction 22
Amplification and sequencing 22
Bioinformatics 23
Population delimitation of selected ant species 23
RESULTS 24
Distribution and habitat preferences of ants 24
New records 25
Metagenome nucleic acids concentration and amplification 26
Microbial community associated to the abdomen of the ants (metagenome) 28
Wolbachia persistence 43
Phylogenetic relationships of COI among ant selected species. 45
Populations of selected ant genera (Atta, Camponotus and Pogonomyrmex) 45
Microbiome among closely related species and populations 55
DISCUSSION 63
Ant's microbiome (the core metagenome) 65
Bacterial diversity patterns among populations of selected ant species 68

Pathogenic bacteria and the importance of Wolbachia 71
CONCLUSIONS 72
APENDDIXES 73
REFERENCES 83

LIST OF FIGURES

Figure 1. Distribution of the collected colonies. Samples were collected across 14 states of México on a habitat gradient. 24
Figure 2. Distribution of the collected colonies from the Camponotus, Atta and Pogonomyrmex genera. Camponotus were mostly found in the central area of the sampled sites, Pogonomyrmex on the northern-central and Atta on the southern-central sites. 25
Figure 3. DNA concentration of ant´s abdomen vs amplification success. On average, positive reactions (n=259) had almost double the DNA concentration of negative reactions (n=42), a significant difference even where there are overlapping deviations (t=-8.9638, p<0.001). 27
Figure 4. Amplification success of the ant subfamilies. Frequencies of most subfamilies are similar on positive and negative reactions; only Dorilyne had 100% positive reactions............................. 28
Figure 5. Frequency histogram of the demultiplexed sequences per sample. Sequence frequency is without any filtering. 29
Figure 6. Q-score average of each sequence position of the demultiplexed sequences. Sequences quality scores are between Q40 and Q30. 29
Figure 7. Shannon´s diversity rarefaction curves for each subfamily. Asymptote is reached at 250 sequences of random sampling. 30
Figure 8. Relative frequency of bacterial orders among ant subfamilies. Black and white bars represent different ant subfamilies, from top to bottom (Dolichoderinae, Dorylinae, Ectatomminae, Formicinae, Myrmicinae, Ponerinae and Pseudomyrmecinae). The last 5bars represent the bacterial orders in the controls. Color bars represent the relative frequency of bacterial orders in each sample, 8 most abundant sOTUs color code is shown. 31
Figure 9. Relative frequency of bacterial sOTUs among ant species (1. Azteca velox, 2. Dolichoderus bispinosus, 3. Dorymyrmex insanus, 4. Dorymmyrmex smithii 5. Liometopum apiculatum, 6. Pseudomyrmex elongatus, 7. P. ferrugienus, 8. P. gracilis, 9. P. pallidus, 10. P. seminole, 11. P. spinicola) of the subfamilies Dolichoderinae (A) and Pseudomyrmecinae (B). Color bars represent the relative frequency of bacterial sOTUs in each sample, 8 most abundant sOTUs color code is shown. 32
Figure 10. Relative frequency of bacterial sOTUs among ant species (1. Eciton burchelii 2. E. hamatum 3. E. mexicanum 4. Labidus praedator 5. Neivamyrmex cornutus 6. N. melanocephalus 7. N. rugulosus 8. Leptogenys elongata 9. Neoponera apicalis 10. N. villosa 11. Odontomachus clarus 12. O. laticeps 13. O. opaciventris 14. Pachycondyla harpax) of the subfamilies Dorylinae (A) and Ponerinae (B). Color bars represent the relative frequency of bacterial sOTUs in each sample, with color code shown for the 8 most abundant sOTUs. 34
Figure 11. Relative frequency of bacterial sOTUs among ant species of the subfamily Formicinae: Camponotus abscisus (A1), C. andrei (A2) C. atriceps (A3), C. claviscapus (A4), C. conspicuus zonatus (A5) C. coruscus (A6), C. festinatus (A7), C. minus (A8), C. planatus (A9), C. rectangularis (A10), C. rectangularis rubroniger (A11), C. sericeiventris (A12), C. striatus (A13), C. trepidulus (A14), Colobopsis etiolata (B), Formica propatula (C1), Formica subcyanea (C2) Lasius mexicanus (D), Myrmecocystus depilis (E1) M. melliger (E2). Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 8 most abundant sOTUs shown in color code. 36
Figure 12. Relative frequency of bacterial sOTUs among ant species. 1. Ectatomma ruidum 2. E. tuberculatum 3. Acromyrmex obtuspinosa 4. A. versicolor 5. Aphaenogaster mexicana 6. Atta

cephalotes 7. A. mexicana 8. A. texana 9. Cephalotes bimaculatus 10. C. goniodontes 11. C. minutus 12. C. umbraculatus 13. Crematogaster crinosa. 14 C. laeviuscula 15. C. opaca 16. Novomessor encifer 17. Pheidole c.f. gouldi 18. P. obtusospinosa 19. Pogonomyrmex barbatus 20. P. maricopa 21. Temnothorax aztecus) of the subfamilies Ectatomminae (A) and Myrmicinae (B). Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 8 most abundant sOTUs shown in color code.. 39
Figure 18. Group significance boxplots for the PERMANVOVA of Pseudomyrmecinae. Both Ectatomminae and Myrmicinae were not significantlye different from Pseudomyrmecinae. 43

LIST OF TABLES.

Table I. Sampling distribution across the fourteen states in México. Localities and habitats are listed in Appendix 1. 17
Table II. Primers used for V4 16S rRNA gene amplification. 20
Table III. List of new state-based registers of the sampled colonies. New records were found in seven states of México, most of them in Guanajuato state. 26
Table 6. Proposed populations defined by geographic distance and COI distances. 55

INTRODUCTION

In nature there is no organism that lacks bacterial contact. This occurs in almost every phase of the life cycle and is a leading force for many mechanisms that modulate those interactions. Both positive and negative interactions are fixed by means of natural selection in several taxa (Celis et al. 2018, Reveillaud et al. 2018, Stępkowski et al. 2018, Tarnecki et al. 2017).

As an ecosystem, the host's living processes dictate the conditions for the bacterial communities, granting shelter and resources to their guests; nevertheless, there are many examples where the host relies on the microbial activity for its own fitness. For these systems, an evolutive model has been proposed, where the host and their symbiotic microbiome (holobiont) with their hologenome (genome and metagenome) act as a unit of selection in evolution. (Zilber-Rosenberg and Rosenberg 2008) Propose that in this model, (i) there is a symbiotic relationship among the host and diverse microorganisms, (ii) there is an effective vertical transmission of those symbiotic microorganisms (iii) this relationship has a direct impact on the fitness of the holobiont and (4) hologenome variation and genetic variation of the holobiont can be enhanced by incorporating different symbiont populations. The holobiont can also change under environmental demand more rapidly by more processes than the genetic information encoded by the host alone. If speciation is driven by symbiosis, microbes should be the cause of reproductive isolation or involved in the evolution of chromosomal speciation genes, acquired by a specific host-microbiome combination that impacts the nutrition, immunity, development or reproduction. (Brucker and Bordenstein 2012)

Considering the eumetazoan set of shared morphological characteristics, bacteria can be present in several regions of the host. They can be found on every surface that derives from the ectoderm and endoderm, but only in some taxa can bacteria be found on tissues that originally derives from the mesoderm. Such hosts could be the best candidates to test the hologenome theory, because in most

of them a specific organ as the bacteriocyte is developed to host its bacterial guests, and bacteria is necessary to complete the metazoan life cycle (Wollenberg and Ruby 2011, Reveillaud et al. 2018, Wernegreen 2002).

In every ecosystem two principal categories of bacteria can be found: first, autochthonous bacteria, present due to a possible dependency on the fluctuations of the oligotrophic conditions, and second, allochthonous bacteria (whose presence is temporal), possibly affected by transmission vectors (fly and nematodes i.e.) and dependent on the occasional increases of the essential nutrient concentration. The host metabolism or the presence of unusual substrates can allow allochthonous bacteria to persist in adverse conditions by the way of dormant forms or resilient structures (Schlegel and Jannasch 1981).

In recent years, great efforts have been made to describe the microbial community associated with animals (Sherrill-Mix et al. 2018), plants and fungi. These findings suggest that only a fraction of the microbial community is autochthonous to the host, but the methods to define the autochthonous community (core microbiome) are quite diverse (Shade and Handelsman 2012, Hernandez-Agreda, Gates, and Ainsworth 2017, Yeoh et al. 2017, Rodrigues et al. 2018) and are not universal for every taxonomic group of the host.

Accessing the core microbiome is necessary to answer questions related to the shared evolutive history among the host and the microbial community, although allochthonous microbiota can also be used to explore the influence on the environment in the host populations.

Ants as an evolutionary model

One of the mayor investments of the ant`s colony is the nest construction and maintenance; this provides a microhabitat with specific and favorable microclimatic conditions that can be also suitable for bacterial growth inside the structure. The sanitization of the nest is another task that

the colony members have. Alongside the fact that construction materials cannot be re-used (Sudd and Franks 1987), sanitization generates a considerable selective pressure for the bacterial community associated within the ant colony. Nesting is also a high source of variability in ants. Some can build the nest in the plains soil, under a rock, on decaying materials on the ground or directly associated to the vegetation, in dead branches, twigs, or in live vegetation. In some cases tree nesting is specific to a tree taxa, as in the mutualism between *Vachellia* spp. and *Pseudomyrmex ferruginea*. Nesting on enclosed environments and construction with surrounding materials do not apply for every ant species as in *Dorylinae* ants.

Trophallaxis (mutual feeding by food or liquids transfer) is a common practice in ants. Both stomodeal (mouth to mouth) and proctodeal (anus to mouth) processes are used to feed the larva but this behavior has also been reported among adults of the colony (Moreira et al. 2006, Richard and Errard 2009, Liebig, Heinze, and Hölldobler 1997, Provecho and Josens 2009). Sharing of the gut content suggests a mechanism that directly transfers the microbial community inside the ant among nestmates. This can be a potential risk of disease spread, but Trophallaxis has also been reported as a mechanism of social immunity (Hamilton, Lejeune, and Rosengaus 2011) in *Camponotus pennsylvanicus.*

Every ant colony relies on eusociality for its functioning and development. This characteristic is defined by three main factors according to (Sudd and Franks 1987): (i) overlapping adult cohorts inside the nest, (ii) cooperative brood care and (iii) sterile of nearly sterile workers or helpers inside the nest.

Microbiomes of eusocial organisms could provide an interesting model because the colonial lifestyle could generate a potential risk of dispersion of pathogenic microorganisms inside the nest, among nests if the species is polydomic or among different species if interspecific interaction is present.

So far, Camponotinni is the only tribe of ants where we can find well defined specialized cells to host a specific bacteria genus, *Blochmannia*. These bacteria-filled cells or bacteriocytes can be found in the adult digestive gut. (Stoll et al. 2010) tracked those cells in the larval development and (Sauer et al. 2002) found *Blochmannia*-filled bacteriocytes in the ovaries of mature queens, suggesting a highly specialized vertical transference process for this symbiont. Nevertheless, evolutionary time and biogeographical processes lead the separation this ant group and its relationship with the microbiome associated is still unknown.

Sources of variability in ants

All species of ants rely on an enclosed environment (nest) to complete the life cycle. The queen spends most of her life inside the nest structure; thus, the development of mechanisms to keep the nest free of pathogens is crucial for its functioning.

Hymenoptera present a complex system of grooming behaviors (Basibuyuk and Quicke 1999), both self-grooming and mutual grooming, which can prevent the dispersal of a disease in the nest, coupled with such highly diverse mechanisms of nest hygiene as necrophoresis and grooming of the internal walls of the nests (Oi and Pereira 1993).

Physical removal of the possible pathogens is enhanced by the secretions of the ants, which can be produced inside the abdomen (venoms and acid) or those produced by the metapleural or other glands. Metapleural gland secretions are composed mostly of hydrocarbons (carboxylic and fatty acids) with a low protein component and the pH is usually low 2.5 to 3.5), and antiseptic properties have been found in most of the metapleural glands tested (Yek and Mueller 2011). The metapleural gland is found only in ants, but not all species have it; particularly, most species of the megadiverse genus *Camponotus* lack this structure.

Ants’ life stories and phylogenetic history provide a diverse background of different potential selective pressures on the associated microbiome. The mechanisms to retain the beneficial microbes and expulse the pathogens could be driven by different selective pressures and can be inferred by the comparison of the microbial community of different populations and species.

Describing the microbial community of ants and defining the mechanisms that mold it is necessary to understand the mechanisms of microbial dispersion in eusocial organisms, the metabolic input provided by the mutualistic bacteria and the potential pathogens.

Here we propose to explore the factors that could affect the composition of the bacterial community inside the ant´s abdomen using the colony as the unit of study due the bacterial transmission processes among nestmates and the dependence of the enclosed environment. We will perform this comparison at different taxonomic levels in the ants, trying to answer whether the evolutionary history of the ants and traits derived from their life history (habitat, trophic position) affect the microbiome at higher taxonomic levels (subfamily and genus) while at population, species and above species level (closely related selected species lineages) we will explore traits as environmental gradient and spatial distribution.

HYPOTHESIS

Because the processes that act as a selective pressure towards the microbiome associated with the different ants are directly related to the evolutionary history of the host, at higher taxonomic levels (subfamily, genus) the phylogenetic history of the host will be the main factor determining the composition of the bacterial community in the abdomen of the host, at the population level internal factors such as the host´s genetic distance may determine the composition of the bacterial community associated with the abdomen of the ants.

At host higher taxonomic levels:

Ho: phylogeny = habitat

Ha: phylogeny ≥ habitat

At host population level:

Ho: Host´s genetic distance = spatial distribution

Ha: Host´s genetic distance ≥ spatial distribution

GENERAL OBJECTIVE

Describe the metagenome of selected ant species collected across an environmental gradient and compare it with the host's, nesting preferences, habitat preferences and phylogenetic relationship.

SPECIFIC OBJECTIVES

Define and describe the core metagenome in the abdomen of selected ant species collected in Mexico and determine the main factors that influence the bacterial community composition.

Define the traits that influence the bacterial community composition on the metagenome across the phylogeny of ants collected in México.

Describe the metagenome variability at a population level along space and the environmental gradient in ants from the genera *Camponotus, Atta* and *Pogonomyrmex*.

MATERIALS AND METHODS

Ant colonies collection and preservation

We aimed to retrieve abundant and/or ecologically dominant ant species in each habitat, that represent different trophic levels and phylogenetic lineages to allow comparison of the host microbiome within these features. To reduce the microbial contamination of the samples, no bait trap or pitfall traps samples were used in this study; instead, all samples were hand collected *ad libitum* by ethanol disinfected forceps. Whenever possible ants from the same colony were collected directly at the nest entrance (i.e. *Pogonomyrmex* spp.) or at the colony walking path (i.e. *Atta* spp.). No ants from different colonies were pooled for further analysis. Each colony was geopositioned using a Garmin GPSmap 62s or by the app GPS Essentials Ver. 4.4.25. Habitat type associated with each sample was retrieved by the vectoral metadata from INEGI´s Land-Use and Vegetation Chart Series IV (2017 edition) using ArcMap 10.2.0.3348 and corroborated by field notes.

Field collections were performed from March of 2016 to November of 2017 in 14 states of México. Collection habitat sites (TABLE I) represented a diverse ecological community that allowed us to compare this feature to the host metagenome. In addition, seven *Atta mexicana* and one *Atta cephalotes* colonies were also retrieved from the social insects functional bioecology laboratory at INECOL, Xalapa.

Table I. Sampling distribution across the fourteen states in México. Localities and habitats are listed in Appendix 1.

State	Collected colonies	Localities	Habitats
Coahuila	27	1	2
Colima	4	1	1
Guanajuato	68	8	8
Jalisco	21	3	5
Michoacán	23	5	5
Morelos	30	1	2
Nayarit	1	1	1
Nuevo León	21	5	6
Oaxaca	17	2	3
Puebla	9	5	5
Queretaro	16	2	6
Quintana Roo	22	5	5
Tamaulipas	39	2	5
Veracruz	32	3	5

Ant identification

Ethanol preserved samples were processed in the Laboratory of Molecular Ecology and Biodiversity at LANASE, ENES, Morelia. At least one ant of each different morphospecies was mounted, and diagnostic morphology was compared to those in the identification keys (Bolton et al. 2006, MacKay and Vinson 1989). Images from the AntWeb database (https://www.antweb.org) were used to compare specific morphology features of each identified species, and previous records of each species were corroborated using the AntMaps database (*www.*antmaps.org), the ant diversity of the Mesoamerican corridor (ADMAC, https://sites.google.com/site/admacsite/home) species list, and the (Vásquez-Bolaños 2015) report for Mexican ants.

Metagenomic analysis

DNA extraction

DNA extraction protocol was adjusted from a previously modified Power Soil (Qiagen©) protocol (Rubin et al. 2014). Whenever possible, seven ants from the same caste of each colony were

transferred to a 0.5 % NaClO solution for 30-60 seconds and then washed with sterile ddH_2O. Abdomens were immediately dissected using sterile forceps, dried at room temperature for two hours in a laminar flow cabinet and transferred to a PowerBead tube with acid washed glass beads (150-212 µm). Tissue was first homogenized using new sterile pestles then ground at 30 Hz for 45 seconds in a Mixer Mill 400 (Retsch ©) adapted with the Qiagen© TissueLyser adapter sets. The tubes were centrifuged at 10000 rcf for 30 seconds and 60 µL of C1 solution (with 20 mg/mL of proteinase K) was added. Samples were incubated overnight (15 h approx.) at 56 °C and a second grinding was performed under the same conditions. The remaining extraction steps were performed as the power soil protocol. DNA was eluted in 100 µL of C6 solution and stored at -20 °C.

Primary PCR (quality control)

In order to test the quality of the DNA extractions, a primary PCR was performed using the 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) primers targeting the V4 region of the bacterial 16S SSU rRNA (Caporaso et al. 2011). This primer set was used without any adapter or barcode. PCR reactions were performed in a 25 µL reaction volume containing 200 nM of each primer, 1X Multiplex PCR Master Mix (QIAGEN) and 2 µL of template DNA. PCR conditions started with an initial melting step at 94° C (15 min), followed by 35 cycles of 94° C (45 sec), 50° C (60 sec) and 72° C (90 sec) and a final extension of 72° C for 5 min.

Amplifications were verified by Electrophoresis Gel and SYBR Safe (ThermoFisher) or by capillarity electrophoresis using the QIAxcel Advanced System (Qiagen). Amplification success was evaluated by DNA concentration using a t-test on R 3.5.1.

Amplicon library preparation and sequencing

We selected 265 samples from those amplified with the 515F-806R primers, along with 4 blank extractions and a user sample. An aliquot of 20 µL of each sample was pipetted in a 96 well of a full skirted PCR plate. Plates were foil sealed with AlumaSeal II Aluminum (Excel Scientific), wrapped and shipped according to the earth microbiome project standards. Plates were sent to the Environmental Sample Preparation and Sequencing Facility from the Argonne National Laboratory, Illinois, U.S.A., where the amplicon library preparation and sequencing were performed.

Amplicon library preparation was performed by the earth microbiome project standards using a construction of the 515FB (Parada, Needham, and Fuhrman 2016) and 806RB (Apprill et al. 2015) primers as follows:

515FB:

AATGATACGGCGACCACCGAGATCTACACGCT-XXXXXXXXXXXX-TATGGTAATT-GT-GTGYCAGCMGCCGCGGTAA

The first part of the construction is the 5′ Illumina adapter followed by the golay barcode to track the sample origin of the sequence (represented with 12 X letters). This sequence was different for each of the 265 samples that we previously selected by using a forward primer pad followed by a two base pair linker and the 515FB primer.

806RB:

CAAGCAGAAGACGGCATACGAGAT-AGTCAGCCAG-CC-GGACTACNVGGGTWTCTAAT

The first part of the construction is the reverse complement of 3′ Illumina adapter followed by the reverse primer pad, the two base pair primer linker and the 806RB primer.

PCR reactions were performed in a 25 µL reaction volume containing 100 nM of each primer, 1X PCR Master Mix and 1 µL of template DNA. PCR conditions started with an initial melting step at 94° C (3

min), followed by 35 cycles of 94° C (45 sec), 50° C (60 sec) and 72° C (90 sec) and a final extension of 72° C for 10 min.

Each sample was amplified in triplicate and pooled in a single volume (75 µL), and amplicons were verified by electrophoresis agarose gel. In each 96 well plate, an equal amount of each sample (240 ng) were combined in a single sterile tube and cleaned using UltraClean PCR Clean-Up Kit (MoBio).

Sequencing was performed by Argonne National Laboratory (Illinois, U.S.A.) in an Illumina MiSeq sequencer using the primers listed in Table II.

Table II. Primers used for V4 16S rRNA gene amplification.

Primer name	primer pad	linker	primer
16SF	TATGGTAATT	GT	GTGYCAGCMGCCGCGGTAA
16SR	AGTCAGCCAG	CC	GGACTACNVGGGTWTCTAAT

Index sequencing primer: AATGATACGGCGACCACCGAGATCTACACGCT

Bioinformatic analysis

Sequences retrieved by Illumina MiSeq were analyzed using QIIME2 (http://qiime2.org/). The pipeline for these methods is available in Appendix 2. Sequences were demultiplexed using only the barcodes from this project on the constructed metatable.

Denoising of the sequences was performed using a 151 bp length for bad quality truncation for both forward and reverse sequences to allow enough overlapping bp. Quality control and chimera were removed using DADA2.

Taxonomy assignation was performed using the SILVA database for the 515-806 region of the 16S ARNr gene. This database was previously trained as a classifier by (Caporaso 2018).

Chloroplast and mitochondrial 16S sequences were removed for the analysis as well as those classified as eukaryotic and unassigned at the first taxonomy level (Domain).

It has been pointed out (Salter et al. 2014) that sample contamination is an important issue to consider during the analysis of sequences retrieved by next generation systems. This is particularly relevant when a bacterial universal primer set is used due their ubiquity (Lachance 2004). Besides the “environmental” contamination (reagents and laboratory based), there is a high chance of cross sample contamination (Sanders pers. com); thus, a scheme for tracking and removing those external sequences should be applied before any bioinformatic analysis.

Using the average of the maximum sequence reads per sOTU of the blank samples we delimitate a lower limit of 751 reads per sOTU all the prior analysis were performed with sOTUs with 721 or more reads.

To track cross sample contamination during manipulation and DNA extraction, batches of 10 samples were used. The order of each sample was registered, and four empty tubes (blanks) were randomly treated as samples in four different batches. To track cross sample contamination during the library preparation and sequencing, the samples were reordered in the PCR plates that we sent to Argonne labs for sequencing. The order was planned to have the maximum inter-well distance between samples from the same species and location.

Filtered sequences were aligned using multiple alignment with fast Fourier transform (MAFFT), with default parameters(Katoh and Standley 2013), non-conserved and highly gapped columns filtered. A gene tree was constructed with Fast tree algorithm with default parameters (Price, Dehal, and Arkin 2010) and rooted on the longest branch.

Alfa diversity metrics were calculated using a sampling depth of 800 sequences per sample to retain most of the blanks for further analysis sOTUs richness. Shannon diversity, Pielou's evenness and Faith’s Phylogenetic Diversity as well as a principal component analysis by the Jaccard, Bray-Curtis, quantitative UniFrac and Qualitative Unifrac distances, were calculated on each colony.

To test the significance among groups in their beta diversity analysis, we used a permutational multivariate analysis of variance (PERMANOVA) test, grouping the colonies by subfamily and running 999 permutations.

To visualize the shared microbial composition among colonies of the selected genera heatmaps for the relative frequencies of each shared sOTUs were created normalizing the frequencies by a log10 factor. Unique sequences were discarded, leaving 42 of the 89 sOTUs for *Camponotus*, 252 of the 890 sOTUs for *Pogonomyrmex* and 142 of the 509 sOTUs for *Atta-Acromyrmex*. Clustering of both axes (ant colonies and sOTUs) was performed by Euclidean distance metric.

Ant Phylogeny analysis

DNA Extraction

DNA was extracted from the full body of an ethanol preserved ant, in some samples more than a single ant was pooled to have enough material for the extraction. Geneaid Genomic DNA Mini Kit (Tissue) was used according to the manufacturer's protocol recommendations but adding the double concentration of proteinase K (40 µL) during an overnight incubation (18 hrs). Eluted DNA was preserved at -20° C for further analysis.

Amplification and sequencing

Cytocrome C oxidase I fragments were amplified using polymerase chain reaction (PCR) with the primers LCO_T7Prom (TAA TAC GAC TCA CTA TAG GG-G GTC AAC AAA TCA TAA AGA TAT TGG) and HCO_T3 (ATT AAC CCT CAC TAA AG-T AAA CTT CAG GGT GAC CAA AAA ATC A) (Folmer et al. 1994). PCR reactions were performed on a 25 µL reaction volume containing 100 nM of each primer, 1X PPP DNA Polymerase Master Mix (Top-Bio) and 2 µL of template DNA. PCR conditions started whit an initial melting step at 95° C (5 min), followed by 35 cycles of 94° C (30 sec), 50° C (50 sec) and 72° C (90 sec) and a final extension of 72° C for 5 min.

Amplicon size was verified using gel electrophoresis and GelRed DNA Stain (Biotium). Positive amplifications were sequenced by Macrogen Inc. (Amsterdam, Netherlands).

Bioinformatics

Sequences were assembled and edited in GENEIOUS v11.1.5 (Biomatters), in order to have background information for the comparison among species. Sequences of closely related species were downloaded from the Barcode of Life Data System (BOLD), (RATNASINGHAM and HEBERT 2007) and aligned in Multiple Sequence Alignment Software (MAFFT) v7.388 (Katoh and Standley 2013). Alignments were trimmed and all gaps were removed. COI Phylograms were constructed in CIPRES Science Gateway V. 3.1 (Miller, Pfeiffer, and Schwartz 2010) using RAxML (Stamatakis 2006) Blackbox algorithm with standard parameters. Sequences will be deposited in BOLD database.

Population delimitation of selected ant species

We use the genetic and geographic information of specimens of *Campononus atriceps*, *Atta mexicana* and *Pogonomrymex barbatus* to perform a population delimitation analysis. We use only those samples whom information of COI sequence, metagenome and geolocation were retrieved.

To delimitate the geographic distance among and between populations we create distance matrix in Qgis Ver. 3.12 and a UPGMA distance dendrogram using PAST Ver. 4.04.

Multiple alignments of COI genes were performed using MUSCLE algorithm in UGENE-Uniprot Ver. 37.0. Phylogenetic trees were constructed using the algorithm RAxML-BlackBox in Cipres Ver. 3.3. outgroup and standard sequences were obtained either from the International Barcode of Life (iBOL) (http://www.ibol.org/) or the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/).

RESULTS

Distribution and habitat preferences of ants

A total of 334 ant colonies were collected across Mexico in 46 localities (Fig. 1) from a variety of habitats (Appendix 1) representing 74 species from 7 of the 11 subfamilies reported to the country according to AntMaps. Nearly 70% of the colonies belonged to the *Formicinae* and *Myrmicinae* subfamilies. The altitude of the collecting sites ranged from 0 to 2793 meters above sea level (MAMSL). Species from the genera *Camponotus*, *Atta* and *Pogonomyrmex* were retrieved from most of the sampled locations (Fig 2.), and their distribution were selected for a subsequent population analysis.

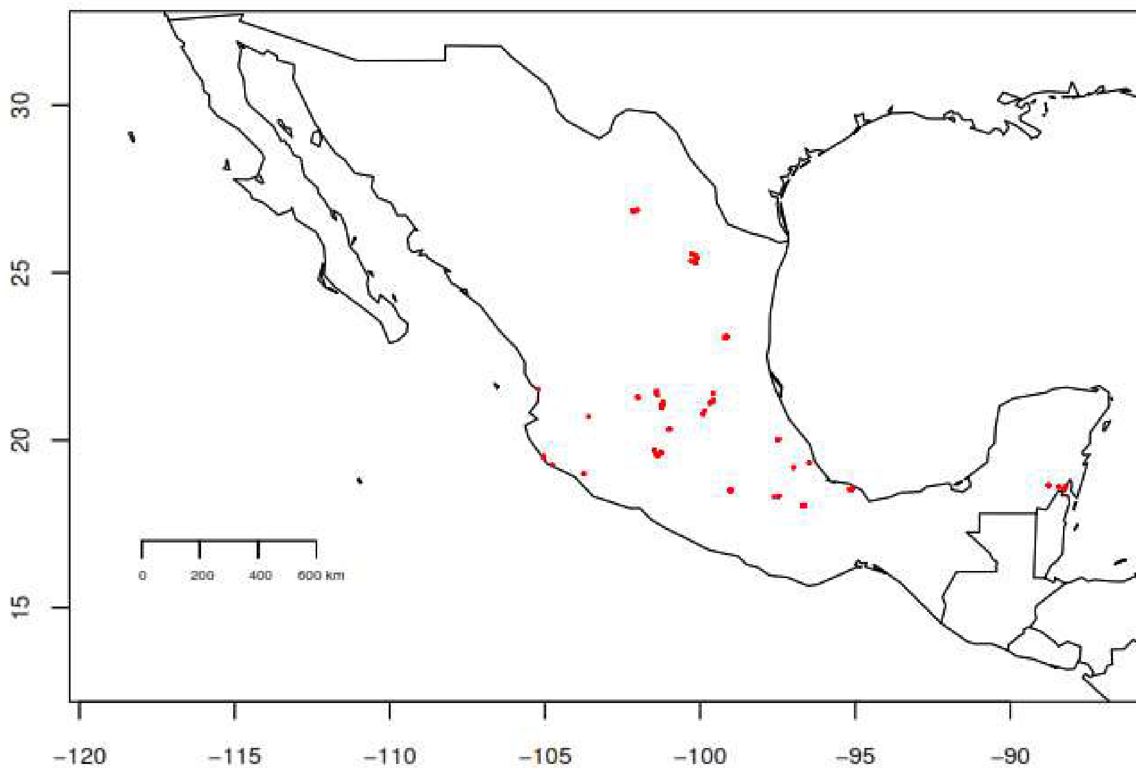

Figure 1. Distribution of the collected colonies. Samples were collected across 14 states of México on a habitat gradient.

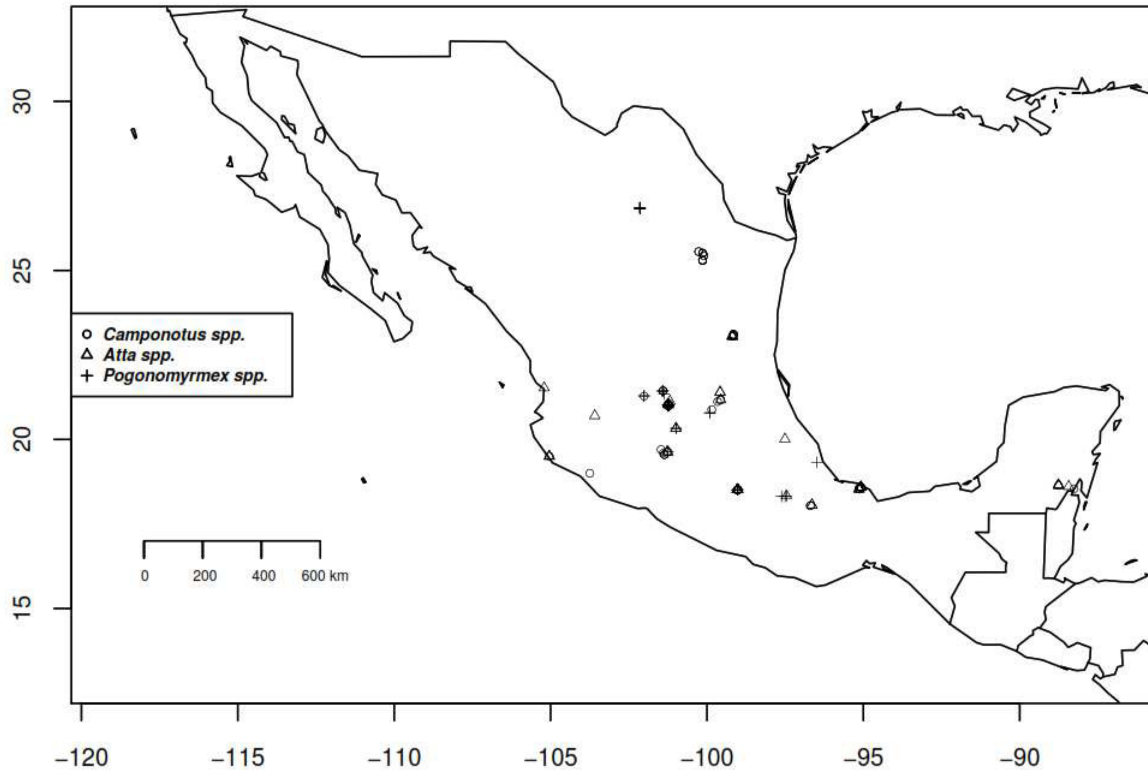

***Figure 2. Distribution of the collected colonies from the Camponotus, Atta and Pogonomyrmex genera**. Camponotus were mostly found in the central area of the sampled sites, Pogonomyrmex on the northern-central and Atta on the southern-central sites.*

New records

After comparing the identified species with the AntMaps.org database, the ADMAC species list and (Vásquez-Bolaños 2015) report of Mexican ants, we found a set of new records for the states of, Guanajuato, Jalisco, Nuevo León, Morelos, Puebla and Queretaro . Identification of those specimens are verified by M. Vazquez-Bolaños (Table III).

__Table III. List of new state-based registers of the sampled colonies__. New records were found in seven states of México, most of them in Guanajuato state.

Genus	Species	State	Elevation (m.a.s.l.)	Latitude	Longitude
Azteca	velox	Morelos	1084	18.5176	-99.006
Dorymyrmex	insanus	Guanajuato	2085	20.32546	-100.997
Dorymyrmex	insanus	Guanajuato	2343.693	20.98923	-101.236
Dorymyrmex	insanus	Guanajuato	2312.664	21.022	-101.224
Dorymyrmex	insanus	Guanajuato	2159.905	20.99989	-101.249
Camponotus	coruscus	Nuevo Leon	422.417	25.44029	-100.096
Camponotus	striatus	Nuevo Leon	628.714	25.55598	-100.265
Formica	propatula	Michoacán	2431.296	19.70182	-101.469
Lasius	latipes	Guanajuato	2462.364	21.43821	-101.406
Lasius	latipes	Queretaro	2575.245	21.11977	-99.6599
Neivamyrmex	melanocephalus	Guanajuato	2405	21.0684	-101.224
Neivamyrmex	rugulosus	Guanajuato	2387	21.06221	-101.227
Syscia	augustae	Queretaro	2575.245	21.11977	-99.6599
Atta	texana	Guanajuato	2296	21.12928	-101.185
Cephalotes	scutulatus	Puebla	615	20.06132	-97.5032
Crematogaster	crinosa	Nuevo Leon	722.906	25.29747	-100.138
Temnothorax	andrei	Guanajuato	2291.956	21.34138	-101.372
Temnothorax	andrei	Jalisco	2054.282	21.28364	-102.011
Stenamma	schmittii	Guanajuato	2291.956	21.34138	-101.372

Metagenome nucleic acids concentration and amplification

DNA extraction was performed on 300 of the collected colonies as described in material and methods. DNA concentration of the dissected abdomens ranged from 47 to 0.7 ng/µL. After running a PCR with the 515F and 806R primers (without linkers) as a primary quality control, only 87% of the samples showed a band on the expected 350 bp size when checked with electrophoresis gels or Qiaxcel runs. Negative reactions had an average of 50% of the DNA concentration of the positive reactions (Figure 3), suggesting that DNA concentration is the main factor for amplification success.

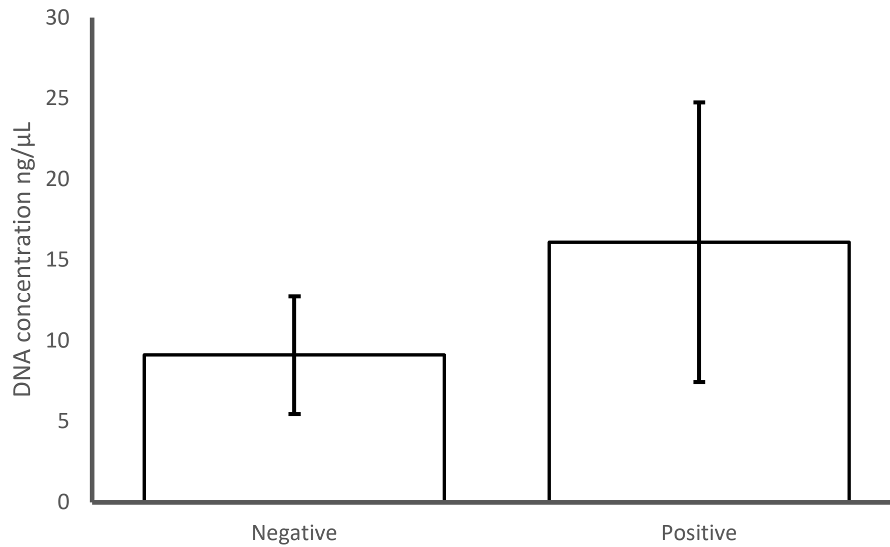

Figure 3. DNA concentration of ant´s abdomen vs amplification success. *On average, positive reactions (n=259) had almost double the DNA concentration of negative reactions (n=42), a significant difference even where there are overlapping deviations (t=-8.9638, p<0.001).*

The amplification success does not seem to be related to the ant subfamily (Figure 4). Most of the ant subfamilies had both positive and negative reactions; only the samples that belong to *Dorylinae* presented 100% positive reactions when amplified with the 515F and 806R primers without linkers.

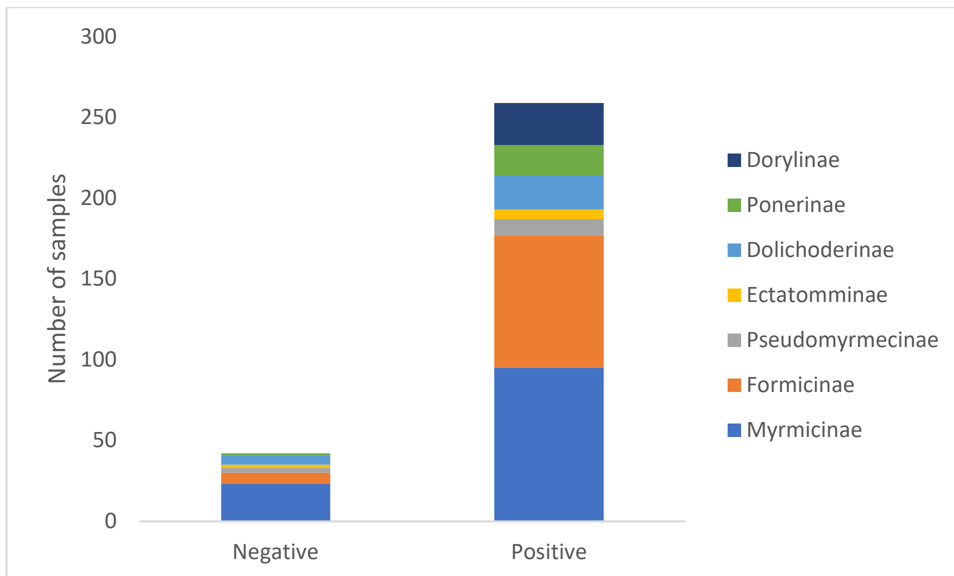

Figure 4. Amplification success of the ant subfamilies. *Frequencies of most subfamilies are similar on positive and negative reactions; only Dorilyne had 100% positive reactions.*

Microbial community associated to the abdomen of the ants (metagenome)

A total of 5.16 X10^6 raw sequences were retrieved after demultiplexing the 266 samples. The highest sequence count for a sample was 45701 sequences, and the minimum two, with a mean of 19403.95 sequences and median of 20545 sequences (Figure 5). Sequence quality score (Q-Score) was on average always higher than 30 (error probability of 0.001, Figure 6.). After removing eukaryotic and non-assigned sequences (at domain level), counts dropped to 4.46 X10^6 sequences with a mean of 16787.53 sequences per sample. Eighteen samples were discarded after limiting the data by a minimum of 800 sequences for the sampling depth. All the blanks remained for further analysis.

Rarefaction curves (Figure 7) show that asymptote is reached at a sampling depth of 250 sequences, even though sampling depth of 800 was used for further analysis to retain all blanks.

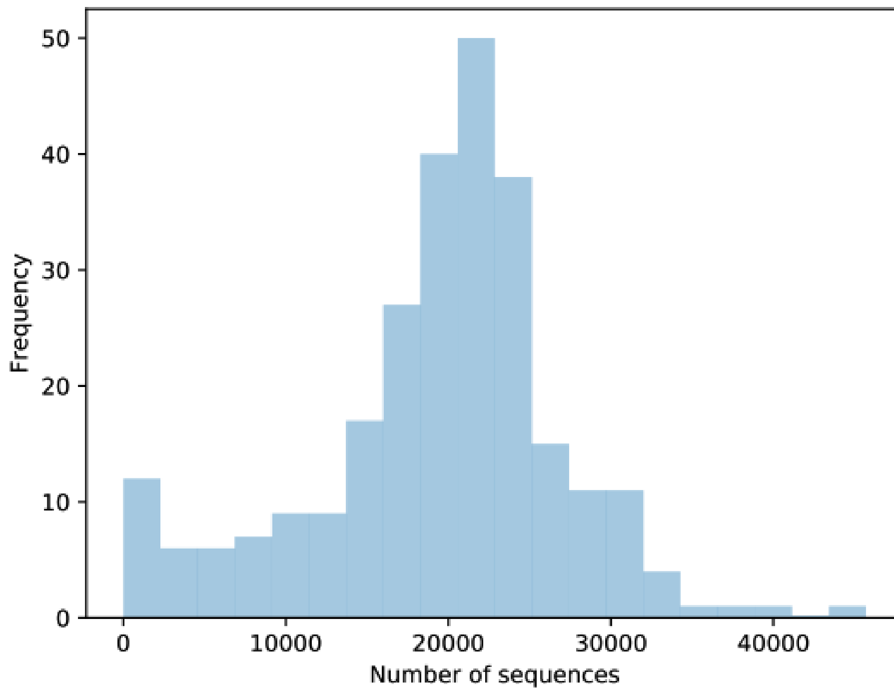

Figure 5. Frequency histogram of the demultiplexed sequences per sample. *Sequence frequency is without any filtering.*

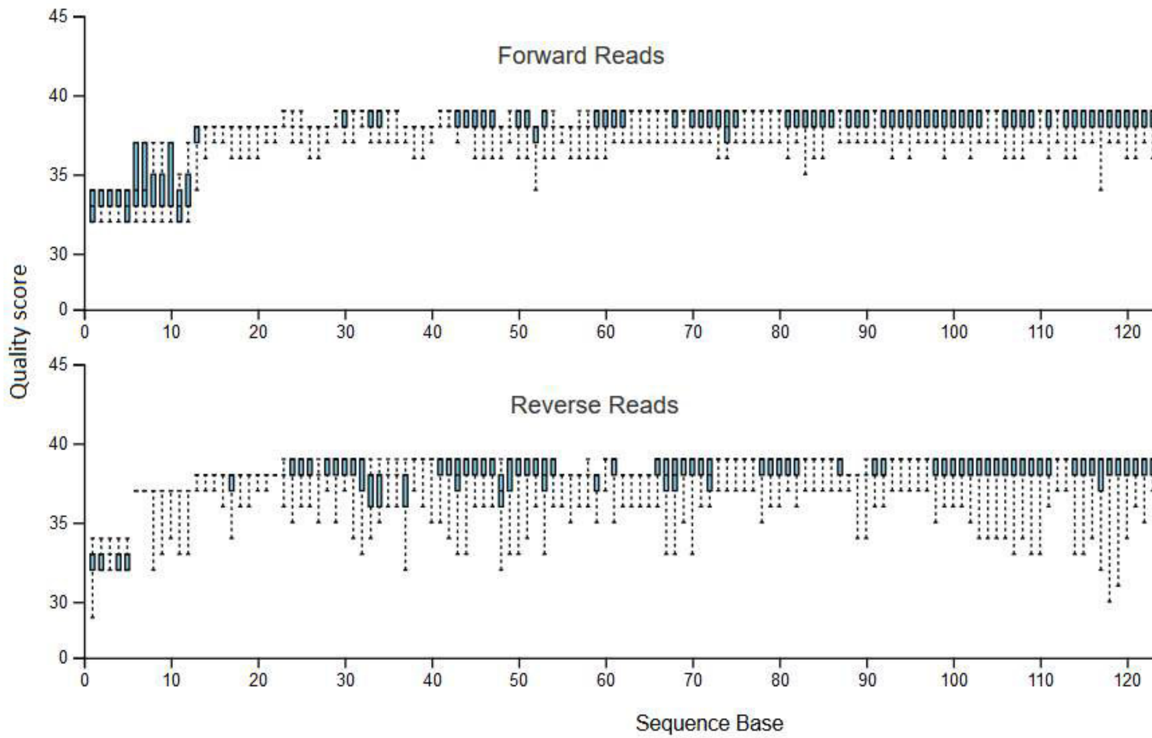

Figure 6. Q-score average of each sequence position of the demultiplexed sequences. *Sequences quality scores are between Q40 and Q30.*

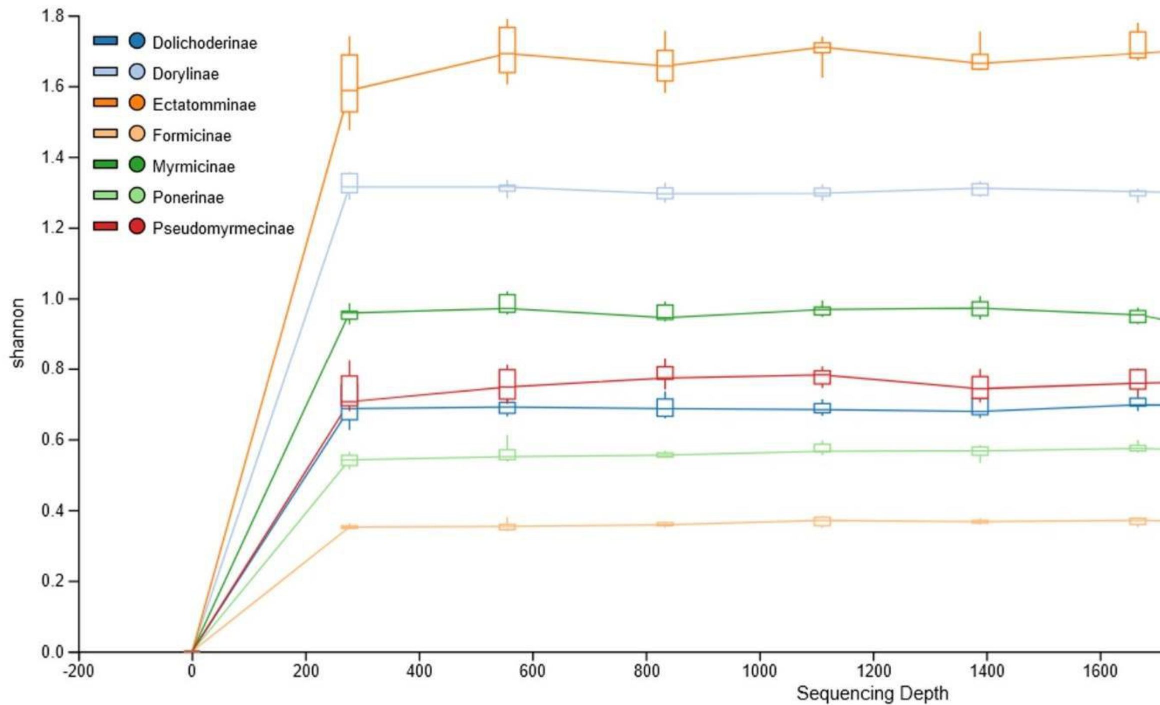

Figure 7. Shannon´s diversity rarefaction curves for each subfamily. *Asymptote is reached at 250 sequences of random sampling.*

When the bacterial order is compared with the ant subfamilies (Figure 8), Rhizobiales appears to be the more frequent bacterial order in *Dolichoderinae,* whereas Enterobacteriales is more frequent in *Formicinae* and Acetobacterales in *Pseudomyrmecinae*. *Dorylinae* have Entomoplasmales, Erysipelotrichales, and Rickettsiales as most frequent bacterial orders. In our samples, at subfamily level there is not a clear pattern on the more frequent bacterial orders in *Ectatomamminae* and in *Myrmicinae*.

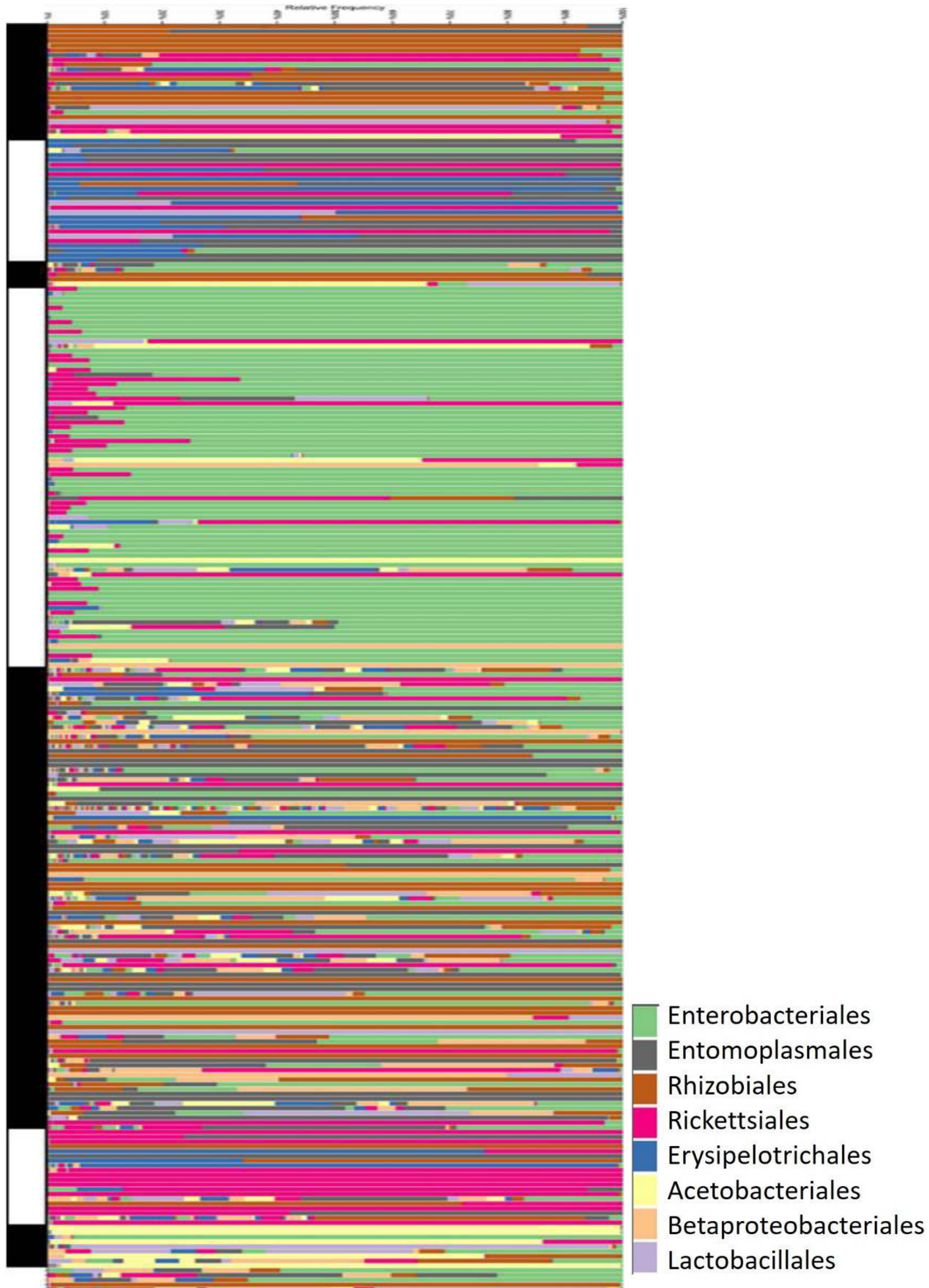

Figure 8. Relative frequency of bacterial orders among ant subfamilies. Black and white bars represent different ant subfamilies, from top to bottom (Dolichoderinae, Dorylinae, Ectatomminae, Formicinae, Myrmicinae, Ponerinae and Pseudomyrmecinae). The last 5bars represent the bacterial orders in the controls. Color bars represent the relative frequency of bacterial orders in each sample, 8 most abundant sOTUs color code is shown.

From the 1624 sOTUs found in all the samples a small fraction appears to be frequent among ant genera; An uncultured sOTU form of *Rhizobiaceae* appears to be present in all the *Dolichoderus bispinosus* colonies (n=12); in most of them relative frequency exceeded 95%. The same pattern is observed in *Dorymyrmex* spp., where all colonies seem to be infected by a specific sOTU of *Wolbachia* sp. Five of the nine *Pseudomyrmex* spp. colonies had the same *Acetobacteriaceae* sOTU at an 89% to 99% relative abundance (Figure 9).

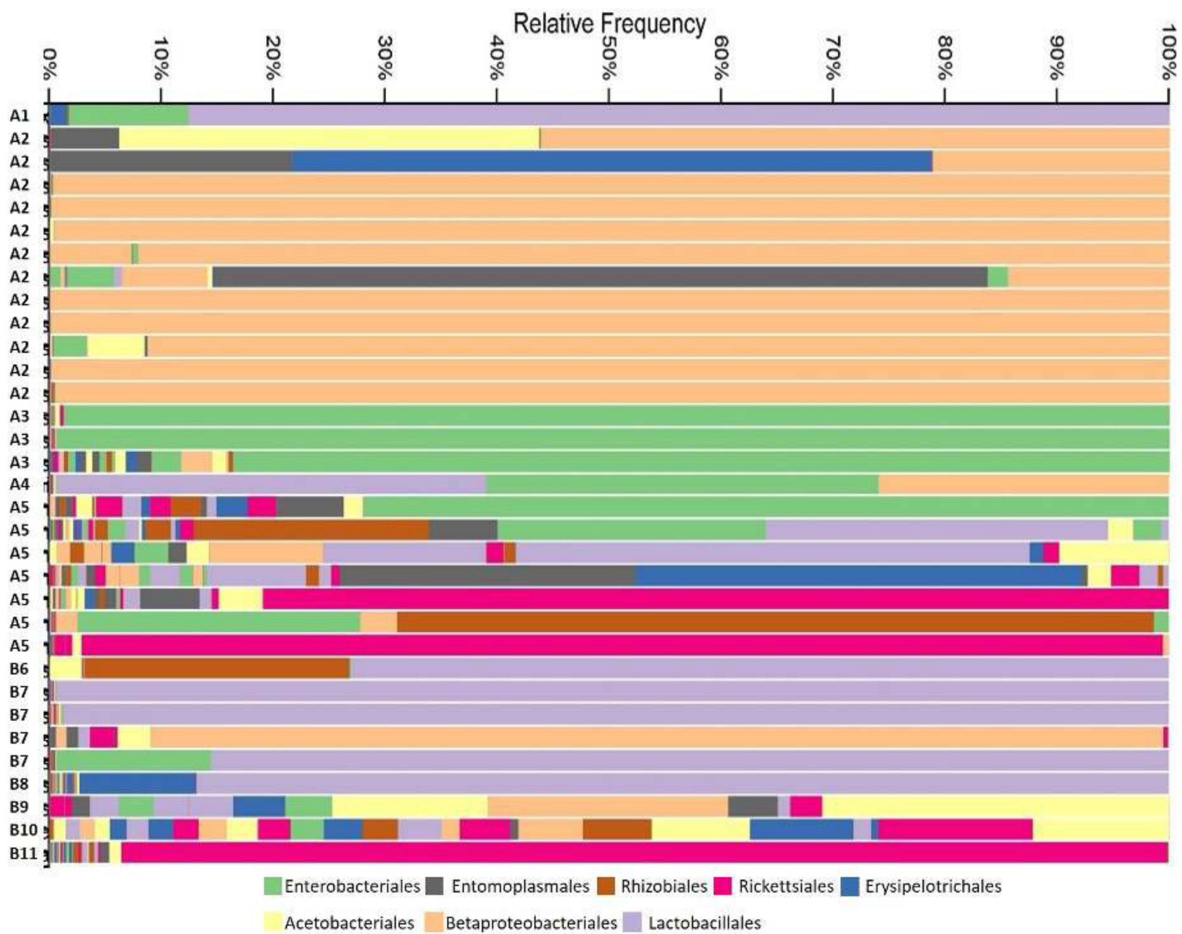

Figure 9. Relative frequency of bacterial sOTUs among ant species (1. Azteca velox, 2. Dolichoderus bispinosus, 3. Dorymyrmex insanus, 4. Dorymmyrmex smithii 5. Liometopum apiculatum, 6. Pseudomyrmex elongatus, 7. P. ferrugienus, 8. P. gracilis, 9. P. pallidus, 10. P. seminole, 11. P. spinicola) of the subfamilies Dolichoderinae (A) *and Pseudomyrmecinae* (B)*. Color bars represent the relative frequency of bacterial sOTUs in each sample, 8 most abundant sOTUs color code is shown.*

A specific sOTU of *Entoplamataceae* is present in most of the *Eciton* spp. colonies at a high relative frequency (22% to 92%) but also a Firmicutes sOTU is present in lower relative frequencies. The *Entoplamataceae* sOTU appears to be the most frequent in *Eciton burchelii* colonies while Firmicutes is most frequent in *Eciton mexicanum* and *Labidus praedator*. Only one colony of *Neivamyrmex* spp. was absent of *Wolbachia* sOTU. (Figure 10)

Both colonies of *Leptogenys elongata* were found infected with a specific Wolbachia sOTU.

Six of the nine colonies of *Odontomachus clarus* were infected with the same Wolbachia sOTU and eight with the *Wolbachia* sOTU previously reported as endosymbiont of the scale insect *Coelostomidia montana* (Dhami et al. 2013). The presence of both *Wolbachia* sOTUs in some cases indicted that the two are not mutually exclusive. In both *Odontomachus opaciventris* colonies, the same Firmicutes sOTU was found on a high relative abundance (75% and 93%, Figure 10).

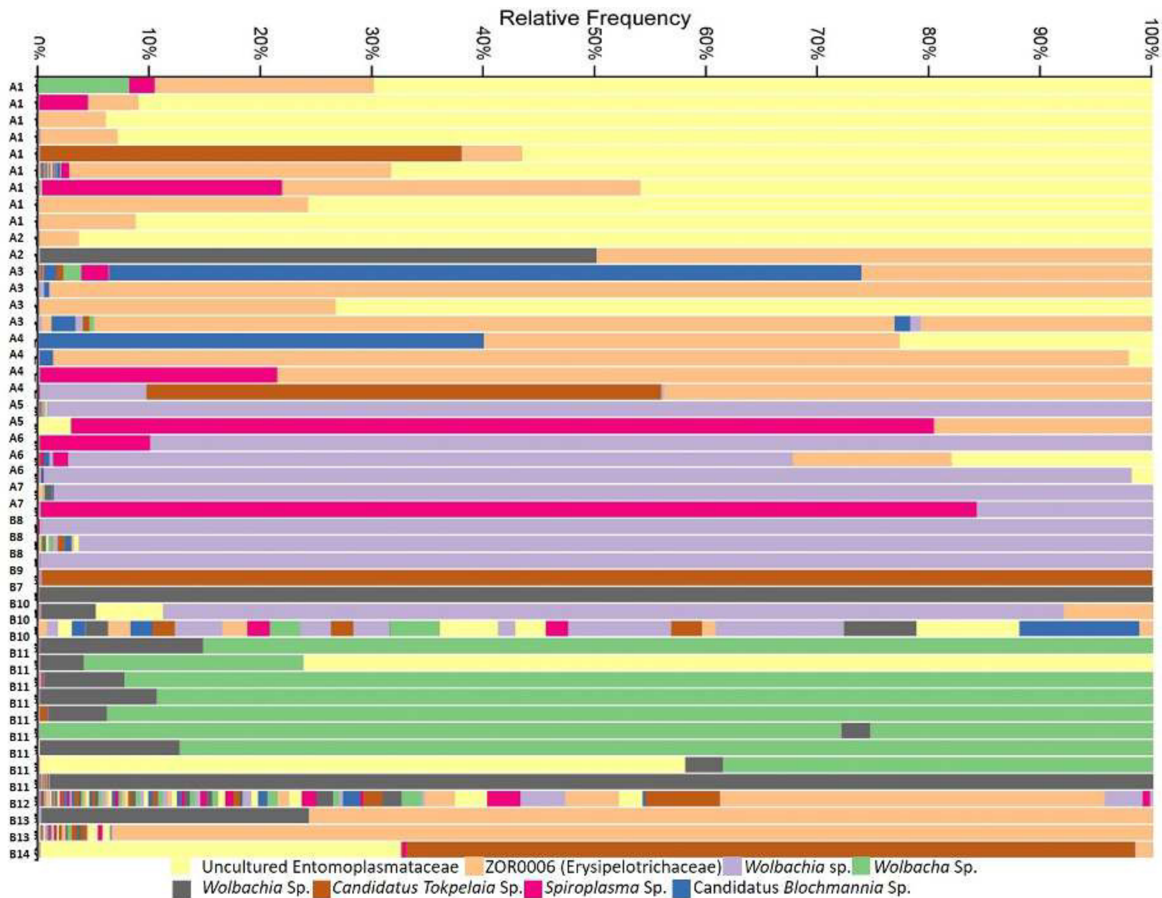

Figure 10. Relative frequency of bacterial sOTUs among ant species *(1. Eciton burchelii 2. E. hamatum 3. E. mexicanum 4. Labidus praedator 5. Neivamyrmex cornutus 6. N. melanocephalus 7. N. rugulosus 8. Leptogenys elongata 9. Neoponera apicalis 10. N. villosa 11. Odontomachus clarus 12. O. laticeps 13. O. opaciventris 14. Pachycondyla harpax)* of the subfamilies *Dorylinae* (A) and *Ponerinae* (B). Color bars represent the relative frequency of bacterial sOTUs in each sample, with color code shown for the 8 most abundant sOTUs.

As expected, most *Camponotus* colonies were associated with a *Blochmannia* sOTU; candidatus *Blochmannia ulcerosus* was found in *C. abscisus*, 3 of the 4 colonies of *C. andreii*, 3 of the 4 colonies of *C. rubrithorax* and in both colonies of *C trepidulus*. Candidatus *Blochmannia rufipes* was found in half of the *C. atriceps* (11 of 22) and *C. festinatus* (1 of 2) colonies. A specific *Blochmannia* sOTU was found in 8 of the 22 colonies of *C. atriceps*, in *C. conspicuus zonatus*, in all the colonies *of C. planatus*, half of the colonies of *C. rectangularis* (1 of 2) in *C. rectangularis rubroniger*, 1 of the 4 colonies of *C. rubrithorax* and half of the *C. sericeiventris* colonies (3 of 6). A different specific *Blochmannia* sOTU

was found in 3 of the 22 colonies of *C. atriceps*, in *C. coruscus* and *C. mina*, half of the *C. festinatus* (1 of 2) and *C. sericeiventris* (3 of 6) colonies as well as in all the *C. striatus* colonies. A *Blochmannia* sOTU previously described as an endosymbiont of *Colobopsis obliquus* was found in *C. claviscapus* colony. Only 2 *Camponotus* colonies were found lacking *Blochmannia*, but this could be a consequence of contamination (Figure 11).

All *Colobopsis etiolata* colonies were found with the presence of an *Enterobacteriaceae* sOTU at high relative abundance (94% to 66%) and a Wolbachia sOTU at lower relative abundance (33% to 4%). A *Wolbachia* sOTU previously reported as an endosymbiont of the scale insect *Coelostomidia montana* (Dhami et al. 2013) was found in 4 of the 5 colonies of *Formica propatula*. A specific *Burkholderiaceae* sOTU was found in all *Lasius mexicanus* colonies. A specific *Wolbachia* sOTU was found infecting 4 of the 5 *Acromyrmex* spp. colonies (Figure 11).

Relative Frequency

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Candidatus Blochmannia sp. *Candidatus Blochmannia* sp. Enterobacteriaceae
Candidatus Blochmannia rufipes *Candidatus Blochmannia ulceosus*
Wolbachia sp. *Wolbachia* sp. Burkholdieriaceae

Figure 11. Relative frequency of bacterial sOTUs among ant species of the subfamily Formicinae: Camponotus abscisus (A1), C. andrei (A2) C. atriceps (A3), C. claviscapus (A4), C. conspicuus zonatus (A5) C. coruscus (A6), C. festinatus (A7), C. minus (A8), C. planatus (A9), C. rectangularis (A10), C. rectangularis rubroniger (A11), C. sericeiventris (A12), C. striatus

(A13), C. trepidulus (A14), Colobopsis etiolata (B), Formica propatula (C1), Formica subcyanea (C2) Lasius mexicanus (D), Myrmecocystus depilis (E1) M. melliger (E2). Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 8 most abundant sOTUs shown in color code.

An uncultured Rhizobiales sOTU was found in 8 of 20 colonies of *Atta mexicana* and 3 of 5 colonies of *Atta texana*; likewise 3 *Atta mexicana* colonies and 1 *Atta texana* colony were found to have present the EntAcro1 Entomoplasmales sOTU. An uncultured Xantomonadales sOTU was found in similar relative abundances (12% to 21%) in each of the *Cephalotes* spp. colonies, with 5 of 7 colonies having present a specific *Burkholderiaceae* sOTU in a similar relative abundance (5% to 15%) and *Ventosimonas gracilis* sOTU (5% to 24% of relative abundance). A *Cephaloticoccus* sOTU was present in 6 colonies of *Cephalotes* spp. (Figure 12).

Relative Frequency

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%

Rhizobiaceae
EntAcro1 (strain)
Entomoplasma sp.
Wolbachia sp.
Candidatus Blochmannia ulcerosus
Rhodanobacteraceae
Spiroplasma platyhelix
RsaHF231 (Filum)

Figure 12. Relative frequency of bacterial sOTUs among ant species. 1. *Ectatomma ruidum* 2. *E. tuberculatum* 3. *Acromyrmex obtuspinosa* 4. *A. versicolor* 5. *Aphaenogaster mexicana* 6. *Atta cephalotes* 7. *A. mexicana* 8. *A. texana* 9. *Cephalotes bimaculatus* 10. *C. goniodontes* 11. *C. minutus* 12. *C. umbraculatus* 13. *Crematogaster crinosa*. 14 *C. laeviuscula* 15. *C. opaca* 16. *Novomessor encifer* 17. *Pheidole c.f. gouldi* 18. *P. obtusospinosa* 19. *Pogonomyrmex barbatus* 20. *P. maricopa* 21. *Temnothorax aztecus*) of the subfamilies Ectatomminae (A) and Myrmicinae (B). Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 8 most abundant sOTUs shown in color code.

There are no clear recurrent sOTUs among *Pogonomyrmex* spp. Colonies. This is even more evident on *P. maricopa* colonies where sOTUs richness appears to be higher than in every other *Pogonomyrmex* sp. sample (Figure 12, 15).

Neither *Ectatomma* spp., *Crematogaster* spp., *Pheidole* spp., *Neoponera* spp., *Liometopum apiculatum* nor *Atta cephalotes* seem to have a highly abundant specific bacterial sOTU across their colonies in our samples. (Figures 9-12)

When comparing the microbiome composition of the different ant genera, there is no clear pattern in the principal component analysis of the UniFrac distances of Bray-Curtis dissimilarity (Figure 13); nevertheless, when subfamilies are plotted (Figure 14), grouping of *Formiciinae* and *Myrmicinae* seems to be driven by axis 1 and 3, which explain 18.67% and 33.86% of the variation, respectively. This can be corroborated when tested with PERMANOVA analysis, which was done for the pairwise of subfamilies (Table IV, Figures 15-18). Only the couplets of *Ectatomminae-Dolichoderinae*, *Ectatomminae-Myrmicinae*, *Ectatomminae-Pseudomyrmecinae*, and *Myrmicinae-Pseudomyrmecinae* have similar beta diversity values, while the other subfamiliy couplets have different overall heterogeneity of species composition.

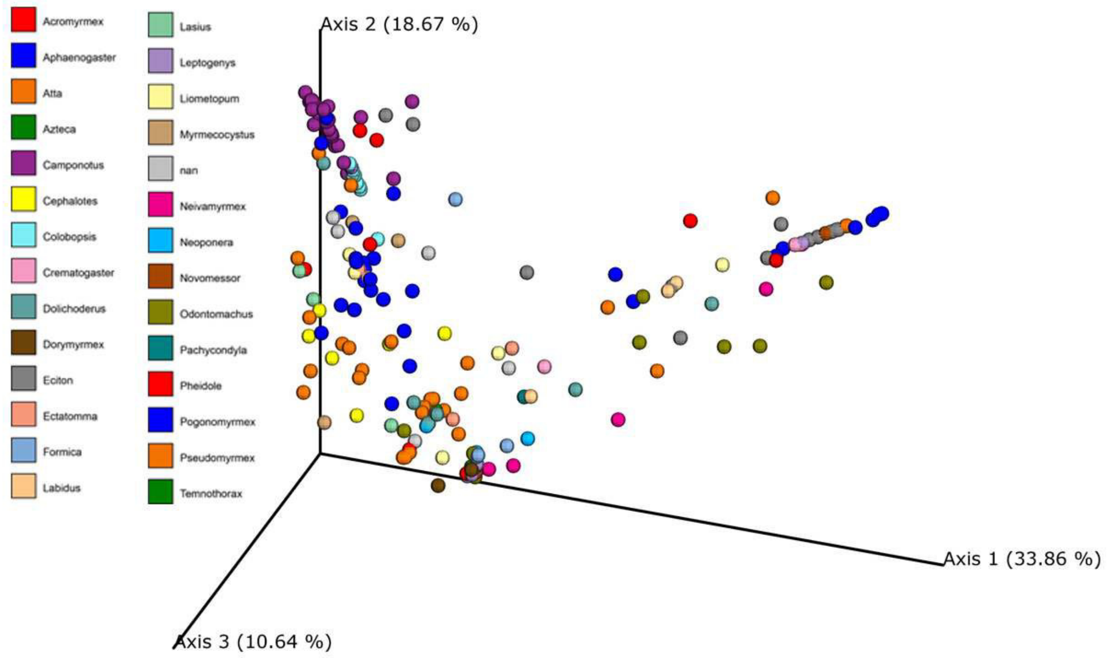

Figure 13. Principal component analysis of the UniFrac distances form the Bray-Curtis dissimilarity plotted by genera.

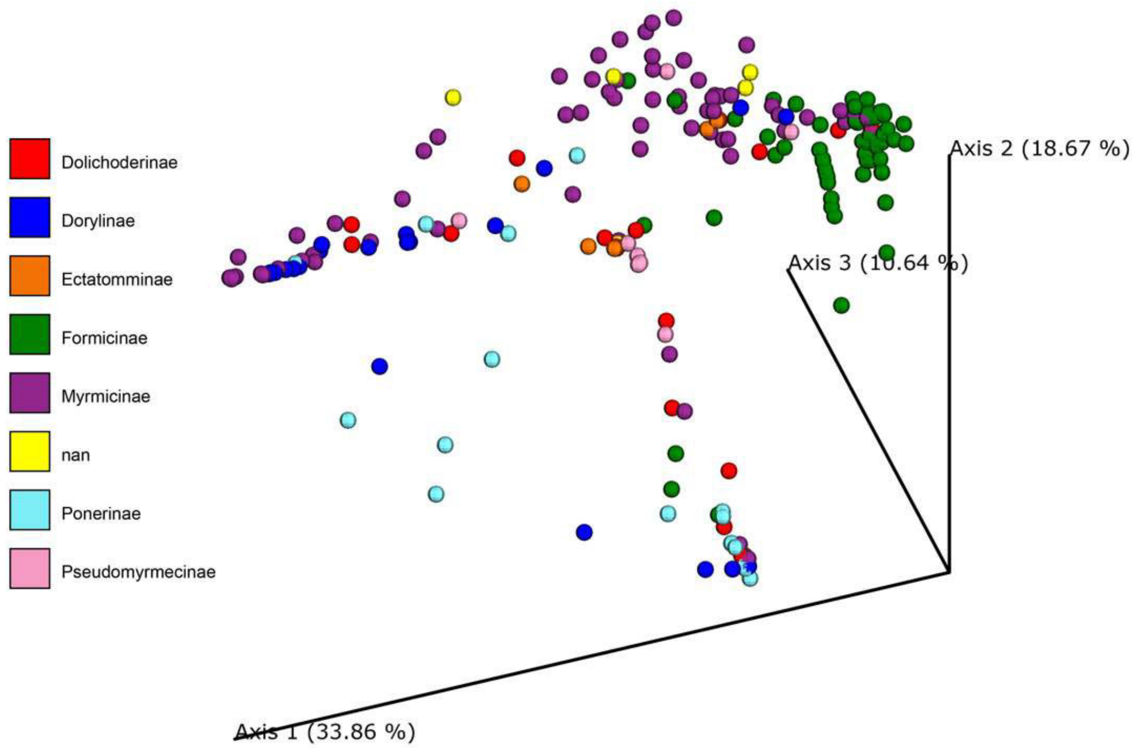

Figure 14. Principal component analysis of the UniFrac distances form the Bray-Curtis dissimilarity plotted by subfamily.

Table IV. PERMANOVA results for the pairwise analysis of the UniFrac distances of the Bray-Curtis dissimilarity. Non significative differences are in bold.

Group 1	Group 2	Sample size	pseudo-F	p-value	q-value
Dolichoderinae	*Dorylinae*	49	13.309	0.001	0.002
Dolichoderinae	*Ectatomminae*	28	1.153	**0.296**	0.311
Dolichoderinae	*Formicinae*	98	35.147	0.001	0.002
Dolichoderinae	*Myrmicinae*	110	3.339	0.005	0.008
Dolichoderinae	*Ponerinae*	42	4.834	0.001	0.002
Dolichoderinae	*Pseudomyrmecinae*	31	2.247	0.024	0.030
Dorylinae	*Ectatomminae*	31	5.267	0.007	0.010
Dorylinae	*Formicinae*	101	80.229	0.001	0.002
Dorylinae	*Myrmicinae*	113	9.348	0.001	0.002
Dorylinae	*Ponerinae*	45	9.255	0.002	0.003
Dorylinae	*Pseudomyrmecinae*	34	8.985	0.001	0.002
Ectatomminae	*Formicinae*	80	11.499	0.001	0.002
Ectatomminae	*Myrmicinae*	92	0.540	**0.82**	0.820
Ectatomminae	*Ponerinae*	24	3.857	0.009	0.012
Ectatomminae	*Pseudomyrmecinae*	13	1.694	**0.105**	0.116
Formicinae	*Myrmicinae*	162	35.207	0.001	0.002
Formicinae	*Ponerinae*	94	54.106	0.001	0.002
Formicinae	*Pseudomyrmecinae*	83	19.746	0.001	0.002
Myrmicinae	*Ponerinae*	106	7.241	0.001	0.002
Myrmicinae	*Pseudomyrmecinae*	95	2.041	**0.054**	0.063
Ponerinae	*Pseudomyrmecinae*	27	5.696	0.001	0.002

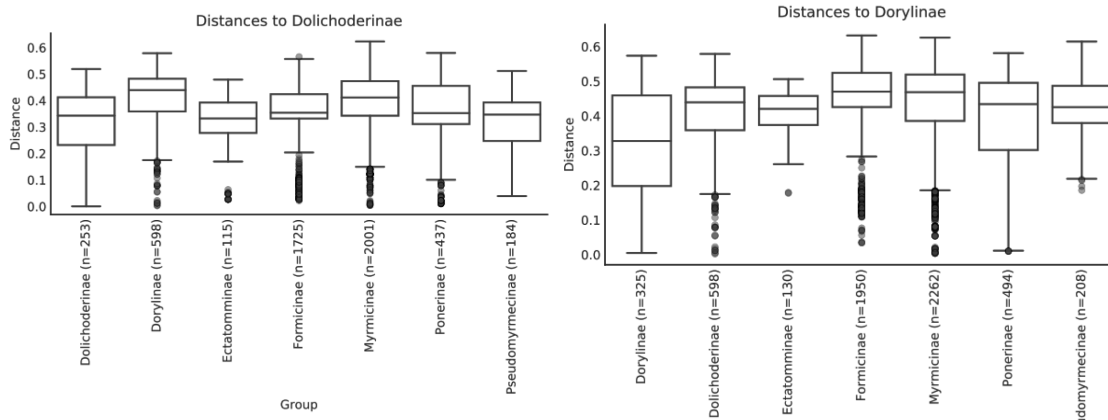

Figure 15. Group significance boxplots for the PERMANVOVA of Dolichoderinae (left) and Dorylinae (right). Only Ectatomminae and Dolichoderinae do not have different overall heterogeneity of species composition among them.

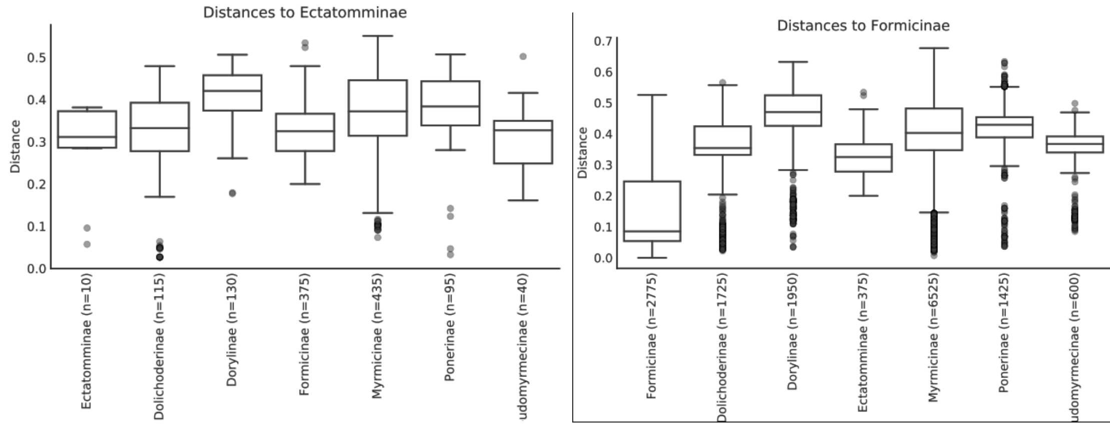

Figure 16.Group significance boxplots for the PERMANVOVA of Ectatomminae and Formicinae. Ectatomminae was not significantly similar to Dolichoderinae, Myrmicinae and Pseudomyrmecinae.

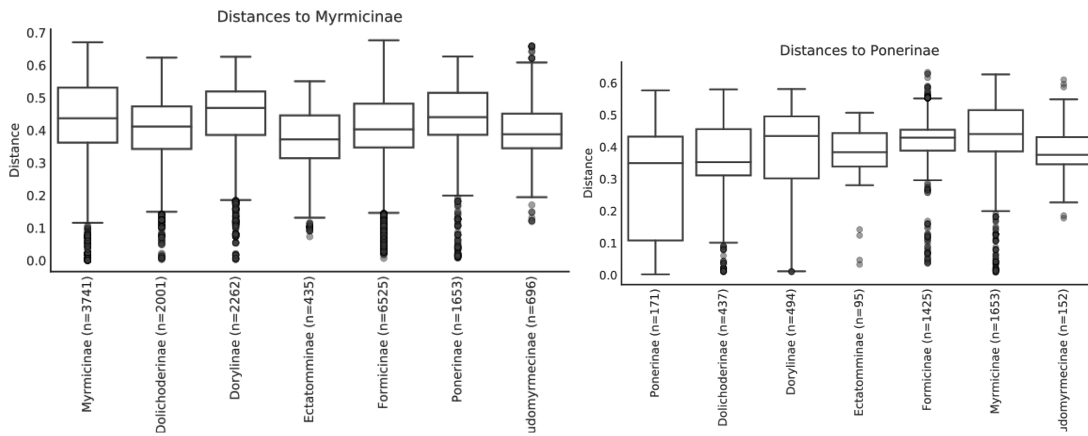

Figure17. Group significance boxplots for the PERMANVOVA of Myrmicinae and Ponerinae. Myrmicinae community composition was not different from Pseudomyrmecinae and Ectatomamminae.

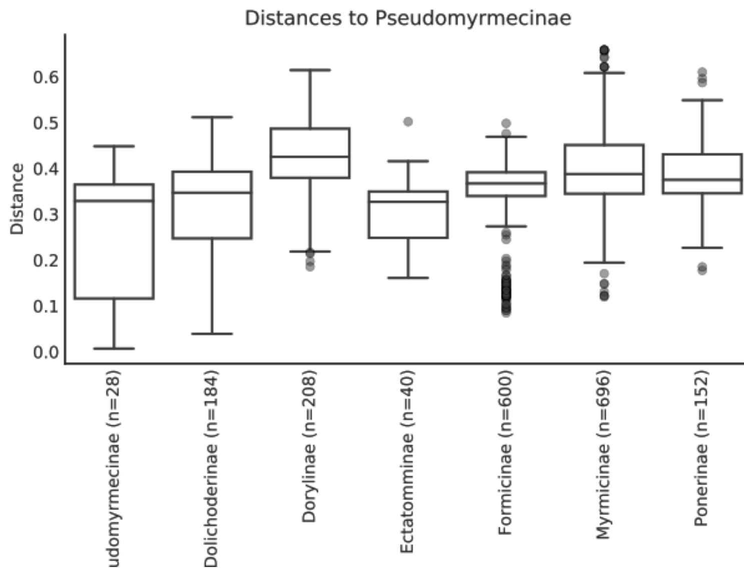

Figure 138. Group significance boxplots for the PERMANVOVA of Pseudomyrmecinae. Both Ectatomminae and Myrmicinae were not significantlye different from Pseudomyrmecinae.

Wolbachia persistence

Remarkably, 188 (72%) of the ant colonies were infected by 3 different sOTUs of *Wolbachia*, 21 of them with the *Wolbachia* sOTU3 previously reported as an endosymbiont of *Coelostomidia montana* (Dhami et al. 2013), 28 of them with the *Wolbachia* sOTU2 “uncultured alpha proteobacterium” and 159 of them with the sOTU1 “*Wolbachia*” (Table V). These three sOTUs are the only members of the family *Anaplasmataceae* found in this study.

Wolbachia was present in all ant subfamilies but some species appear to be free of this bacterial genus: *Myrmecocystus depilis, Aphaenogaster mexicana, Crematogaster laeviuscula, Novomessor ensifer Pheidole cf. gouldi, Pseudomyrmex gracilis, Pseudomyrmex pallidus, Pseudomyrmex Seminole* and *Ectatomma ruidum*. Only one colony of each of the previously listed species was used for this analysis (2 colonies in *E. ruidum*), so more information is needed to ensure that those species are *Wolbachia* free.

Table V. Ant`s species with Anaplasmataceae sOTUs reported. There are 10 species with two Wolbachia *sOTUs, but no species with the 3 sOTUs were recorded.*

Wolbachia sp. sOTU1	
Species	Nests
Acromyrmex octospinosus	1
A. versicolor	3
Atta mexicana	1
A. texana	1
Azteca velox	1
Camponotus abscisus	1
C. andrei	1
C. atriceps	5
C. claviscapus	1
C. conspicuus zonatus	1
C. coruscus	1
C. festinatus	1
C. rectangularis rubroniger	1
C. rubrithorax	3
C. striatus	2
C. trepidulus	1
Cephalotes goniodontus	3
Colobopsis etiolata	10
Dolichoderus bispinosus	1
Dorymyrmex insanus	3
D. smithi	1
Eciton mexicanum	1
Ectatomma tuberculatum	1
Formica propatula	1
F. subcyanea	1
Lasius mexicanus	1
Leptogenys elongata	2
Liometopum apiculatum	2
Neivamyrmex cornutus	1
N. melanocephalus	3
N. rugulosus	2
Neoponera villosa	1
Pheidole obtusospinosa	1
Pogonomyrmex barbatus	1
Pseudomyrmex ferrugineus	1

Wolbachia sp. sOTU2	
Species	Nests
Camponotus. striatus	1
C. planatus	6
Temnothorax aztecus	1
Wolbachia sp. sOTU3	
Formica propatula	2
Atta cephalotes	3
Odontomachus opaciventris	1
Odontomachus clarus	1
Neoponera villosa	1
Wolbachia sp. sOTU1 and sOTU2	
Pheidole obtusospinosa	1
Acromyrmex versicolor	1
Pogonomyrmex maricopa	1
Atta mexicana	1
Dolichoderus bispinosus	1
Pogonomyrmex barbatus	1
Liometopum apiculatum	1
Camponotus atriceps	1
Wolbachia sp. sOTU2 and sOTU3	
Odontomachus clarus	8
Wolbachia sp. sOTU1 and sOTU3	
Formica propatula	2

Phylogenetic relationships of COI among ant selected species.

Mitochondrial genes are widely used to explore the population genetics of animals, they are consider highly stable due they low mutation rate, intron absence and mother linage (Deepak & Mahadevan, 2019). We use the COI gene with and expected amplicon size of 658 bp. Cytocrome C oxidase I fragments from 257 colonies were successfully amplified, representing 25 genera from 7 subfamilies (Fig. 19).

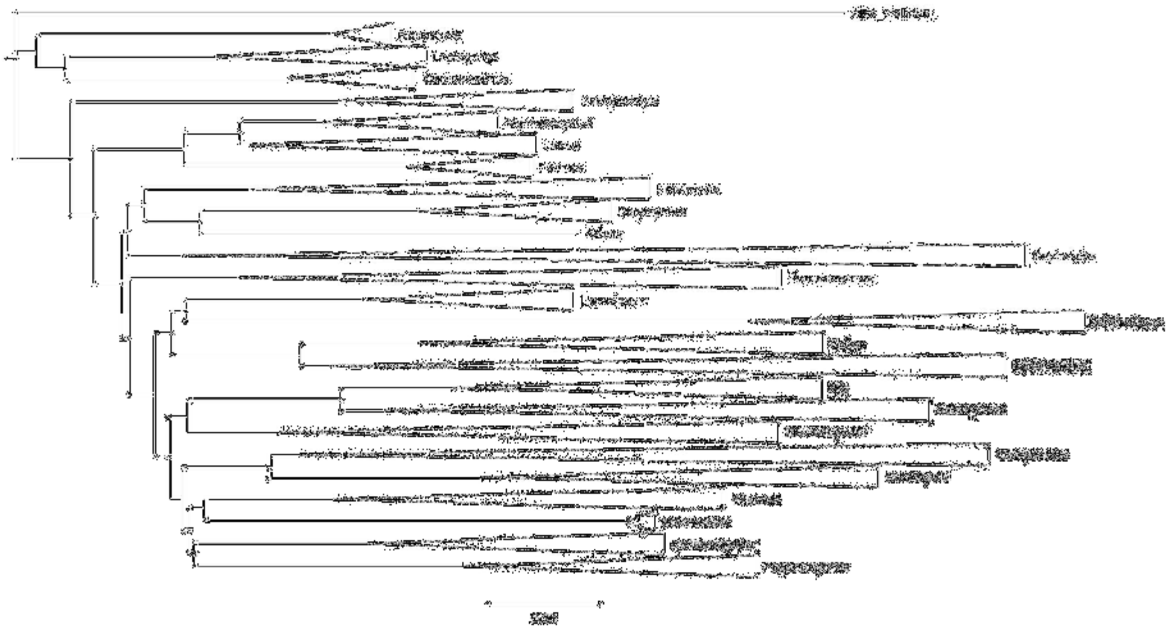

Figure19. Neighbour joining dendogram of COI from the sampled colonies. Tree was rooted with the bee Apis mellifera and sequences are collapsed by genera; scalebar is substitutions per site.

Populations of selected ant genera (Atta, Camponotus and Pogonomyrmex)

Only four species of *Camponotus* appear to be monophyletic in the the RAxML phylogram (Figure 20): *C. striatus*, *C. rectangularis*, *C. planatus* and *C. sericeiventris*, and the single colony of *C. mina.* There is likely a higher diversity of *Camponotus* across México represented by cryptic species that can be identified by morphology using the current identification keys.

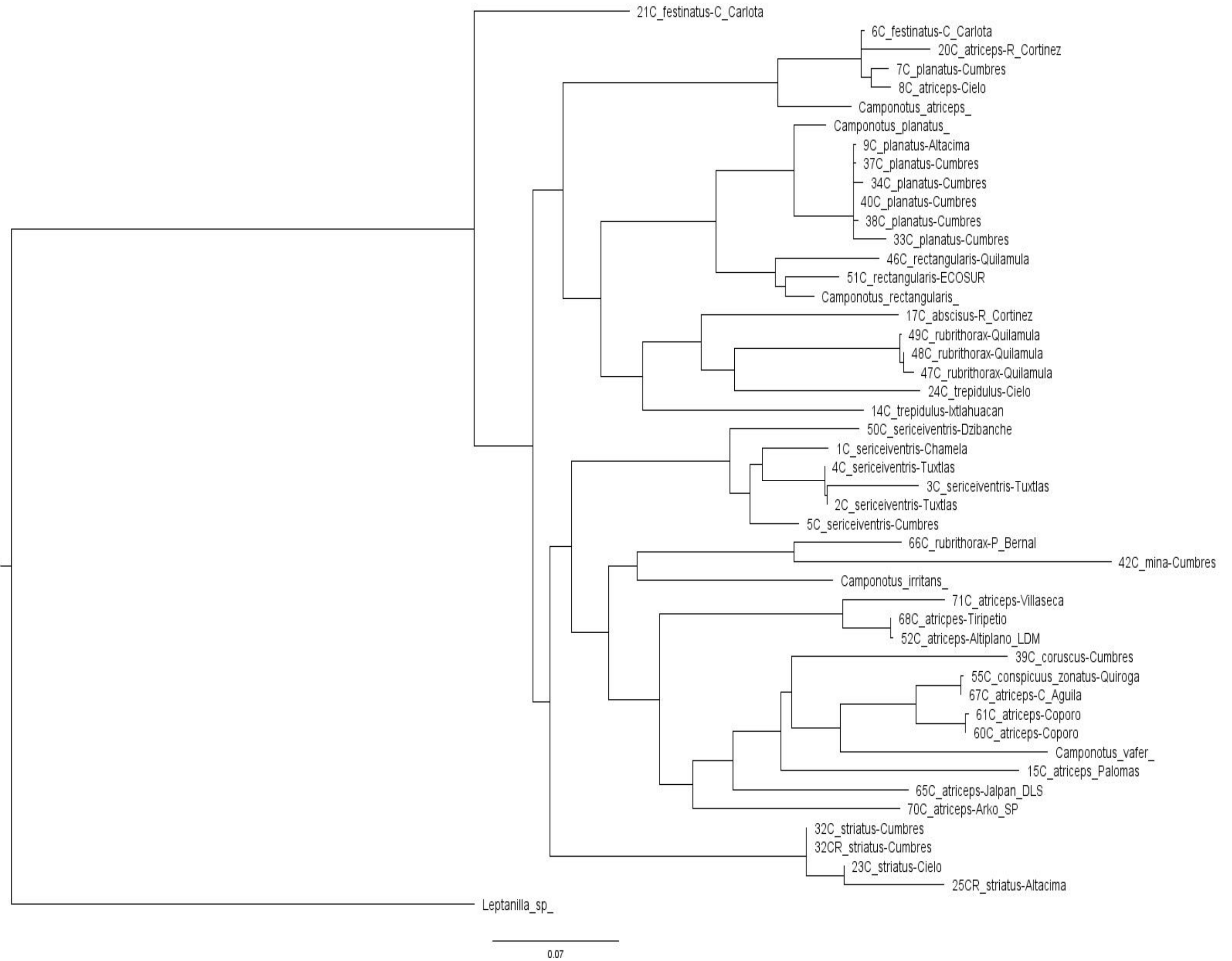

Figure 20. RAxML Phylogram of COI from Camponotus species. Tree was rooted with the Leptanillinae Leptanilla sp.; scalebar show substitutions per site.

We select *C. striatus* nests to perform a population analysis because it was the species of this genus with more samples across the study sites. Geographic distance dendogram of C. atriceps nests group 3 populations separated by at least 300 km and out group the 20C nest by 1000 km. (Fig. 21). Comparisons on the COI sequence of *C. atriceps* samples also show the presence of three principal groups (Fig. 22).

Figure 21. UPGMA Geographic distance dendrogram of selected nests of C. atriceps*.*

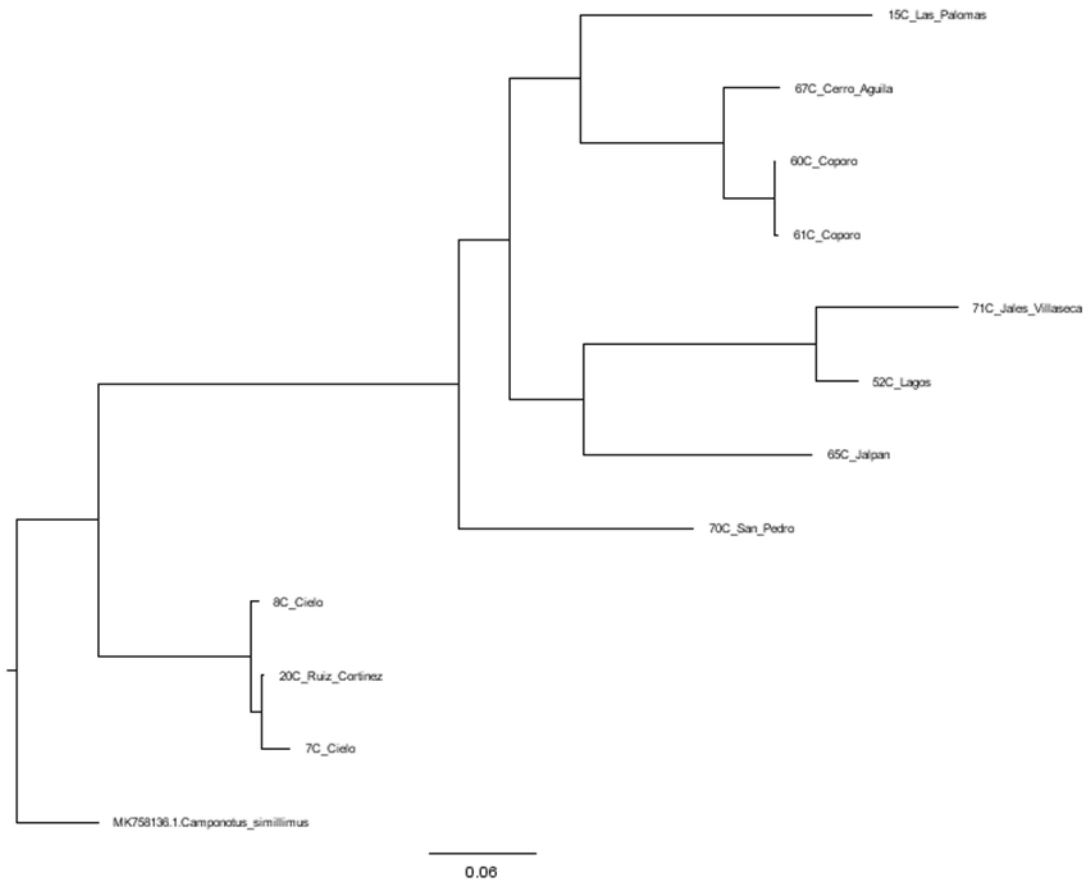

Figure 22. RAXmL Filogram of selected nestos of C. atriceps. Tree was rooted with C. simillimus; scalebar show substitutions per site.

All *A. cephalotes* colonies are monophyletic in the RAxML phylogram (Figure 23). All other colonies form a monophyletic group excluding the *A. texana* COI sequence from BOLD database, suggesting that *A. texana* could be morphologically misidentified and should belong to *A. mexicana*.

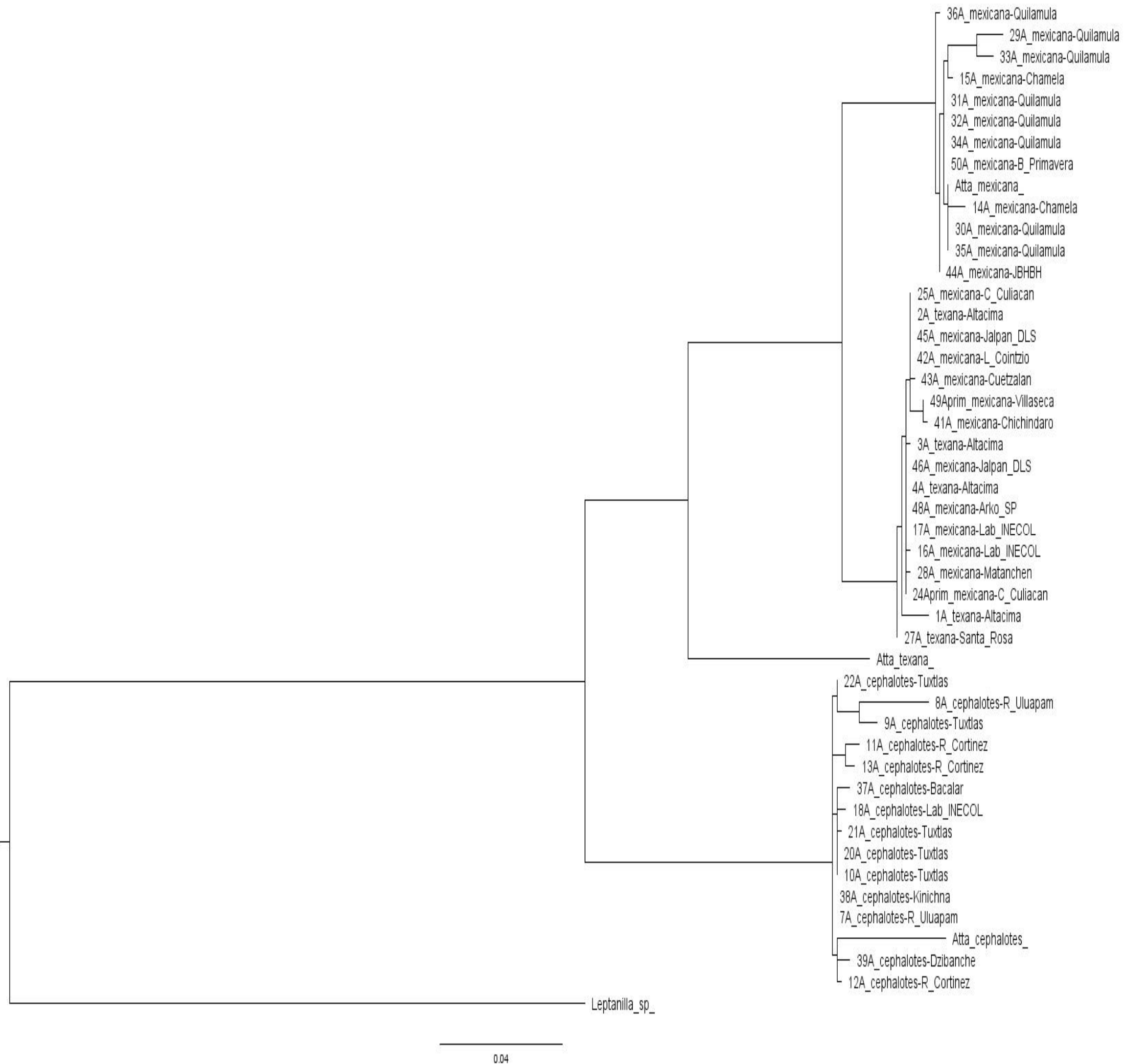

Figure 23. RAxML joining Phylogram of COI from Atta species. Tree was rooted with the Leptanillinae Leptanilla sp.; scalebar is substitutions per site.

Geographic distances of *A. mexicana* nests seem to group two principal populations at longer distances than those found in *C. atriceps* (up to 1000 km) (Fig. 24), this is consistent with the COI phylogram of the same specimens (Fig. 25).

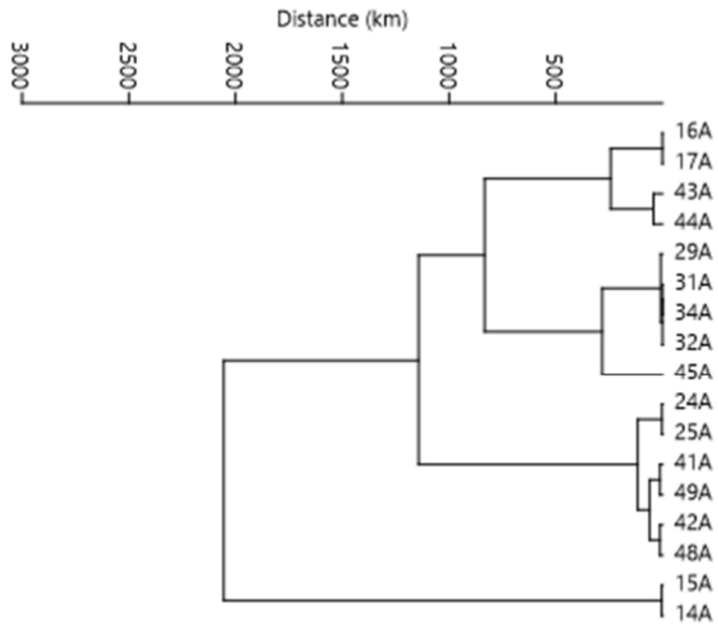

Figure 24. UPGMA Geographic distance dendrogram of selected nests of A. mexicana

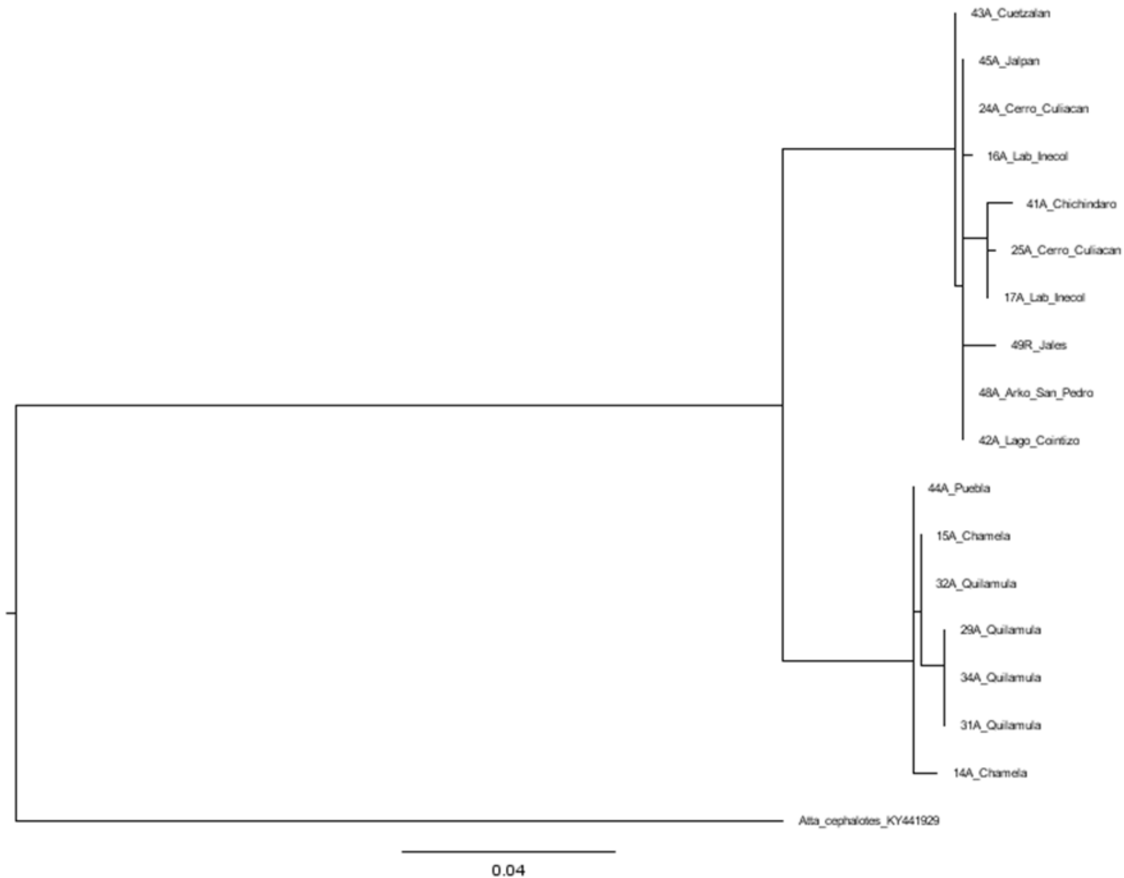

Figure 25. RAxML joining Phylogram of COI from Atta mexicana selected nests. Tree was rooted with Atta cephalotes.; scalebar shows substitutions per site.

A lineage of *P. barbatus* from Cuatro Cienegas (Coahuila) and Puente national (Veracruz) is immediately grouped, and the rest of *P. barbatus* from Cuatro Cienegas are inside a sister group with the rest of the *Pogonomyrmex* colonies sampled in this study. Two clusters are formed with the rest of *Pogonomyrmex*: one within the *P. barbatus* BOLD sequence and another with the *P. rugosus* BOLD sequence.

All *Pogonomyrmex maricopa* colonies from this study form a monophyletic group, among the *P. comanche* sequence from BOLD database, leaving outside the group as a sister branch the *P. maricopa* COI BOLD sequence, (Figure 26).

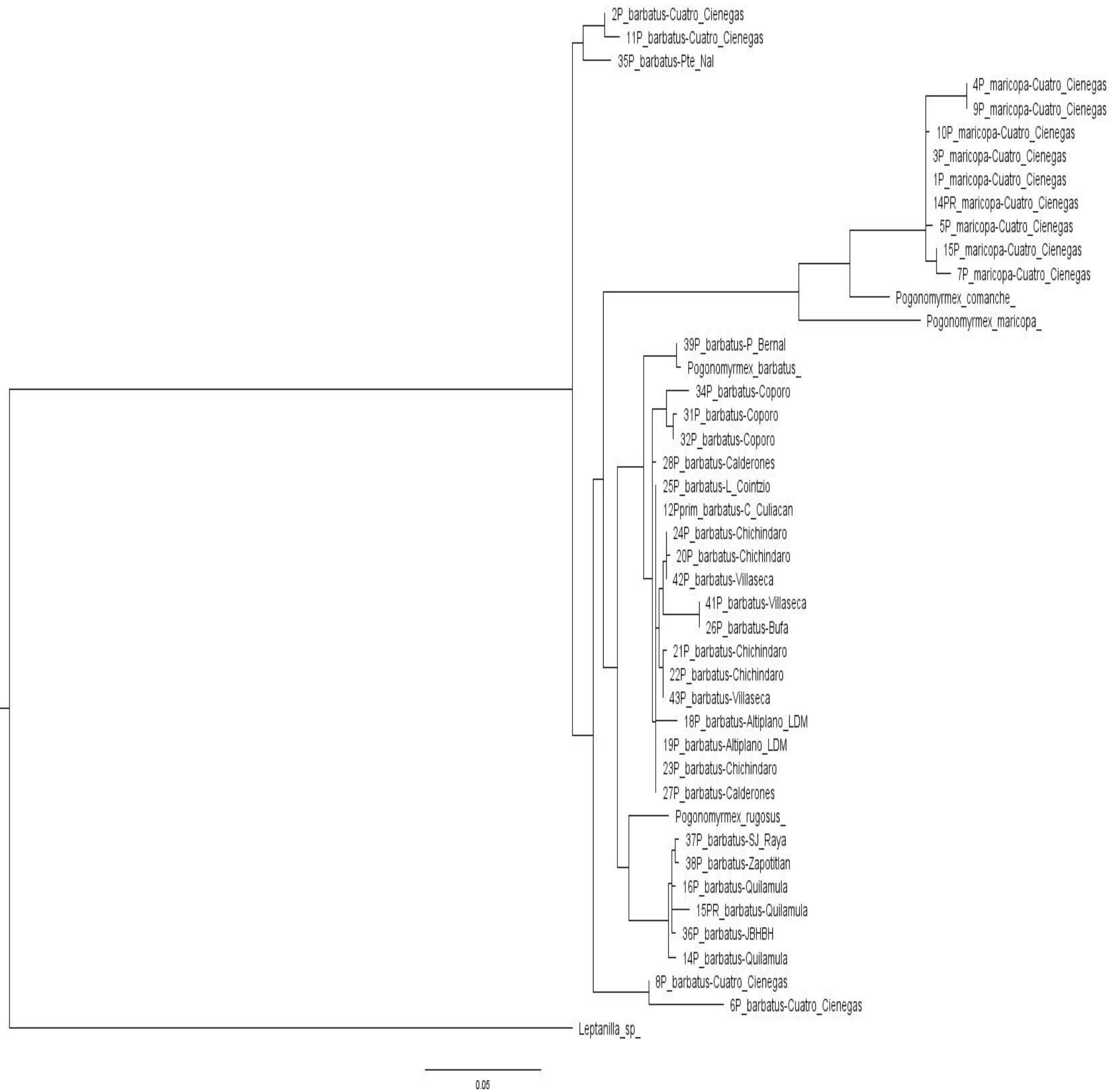

Figure 26. RAxML joining Phylogram of COI from Pogonomyrmex species. Tree was rooted with the Leptanillinae Leptanilla sp.; scalebar is substitutions per site.

Nests of P. barbatus seems to be grouped I three different population within a 700 km distance of separation among each group. (Fig 27.). The same pattern is seen when COI sequences are compared (Fig. 28)

Figure 27. UPGMA Geographic distance dendrogram of selected nests of P. batbatus

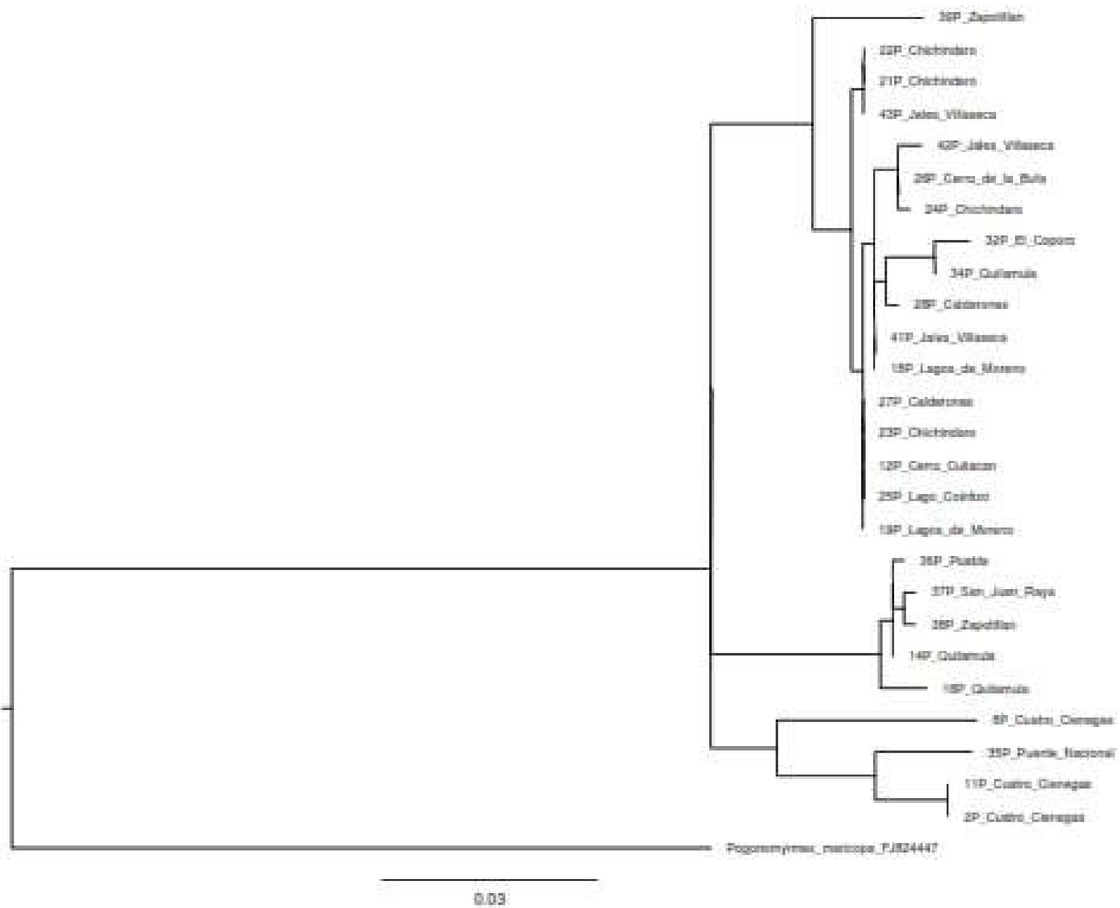

Figure 28. RAxML joining Phylogram of COI from Pogonomyrmex barbatus. Tree was rooted with the P. Maricopa COI.; scalebar shows substitutions per site.

Considering both distances dendrograms and COI phylograms we propose to group 3 populations of *C. atriceps*, 2 populations of *A. mexicana* and 3 populations of *P. barbatus* for the microbiome analysis comparison (Table 6).

Table 4. Proposed populations defined by geographic distance and COI distances.

Species	Population	States	Nests
Atta mexicana	A	Guanajuato, Puebla, Michoacan	8
Atta mexicana	B	Jalisco, Morelos, Puebla	5
Camponotus atriceps	A	Guanajuato, Michoacan	4
Camponotus atriceps	B	Queretaro, Guanajuato, Jalisco	3
Camponotus atriceps	C	Michoacan, Veracruz, Tamaulipas	4
Pogonomyrmex barbatus	A	Jalisco, Guanajuato, Michoacan, Queretaro	17
Pogonomyrmex barbatus	B	Puebla, Morelos	5
Pogonomyrmex barbatus	C	Coahuila, Veracruz	4

Microbiome among closely related species and populations

The microbiome among the sampled species of *Camponotus* results in a notable the low sOTU richness among them is clearly dominated by 3 *Blochmannia* sOTUs and one *Wolbachia* sOTU. Each *Blochmannia* sOTU seems to be mutually exclusive in most of the *Camponotus* colonies. The three populatons are related to those sOTUS: Population A and B microbiomes are mainly dominated by the *Blochmannia* sOTU "Candidatus *Blochmannia*" or the sOTU "Candidatus *Blochmannia,* uncultured", the "*Blochmannia rufipes*" sOTU is only present in population C, all samples oly have the presence of one "*Blochmannia* spp. sOTU, but the presence of *Wolbachia* sp. Can be seen in some samples of all populations (Fig. 29)

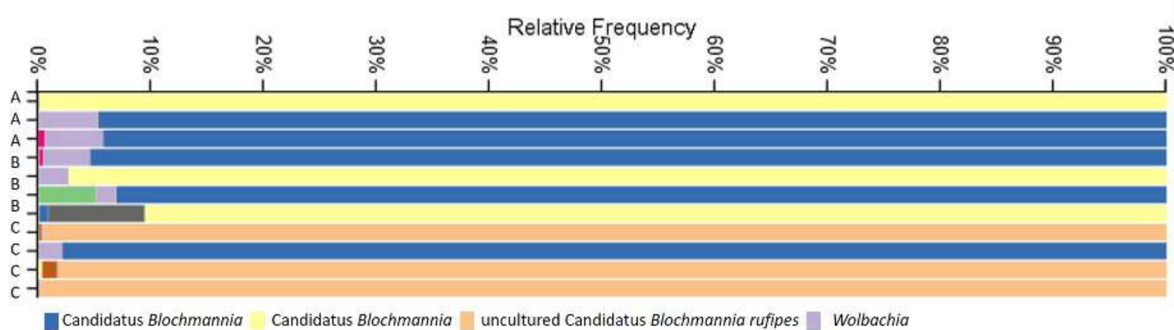

Figure 29. Relative frequency of sOTUS present in C. atriceps populations. Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 4 most abundant sOTUs shown in color code.

C. atriceps popuaions showed a low species richness and a high dominance of *Blochmannia* spp. (Enterobacteriaceae) and Wolbachia spp. (Anaplasmataceae), other bacteria is either in a lower frequency or not shared among populations (Fig 29). There seems to be a diversity pattern among *C. atriceps* populations where population C have almost one tenth of the Shannon`s diversity index than population B (Figure 30).

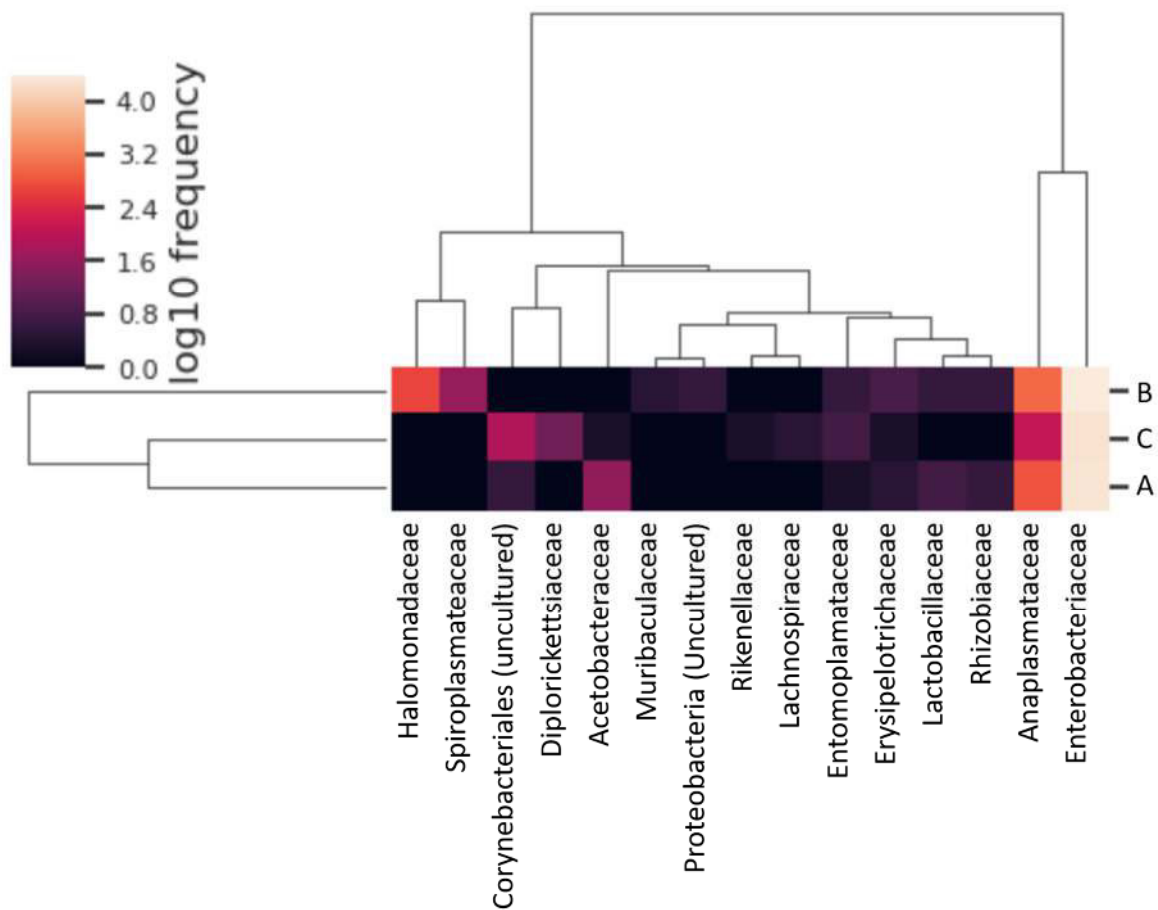

Figure 30. Heatmap of the relative frequency of bacterial families in C. atriceps. Color code represent the logarithmic frecuency of each family

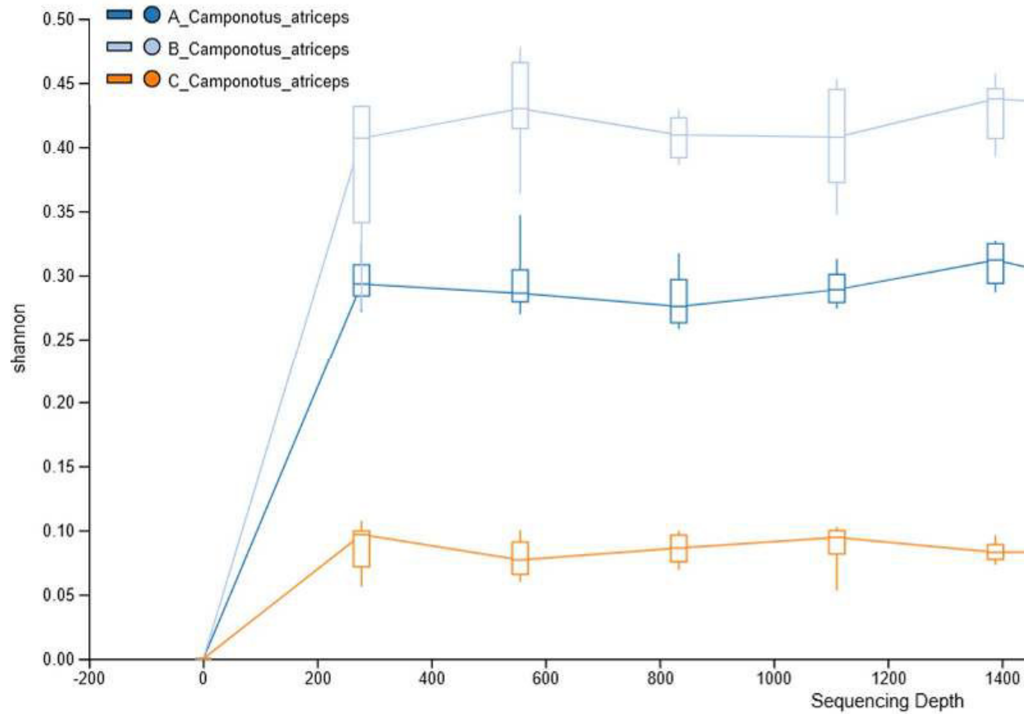

Figure 31. Alpha rarefaction of the Shannon index of the microbiome from three populations of C. atriceps. Color code shows each population.

There is a significative difference of the microbiome of population C when geographic distance is used to group the nests of *C. atriceps* (Weighted UniFrac, P= 0.021), apparently this population have the lowest microbial abundances respect populations A and B (Fig 31)

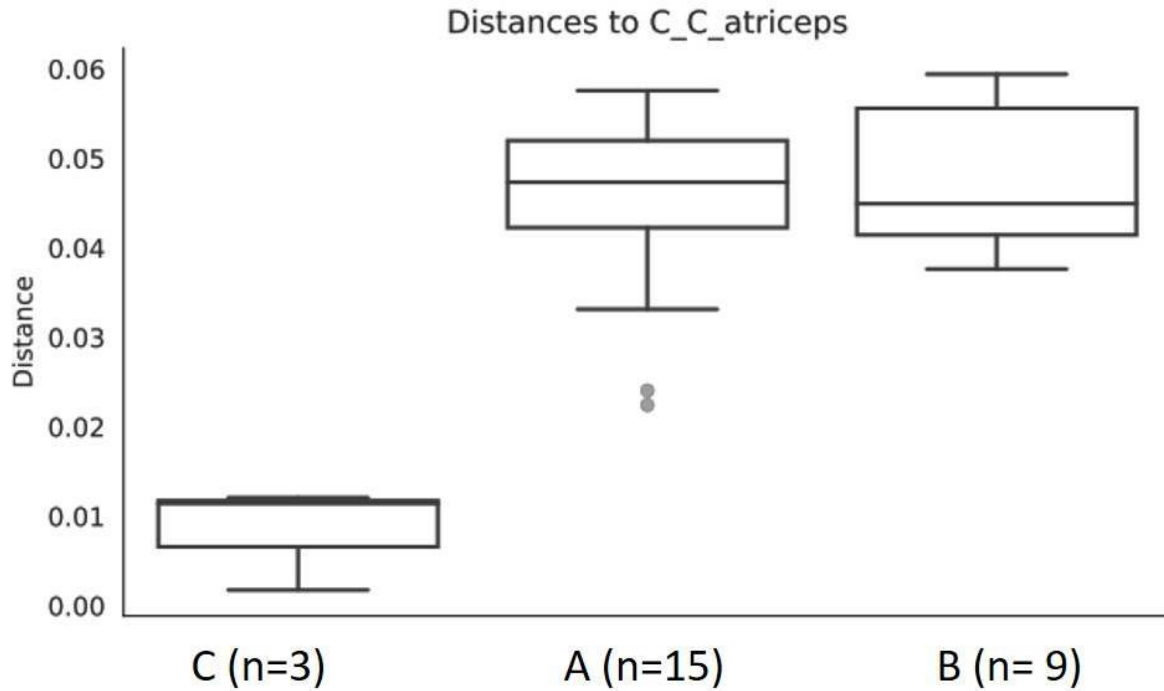

Figure 32 Weighted unifrac of the distances of microbial abudances among populations of C. atriceps. P=0.021)

Atta mexicana exhibit a richer microbiome than *Camponotus atriceps*, and clustering appears to be more related to diversity than a specific sOTU. Most of *A. mexicana* samples are dominated by a single sOTU of uncultured Rhizobiales bacteria and is present in both of the previously delimited populations. 2 samples from population A and one from population B have a much diverse microbiome and one sample of population A is clearly dominated by the EntAcro1 sOTU (Fig.33).

Alfa diversity seems to be similar among both *A. mexicana* populations and asymptote is reached at a relative low Shannon`s diversity index (0.03 bit/indi) in both populations (Fig. 34).

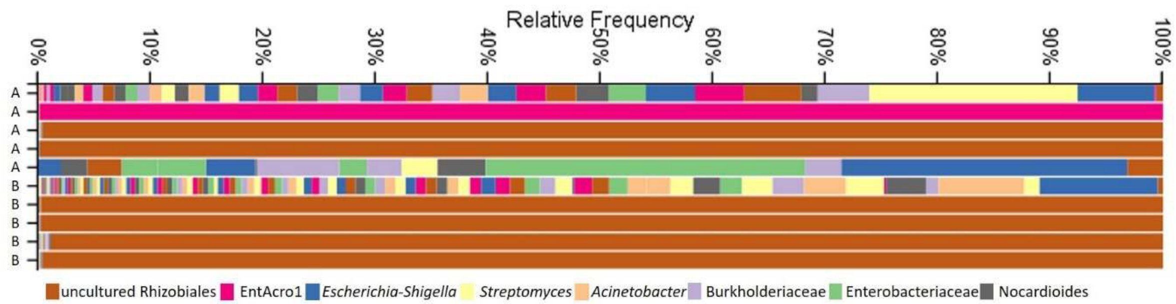

Figure 33. Relative frequency of sOTUS present in A. mexicana populations. Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 8 most abundant sOTUs shown in color code.

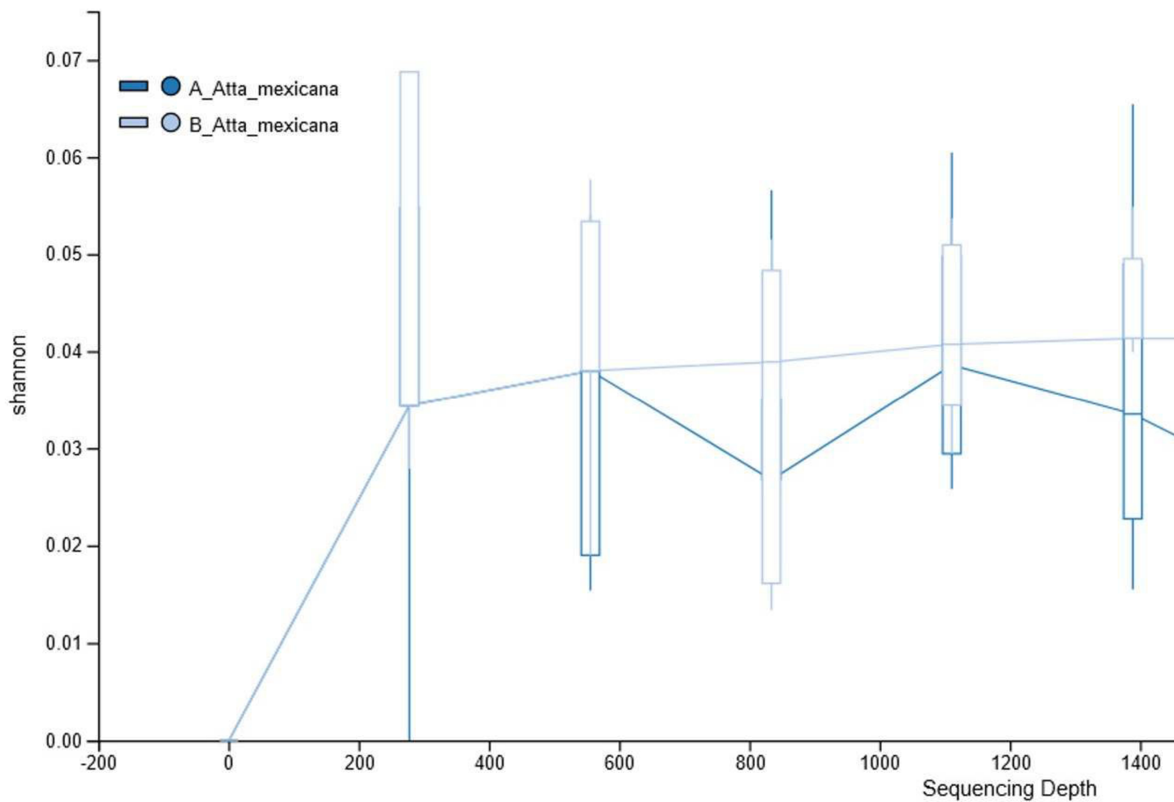

Figure 34. Alpha rarefaction of the Shannon index of the microbiome from two populations of A. mexicana. Color code shows each population.

Alfaproteobacteria is the most abundant class in *A. mexicana* populations, Actinobacteria, Gamma proteobacteria ans Mollicutes are also in high abundances in both populatons, only one quarter of the bacterial classes are shared among the two previously defined *A. mexicana* populations (Fig. 35)

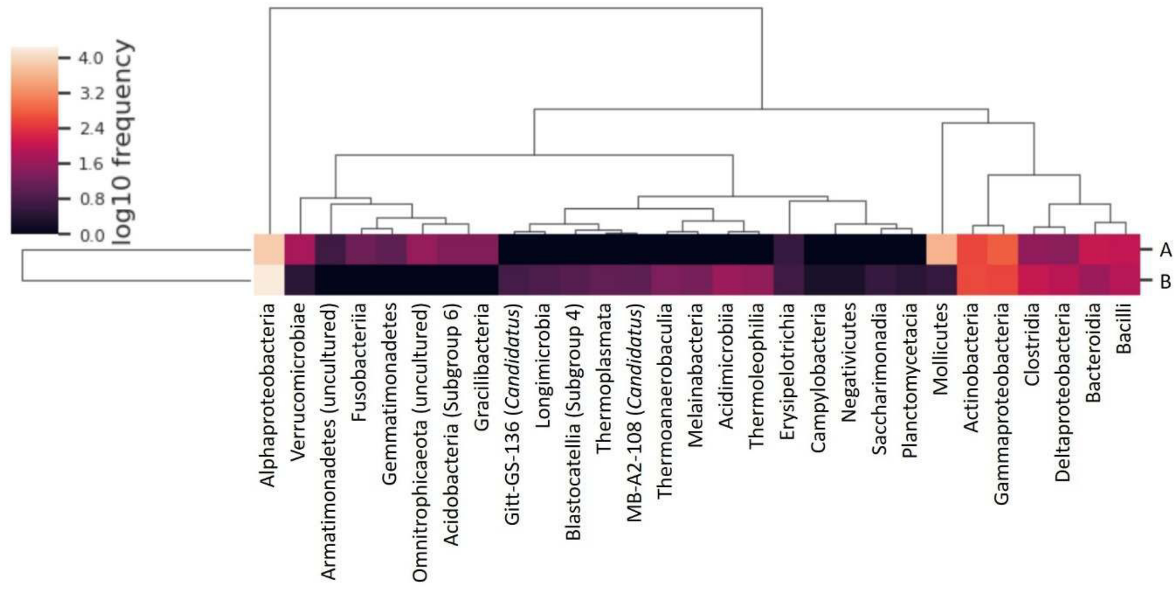

Figure 35 Heatmap of the relative frequency of bacterial classes in A. mexicana. Color code represents the logarithmic fecuency of each class

The microbiome of *Pogonomyrmex barbatus* is even more diverse than those recorded for *A. mexicana* and *C. atriceps*. And there is not a clear evidence of a specific dominant sOTU among samples or populations (Fig. 36), nevertheless a clear difference in the alfa diversity is present between populations where population C is the more diverse followed by population A with half of the Shannon`s diversity index. Population B have the lower alfa diversity with less than 1/10 of population C Shannon`s diversity index (Fig.37)

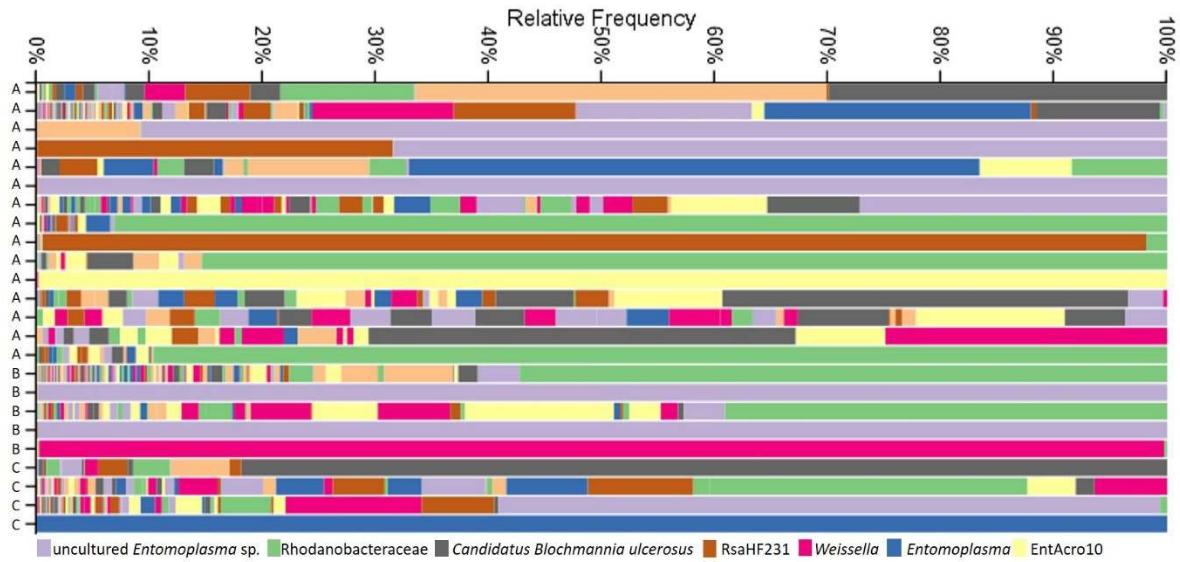

Figure 36. Relative frequency of sOTUS present in P. barbatus populations. Color bars represent the relative frequency of bacterial sOTUs in each sample, with the 7 most abundant sOTUs shown in color code.*g*

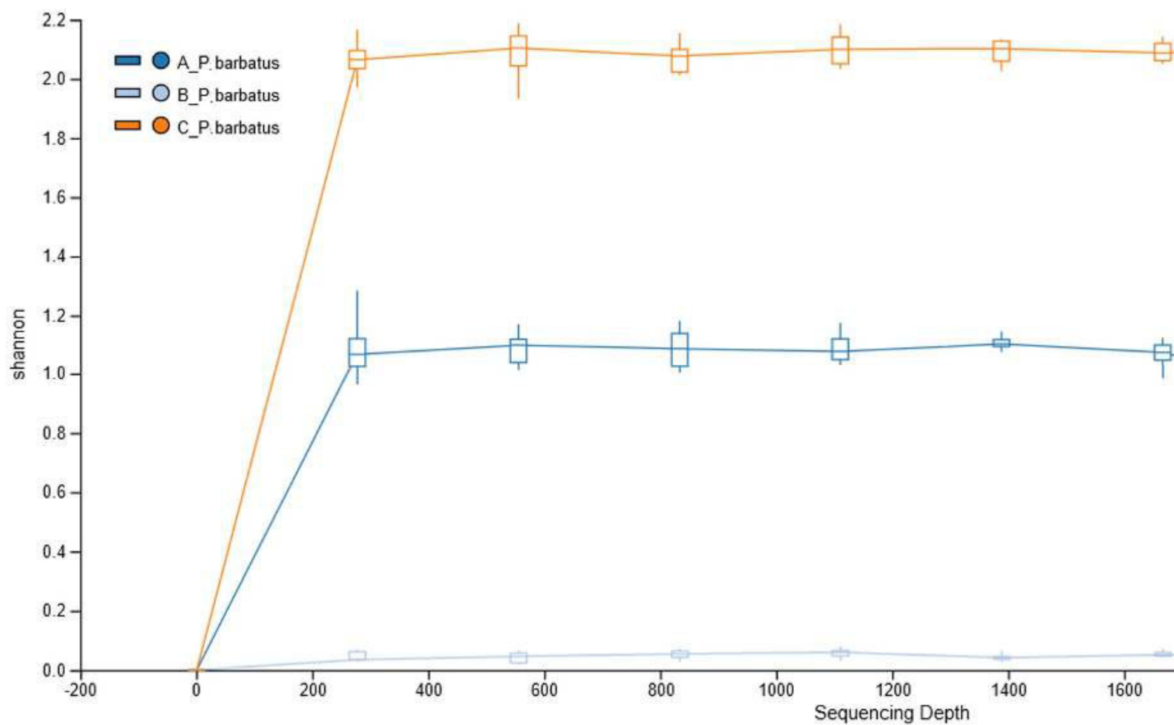

Figure 37. Alpha rarefaction of the Shannon index of the microbiome from three populations of P. barbatus. Color code shows each population.

There are five bacterial fila present in all three *P. barbatus* populations; Proteobacteria, Tenericutes, Firmicutes, Actinobacteria and Bacteroidetes. Population C is the only one that dones not have a exclusive Filum besides that is also the population with lower richness at this taxonomic level (Fig. 38).

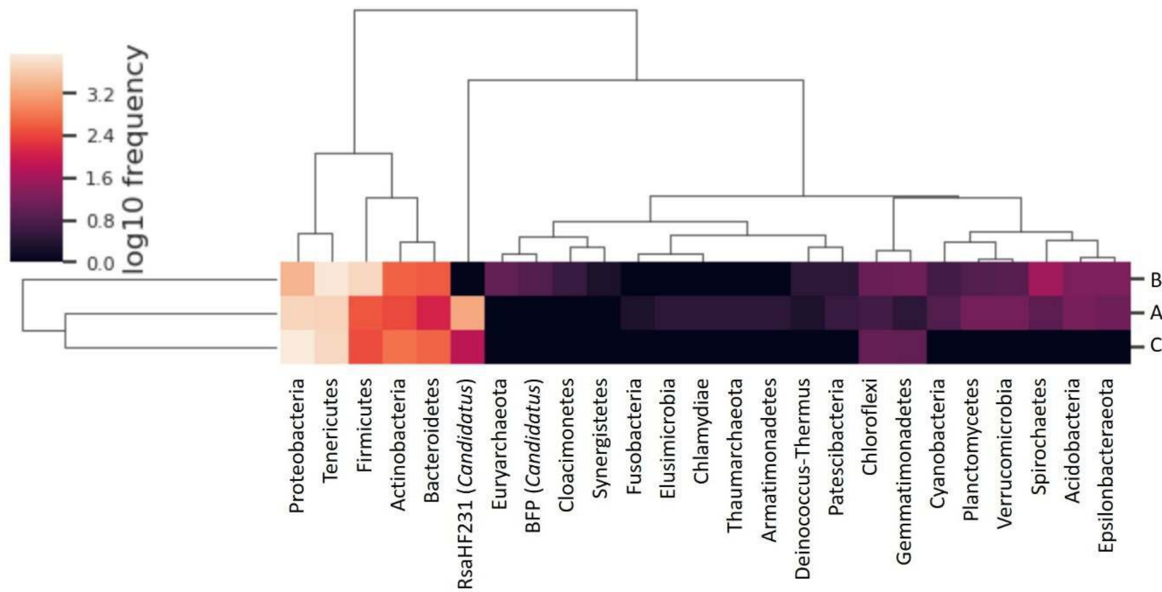

Figure38. Heatmap of the relative frequency of bacterial fila in Pogonomyrmex barbatus. Color code represents the logarithmic fecuency of each filum.

DISCUSSION

New generation sequencing techniques allow to detect the presence of low-density microbes in almost any environmental sample, an unwanted consequence of his high sensitivity is the detection of contaminant sequences that usually are in low densities. Bacterial DNA is everywhere, even if the equipment is properly sterilized DNA removal can only be achieved using an enzymatic or chemical digestion procedure (i.e. with dnase or strong acids).

Contamination in metagenomic analysis could have different sources and there are some critical issues to address in the experimental procedure. The impact of this exogenous DNA on the data interpretation seems to be critical in low biomass samples (Glassing et al. 2016) therefore should be considered in metagenomic studies of small size insects.

Exogen microbial DNA could come from the environment; during sample collection, or processing (DNA extraction or amplification) or from the lab reagents (Salter *et al*., 2014). On this study we use alcohol-fire sterilized foceps for the collection of every ant specimen, and laboratory precautions were taken on the handling of the samples. DNA extraction was performed in batches of 10 samples always using a facemask, sterile nitrile gloves and inside a UV sterilized laminar flow cabin.

Another important source of exogenous DNA comes from sample cross contamination; this can happen during the sample DNA extraction, amplification or during the DNA sequencing (Wright and Vetsigian 2016, Edgar 2016).

We used an a priori approach to diminish the probability of sample contamination by handling and manipulating the samples in aseptic or sterile conditions and by extracting the DNA in batches of 10 samples and an a posteriori approach by using blanks in most of the DNA extraction batches and analyzing the metagenome of those blank samples; Using an statistical approach of the average of the maximum reads per sOTU of each blank as a cut off number for previous analysis.

Microbial communities of ants have been studied for more than a century (Grabham 1917), mainly to explore the potential risk of ants being vectors of pathogenic bacteria. Recently, culture-independent techniques have been used to retrieve more complete information on the bacterial community composition (Russell, Sanders, and Moreau 2017), and approaches tend to answer questions about the possible benefits that ants can acquire from mutualistic bacteria and explore the holobiont theory in some groups of Formicidae. Here, we explore the possible ecological and evolutionary factors that can be responsible for associations of ants and their microbiome. This is the first study that compares the microbiome of ants in different ecosystems of México.

There have been efforts to study ant diversity in México (Vásquez-Bolaños 2015), particularly in such states as Veracruz, but states from the north central part of México (such as Guanajuato and Queretaro, Table III) are apparently undersampled It is important to increase the sampling efforts in this region to test the connectivity alongside the northern and southern ant populations. A recent metadata study shows the ant records across all the country with their abundance and distribution patterns, they conclude that more than half of the ant records in México correspond to samples from Chiapas and Veracruz (Dáttilo et al. 2020).

Most of the ant species in México can be identified using their morphological characteristics, but identification guides (Bolton et al. 2006, MacKay and Vinson 1989) are outdated and lack sufficient information to distinguish between some groups, particularly inside the *Camponotus* genus (Figure 20), between *Atta mexicana* and *Atta texana* (figure 23), between *Pogonomyrmex barbatus* and *Pogonomyrmeex rugosus, and* between *Pogonomyrmes comanche* and *Pogonomyrmex maricopa* (Figure 26).

Interspecific hybridization can be a plausible explanation for the species mixed clades of *Pogonomyrmex*. Hybridization has been reported between colonies of *P. barbatus* and *P. rugosus*

(Anderson et al. 2006, Helms Cahan and Keller 2003, SCHWANDER, CAHAN, and KELLER 2007) but neither for *P. maricopa* nor *P. comanche*. If we can identify those hybrid populations comparing their microbiome among the non-hybrid, it could help to explain the complex patterns seen in *P. barbatus* (Figure 36).

Ant's microbiome (the core metagenome)

A specific Entomoplasmatales clade has been proposed (Funaro et al. 2011) as the principal component of the army ant's microbiome (*Aenictinae*, *Dorylinae*, and *Ecitoninae*), suggesting coevolution among this bacterial clade and army ants. We found Entomoplasmatales as the dominant order in most of the *Eciton* colonies (10 of 14) and 3 of 5 of the *Neivamyrmex* colonies but not in *Labidus* colonies where the most frequent clade belonged to Firmicutes (Figure 10). The new world army ants study of (Łukasik et al. 2017) suggests that an uncultured Firmicutes clade is dominant for *Labidus*.

Dorylinae is an interesting candidate to explore the evolutionary relationship with Entomoplasmatales and Firmicutes due the potentially ancient relationship.

Turtle ants (*Cephalotes*) have been used as a model to study ant-bacteria coevolution (Lanan et al. 2016). The most abundant clades in which they were found were Burkholderiales, Pseudomonadales, Verrucomicrobiales, Xanthomonadales and Rhizobiales, the latter being a bacterial order that helps the ants to overcome nitrogen input deficiencies. In all *Cephalotes* colonies of this study, we found the same clades as the principal component (more than 95%) of the bacterial community (Figure 12). This suggests that coevolution could apply not only for Rhizobiales, but for all the previously listed orders; nevertheless, metabolic input or fitness impact on the host of the other bacterial orders must be investigated.

Two sOTUs found in our *Cephalotes* colonies were *Ventosimonas gracilis* (5% to 24% of relative abundance) and a *Cephaloticoccus* sOTU, present in 6 colonies of *Cephalotes* spp. (Figure 12). A *Ventosimonas gracilis* strain was isolated from the gaster of *C. varians* in Florida U.S. (Lin, Hobson, and Wertz 2016) and the Genus *Cephaloticoccus,* two unique species that were isolated from the guts of *Cephalotes rohweri* and *Cephalotes varians* in Arizona, U.S. (Lin et al. 2016) The presence of this sOTUs suggests a widespread distribution of both genus and an ancient shared evolutionary history.

As previously reported by (González-Escobar et al. 2018), we found that Proteobacteria and Firmicutes were the dominant bacterial Phyla on *Liometopum apiculatum* adults (Figure 8), but we found a more diverse pattern at the bacterial order level, and there is no clear pattern on the order relative frequency (nor is there at the sOTU level, Figure 9). This is probably because our samples have a higher spatial distribution than those reported by (González-Escobar et al. 2018), suggesting that the *Liometopum apiculatum* microbiome could be driven more by ecological than evolutive factors.

There are no previous reports on the *Dolichoderus bispinosus* metagenome. We found an uncultured sOTU from *Rhizobiaceae* in all the *Dolichoderus bispinosus* colonies as the dominant sOTU. Colonies from this species were sampled in different localities of Oaxaca, Veracruz and Quintana-Roo, suggesting that the relationship with this sOTU could be driven by the evolutive history of the ant.

The Entomoplasmales (Mollicutes) sOTU EntAcro1 seems to be present in most of the leaf cutter ants. It was previously reported as part of the microbiome of *Atta cehpalotes* (Zhukova et al. 2017), *Acromyrmex echinatior* and *Acromyrmex octospinosus* (Sapountzis et al. 2015) We found EntAcro1

in all the species of *Atta* and *Acromyrmex* but not in all their colonies, suggesting that this OUT could be part of the Leaf cutter ant's core microbiome as a probable mutualistic bacteria.

There are no previous reports on the metagenome of *Pogonomyrmex,* and we did not find any clear association of a specific sOTU with the ant species nor the locality of the colony (Figure 14). If we can detect those probable hybrid colonies, we can compare its microbial community among those not hybrid, and we must seek not only the most abundant sOTUs but those in lower relative frequencies. Some of the more bacterial diverse colonies were found in this genus, particularly in *P. maricopa* from Cuatro Ciénegas, Cohahuila.

It seems that *Camponotus* have more cohesion among groups by their microbiome (specifically *Blochmannia*) than by their COI sequence. Genetic diversity in *Camponotus* has been characterized as highly polymorphic in such species as *C. floridiauns* (Heinze et al. 1994), *C. herculeanus* and *C. ligniperda* (GERTSCH, PAMILO, and VARVIO 1995), and probably this also occurs in the species that we sampled in this study. Colonies from this genus presented the lower bacterial diversity in comparison with the other genera and were dominated by 4 *Blochmannia* sOTUs (Figure 13). Its known that *Camponotus* have specialized cells called bacteriocytes that harbor *Blochmannia* (Stoll et al. 2010, Wolschin et al. 2004, Schröder et al. 1996). While we used the whole abdomen to the analysis, the *Blochmannia* sequences can obfuscate other sOTUs present; another explanation is that *Camponotus* guts can have a low bacterial richness due the low pH inside the digestive tube (Heike Feldhaar, personal communication, 2018).

Previous reports on *Camponotus textor* (Ramalho et al. 2017) suggest that *Blochmannia* is not species specific. This is coherent with the *Blochmannia* sOTU distribution among our sampled colonies where none of them seems to be related to a specific ant species.

According to (Zilber-Rosenberg and Rosenberg 2008) there is a continuum of mechanisms for the vertical transmission of the microbial symbionts. Even though they propose that a categorization of those mechanisms could be simplistic, a clear pattern in the more direct or more indirect mechanisms is presented. In social and gregarious insects, transmission routes are clearly direct (Onchuru et al. 2018). This suggests that the whole colony and not the individual should be treated as the host factor of the holobiont.

Kin selection (Smith 1964) has been proposed as an evolutive strategy to explain the success of social behavior (Eberhard 1975) in all members of Formicide. Nevertheless, if the colony is considered as a holobiont, kin selection is acting not only on gene copies but also on the microbial component transmitting the core microbiome to future generations by rearing the siblings more than the offspring.

Bacterial diversity patterns among populations of selected ant species

We selected three ant species to inquire the microbiome patterns at population level: *Camponotus atriceps, Atta Mexicana* and *Pogonomyrmex barbatus*. The selection of those species' rests in their wide geographic distribution in Mexico besides the high population densities inside their nests. We were able to collect enough samples to perform a genetic and geographic analysis to delimitate the distance among the populations of each species (Fig. 21-28, Table 6.) to compare the bacterial community of each population and compare it between populations as well as the comparison between species.

The dominance of *Blochmannia* spp. In the microbiome of carpenter ants (*Camponotus* spp.) have been previously explored and the ubiquitous presence of this bacteria is explained by its vertical transmission (Schröder et al., 1996). We found 3 *Blochmannia* sOTUs in all *C. atriceps* samples. Each sample seems to have an exclusive *Blochmannia* sOTU that dominate the whole bacterial

community inside the gaster. there is not a clear pattern among the presence of each sOTU among populatios but sOTU "*Candidatus Blomachnnia rufipes*" that is exclusive of population C (Fig. 29). This sOTU have been found in not only in *C. atriceps but C. floridianus and C. rufipes* and their geographic distribution does not correspond to the host`s suggesting that long distance migrations of ants belonging to this genus could occur before the *Blochmannia* phylogenetic differentiation (Sauer et al. 2000).

Due the high dominance of *Blomachnnia* sOTUs in this genus bacterial diversity is usually low. lhe lower bacterial diversity was found in C. atriceps population C. (Fig. 31) as the lower bacteria abundance (Fig. 32) where *Candidatus B. rufipes* was exclusively found. When cultured, we suggest exploring the antimicrobial proprieties of this bacteria as a possible explanation on this low diversity.

Microbial community of *Atta mexicana* samples is clearly dominated by a single sOTU from the Rhizobiales bacterial family followed by the sOTU EntAcro1. Both microbial taxa have been previously reported for leaf cutting ants (Zhukova *et al*., 2017). A bacterial biofilm of Rhizobiales bacteria was described forming a microbial biofilm in the midgut of *Acromyrmex echinatior* serving as a nitrogen fixing bacteria and/or for protection (Sapountiz *et al*., 2015).

A single colony from the population A of A. mexicana was mostly dominated by the sOTU EntAcro1 (Fig. 33). This bacterium was firstly discovered by (Sapountiz *et al*., 2015) where they propose that it´s presence is also dominant and a mutually exclusive synergism with the sOTU EntAcro2 was proposed. (Zhukova *et al*., 2017) monitored the transmission of bacteria in laboratory-maintained nests of Acromyrmex echinatior and they confirm that the bacterial diversity was significantly lower in those workers with EntAcro1 inside the gut. This can explain the low microbial diversity in

our *A. mexicana* samples and the significant difference in the alpha diversity between those bacteria without this sOTUs (Fig. 33-34).

The alfa diversity between both A. mexicana populations seems to be similar (fig. 34) only when comparing the bacterial classes a notable difference can be seen between populations; Both populations are dominated by Alfaproteobacteria (this is mainly represented by the uncultured Rhizobiales sOTU) but Population A have a considerable abundance of Mollicutes, and a lower class richness (Fig. 35), probably another Mollicutes bacteria with the same effect as EntAcro1 could be present in that population. Nutrient providing and Virus protection are the main proposals to explain the evolutive mainteinance of Mollicutes in leaf cutter ants (Sapountzis et al. 2018)

This is the first report on the metagenome associated to any species of harvester ant (*Pogonomyrmex* spp.). There is not a clear pattern on the main microbial sOTUs among each population of P. barbatus, nevertheless a sOTU of *Entomoplasma* sp. Seems to dominate several samples (Fig. 36), Entomoplasmatales have been widely reported in ants` metagenome the genus *Entomoplasma* have only been reported in Ponerinae ants (Wong 2015) *Formica sanguinea* and its servant *Serviformica* sp. (Haapaniemi and Pamilo 2015).

The second most abundand sOTU in P. barbatus samples belongs to the family Rhodanobacteraceae and this is the first report of any member of this bacterial family in Formicidae, as well as the report of the sOTU from the *Candidatus* of phylum RsaHF231 that is present in populations A and C, this sOTU is the third more abundant sOTU in *P. barbatus*.

Another interesting sOTU inside the abdomen of P. barbatus is Candidatus Blomachnnia ulcerosus, as previously discussed, the Genus *Blomachnnia* have been exclusively reported associated with *Camponotus* ants. Most of the diet of P barbatus is based on seeds but insect parts are also

foraged (Morehead and Feener Jr 1998). This can explain the presence of *Blomachnnia* due the possible foraging of *Camponotus* corpses.

Alfa diversity of *P. barbatus* populations are dramatically different, even though in general P. barbatus hve a greater microbial biodiversity than *C. atriceps* and *A. mexicana*, comparisons among the three populations of *P. barbatus* shows that population C have the higher shannon´s biodiversity index, while population B have less than one tenth of the biodiversity of population C (Fig 37.). A complete inverse pattern is presented when we compare the bacterial fila inside each *P. barbatus* population where population C have the less fila present (Fig 38).

Pathogenic bacteria and the importance of Wolbachia

A remarkable frequency of 3 sOTUs of *Wolbachia* infection was found in 72% of the sampled colonies (Figure 22). The presence of *Wolbachia* seems to be independent of the ants' phylogeny and locality. Note that other studies report a lower frequency; (Russell et al. 2012) found 40% infection rates from two or more *Wolbachia* strains across their Formicidae samples, suggesting that *Wolbachia* can be found in high frequencies within some ant species. (Russell, Sanders, and Moreau 2017) report the presence of Wolbachia in 34-35% of ant species. This genus is a well-known reproductive parasite of insects where parthenogenic induction to kill males and cytoplasmatic incompatibility have been reported in Hymenoptera (Werren, Baldo, and Clark 2008). There is no clear explanation on how *Wolbachia* can be as widespread among insects. Some suggestions of fitness increase by virus protection have been made in *Drosophila melanogaster* (Hedges et al. 2008) and on *in vitro* infected *Aedes aegypti* (Carrington et al. 2018). In Formicinae, *Wolbachia* has been proposed as a possible mutualistic bacteria of fungus growing ants (ANDERSEN et al. 2012), but the mechanism of mutual benefits is undescribed.

It is important to highlight the implications of using *Wolbachia* as a biological control to reduce the spread of mosquito borne diseases which could be underestimated (Niang et al. 2018). This method is already implemented in Baja California Sur, México (http://www.eliminatedengue.com/mx) as well as in other countries, but the crossed infection of *Wolbachia* is still not fully understood.

CONCLUSIONS

The described metagenome of 60 % of the ant subfamily diversity of México shows that hosts´ evolutive history is the main factor to maintain the microbiome for most of the subfamilies, but not in *Ectatomamminae* or *Myrmicinae*, where ecological factors could play a more important role in the associated microbial community.

Camponotus is clearly dominated by 4 *Blochmannia* sOTUs (*Blochmannia* "Uncultured bacterium", *Blochmannia* "Candidatus *Blochmannia*", "*Blochmannia ulcerosus* and "*Blochmannia rufipes*").

Atta and *Acromyrmex* could be dominated by EntAcro1, but a single sOTU of an uncultured Rhizobiales bacteria dominate the microbial community of A. mexicana.

There is no clear pattern of sOTUs associated with a particular *Pogonomyrmex* species., *P. maricopa* bacterial diversity is the higher among all *Pogonomyrmex* species. P. barbatus metagenome shows a dominance of the bacterial family Rhodanobacteraceae and filum RsaHF231, this is the first report of those bacterial taxa in Formicidae

Apparently at family level bacterial composition is driven by evolutive factors. Coevolution is clear in the Camponatinni tribe and is plausible in Dolicoderine and Army ants, as well as in XXX because of the widespread of the symbiont-host relationship.

We still must explore the environmental-gradient and spatial distribution in most of the populations, but in *Liometopum apiculatum* spatial distribution seems to be the factor that molds the bacterial community.

APENDDIXES

Appendix 1. List of habitats and localities sampled in each state of México in this study. Habitat information was retrieved by the vectoral metadata of INEGI´s Vegetation and land use series IV chart (2017 edition). Elevation shows maximum and minimum in each state.

State	Localities	Habitats	Species	Elevation (MASL)
Coahuila	Cuatro Cienegas	Halophilic grassland	6	718-792
		Sand desert vegetation		
Colima	Potrero in Ixtlahuacan	Seccondary brushland from deciduous forest	4	171-176
Guanajuato	Calderones	Natural grassland	17	2064-2793
	Camino a chichindaro	Induced grassland		
	Cerro Culiacán	Oak forest		
	Cerro de la Bufa	Secondary tree vegetation from deciduous forest		
	Jales Villaseca	Secondary tree vegetation from deciduous forest		
	Las Palomas	Urban zone		
	Rancho Cerca del Coporo	Pine forest		
	Santa Rosa	Secondary brushland from oak forest		
Jalisco	Altiplano Lagos de Moreno	Secondary brushland from deciduous forest	12	23-2064
	Bosque Primavera	Secondary brushland from natural grassland		
	Chamela	Pine-Oak forest		
		Medium deciduous forest		
		Low deciduous forest		
Michoacán	Arko San Pedro	Seasonal annual agricultural plantation	11	2015-2431
	Cerro del Aguila	Seccondary brushland from oak forest		
	Encinar norte Quiroga	Oak forest		
	Lago Cointizo	Permanent forest plantation		
	Tiripetío	Secondary tree vegetation from deciduous forest		
Morelos	Quilamula	Seccondary brushland from oak forest	11	1076-1250
		Seasonal annual and semipermanent agricultural plantation		
		Seasonal and semipermanent annual agricultural plantation		
Nayarit	Cocodrialio Kiekari, Matanchen	Wetland vegetation	1	N/A
Nuevo León	Cumbres	Oak forest	13	422-1697
	Cumbres (El Mezcal)	Permanent irrigation agricultural plantation		
	Cumbres (estanzuela)	Submontante scrub		
	Cumbres (las Adjuntas)	Pine-oak forest		

	Cumbres (Quinta villas de boca)			
Oaxaca	Cafetal Carlota Río Uluapam	Secondary shrubland from mesophyll forest Seasonal annual agriculture Evergreen high forest	10	350-929
Puebla	Cascada las brisas, Cuetzalan Cascada las Hamacas Jardín botánico Helia Bravo Hollis Las cañadas San Juan Raya Zapotitlan	Temporal agricultural plantation Permanent grassland plantation Crasicaule scrub Mezquite forest	7	281-1432
Queretaro	Cerca de Peña de Bernal Jalpan de la sierra	Crasicaule scrub Secondary forest from submontane scrub Annual irrigation agricuture plantation Deciduous forest Induced grassland Seccondary brushland from oak forest	10	511-2575
Quintana-Roo	Botadero Bacalar Dzibanche Ecosur Chetumal Kinichna Oxtankah	Secondary shrubland from sub-evergreen medium forest Permanent grassland plantation Secondary tree from sub-evergreen medium forest Human settlements Seasonal annual agriculture plantation	9	0-101
Tamaulipas	Altacima Cielo	Mesophyll mountan forest Seasonal annual agriculture plantation Induced grassland Medium deciduous forest	18	123-940
Veracruz	Los Tuxtlas Puente nacional Ruiz Cortinez	Seccondary tree from evergreen high forest Permanent grassland plantation Seasonal annual and semipermanent agriculture Seasonal annual agriculture Permanent grassland plantation Evergreen high forest	14	87-1088

Appendix 2. Pipeline used for sequences treatment in QIIME2. Pipelines have annotations marked with # at the beginning of the paragraph.

#Import:

```
# NOTE files must be previously compressed and renamed as following:

barcodes.fastq.gz, forward.fastq.gz and reverse.fastq.gz, the three files must be in the same folder

# NOTE inside that folder there must be no other file tan the three previously described, any other file will cause a reading error.

qiime tools import --type EMPPairedEndSequences --input-path /media/sf_P1/fastq --output-path /media/sf_P1/fastq/fastqs.qza
```

#Demultiplex

```
#NOTE if the sequences come from a multiple project file, here is where filter by Project must be done. This is by using a metatable only with one project data, (use only the codes and barcodes of one Project excluding the barcodes and codes of other projects) metatable is named as MJAM_samples2.tsv

# NOTE correspondence table must be in a *.tsv format with al least 2 columns, the first one with the heading #SampleID and the second one with the heading Barcodes.
# NOTE in the earth microbiome protocol reverse complementary sequences does not have barcodes, thus the command --p-rev-comp-mapping-barcodes must be removed
# NOTE the second script serves to generate a visualization file of the summary of the demultiplexed sequences
# NOTE the metatable in a *.tsv file is generated in ASCII or UTF-8 code, there is a google sheet plug in to do it.

qiime demux emp-paired --m-barcodes-file /media/sf_P1/P2/MJAM_samples2.tsv --m-barcodes-column BarcodeSequence --i-seqs /media/sf_P1/fastq/fastqs.qza --o-per-sample-sequences demux

qiime demux summarize --i-data /media/sf_P1/p2/demux3.qza --o-visualization /media/sf_P1/p2/demux3.qzv
```

#denoise:

```
# NOTE: remove the command –verbose, this is only useful to see the log activity of this command

## NOTE the command run dada2 does sequence quality control, chimera removal and feature table construction

qiime dada2 denoise-paired --i-demultiplexed-seqs /media/sf_P1/P2/demux3.qza --p-trunc-len-f 151 --p-trunc-len-r 151 --o-representative-sequences rep_seqs_dada3.qza --o-table /media/sf_P1/P2/feature_table_dada3.qza --o-denoising-stats /media/sf_P1/P2/denoising-stats3.qza

# NOTE to view the representative sequence file for all samples
```

qiime feature-table tabulate-seqs --i-data /media/sf_P1/P2/rep_seqs_dada3.qza --o-visualization /media/sf_P1/P2/rep_seqs_dada3.qzv

NOTE to have the visualization of the summary of identified features

qiime feature-table summarize --i-table /media/sf_P1/P2/feature_table_dada3.qza --o-visualization /media/sf_P1/P2/feature_table_dada3.qzv --m-sample-metadata-file /media/sf_P1/P2/Metadata_table.tsv

#visualize the denoising stats

qiime metadata tabulate --m-input-file /media/sf_P1/P2/denoising-stats3.qza --o-visualization /media/sf_P1/P2/denoising-stats3.qzv

#Assign taxonomy

#NOTE: to assign taxonomy it is recommended to use a classifier trained with the project data, but in this analysis I am using a previously trained classifier using silva database

qiime feature-classifier classify-sklearn --i-classifier /media/sf_P1/Silva/silva-132-99-515-806-nb-classifier.qza --i-reads /media/sf_P1/P2/rep_seqs_dada3.qza --o-classification /media/sf_P1/P2/taxonomy.qza

qiime metadata tabulate --m-input-file /media/sf_P1/P2/taxonomy.qza --o-visualization /media/sf_P1/P2/taxonomy.qzv

#Remove mitocondrial and chloroplast sequences from sequences

qiime taxa filter-seqs --i-sequences /media/sf_P1/de_LANASE/rep_seqs_dada.qza --i-taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --p-exclude mitochondria,chloroplast --o-filtered-sequences /media/sf_P1/de_LANASE/sequences-with-phyla-no-mitochondria-no-chloroplast.qza

#Remove mitocondrial and chloroplast sequences from table

qiime taxa filter-table --i-table /media/sf_P1/de_LANASE/feature_table_dada.qza --i-taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --p-exclude mitochondria,chloroplast --o-filtered-table /media/sf_P1/de_LANASE/table_no_mit_no_chl.qza

#Remove Unassigned (at level 1) and D_0__Eukaryota from sequences

qiime taxa filter-seqs --i-sequences /media/sf_P1/de_LANASE/sequences-with-phyla-no-mitochondria-no-chloroplast.qza --i-taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --p-exclude Unassigned,D_0__Eukaryota --o-filtered-sequences /media/sf_P1/de_LANASE/filter2/sequences-with-phyla-only-bacteria.qza

#Remove Unassigned (at level 1) and D_0__Eukaryota from table

qiime taxa filter-table --i-table /media/sf_P1/de_LANASE/table_no_mit_no_chl.qza --i-taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --p-exclude Unassigned,D_0__Eukaryota --o-filtered-table /media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza

#Barplots

```
qiime taxa barplot --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --i-taxonomy
/media/sf_P1/de_LANASE/taxonomy.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization
/media/sf_P1/de_LANASE/filter2/taxa-bar-plots_only_bacteria.qzv
```

```
#Filter for Dolichoderinae and Pseudomyrmecinae
```

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where "Subfamily IN
('Dolichoderinae', 'Pseudomyrmecinae')" --o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Dolichoderinae_and_Pseudomyrmecinae.
qza
```

```
#Filter for Dorylinae and Ponerinae
```

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where "Subfamily IN
('Dorylinae', 'Ponerinae')" --o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Dorylinae_and_Ponerinae.qza
```

```
#Filter for Ectatomminae and Myrmicinae
```

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where "Subfamily IN
('Ectatomminae', 'Myrmicinae')" --o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Ectatomminae_and_Myrmicinae.qza
```

```
#Filter for Formicinae
```

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where
"Subfamily='Formicinae'" --o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Formicinae.qza
```

```
#Barplots for each subfamily (group)
```

```
#Barplots for Dolichoderinae and Pseudomyrmecinae
```

```
qiime taxa barplot --i-table
/media/sf_P1/de_LANASE/filter2/table_Dolichoderinae_and_Pseudomyrmecinae.
qza --i-taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization
/media/sf_P1/de_LANASE/filter2/taxa-bar-
plots_Dolichoderinae_and_Pseudomyrmecinae.qzv
```

#Barplots for #Filter for Dorylinae and Ponerinae

```
qiime taxa barplot --i-table
/media/sf_P1/de_LANASE/filter2/table_Dorylinae_and_Ponerinae.qza --i-
taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization
/media/sf_P1/de_LANASE/filter2/taxa-bar-plots_Dorylinae_and_Ponerinae.qzv
```

#Barplots for #Filter for Ectatomminae and Myrmicinae

```
qiime taxa barplot --i-table
/media/sf_P1/de_LANASE/filter2/table_Ectatomminae_and_Myrmicinae.qza --i-
taxonomy /media/sf_P1/de_LANASE/taxonomy.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization
/media/sf_P1/de_LANASE/filter2/taxa-
bar_plots_Ectatomminae_and_Myrmicinae.qzv
```

#Barplots for Formicinae

```
qiime taxa barplot --i-table
/media/sf_P1/de_LANASE/filter2/table_Formicinae.qza --i-taxonomy
/media/sf_P1/de_LANASE/taxonomy.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization
/media/sf_P1/de_LANASE/filter2/taxa-bar-plots_Formicinae.qzv
```

#**Tree construction**

#NOTE: to carry out a multiple sequence alignment using Mafft:

```
qiime alignment mafft  --i-sequences /media/sf_P1/de_LANASE/filter2/sequences-
with-phyla-only-bacteria.qza --o-alignment
/media/sf_P1/de_LANASE/filter2/aligned_seqs_bacteria.qza
```

#NOTE: to mask (or filter) the alignment to remove positions that are highly variable. These positions are generally considered to add noise to a resulting phylogenetic tree.

```
qiime alignment mask  --i-alignment
/media/sf_P1/de_LANASE/filter2/aligned_seqs_bacteria.qza --o-masked-alignment
/media/sf_P1/de_LANASE/filter2/masked_aligned_seqs_bacteria.qza
```

#NOTE: to create the tree using the Fasttree program

```
qiime phylogeny fasttree  --i-alignment
/media/sf_P1/de_LANASE/filter2/masked_aligned_seqs_bacteria.qza --o-tree
/media/sf_P1/de_LANASE/filter2/unrooted-tree.qza
```

#NOTE: to root the tree using the longest root

```
qiime phylogeny midpoint-root  --i-tree /media/sf_P1/de_LANASE/filter2/unrooted-
tree.qza  --o-rooted-tree /media/sf_P1/de_LANASE/filter2/rooted-tree.qza
```

#Alpha rarefaction

#NOTE: metadata columns will be been omitted if they didn't contain categorical data, or the column consisted only of missing values

#NOTE: a value of sampling depth is needed to applied in the next analysis. This value can be found analysing the rarefaction curves, in this case I use observed OTUs as a parameter and not Shannon diversity, the value was 1750

qiime diversity alpha-rarefaction --i-table /media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --i-phylogeny /media/sf_P1/de_LANASE/filter2/rooted-tree.qza --p-max-depth 2500 --m-metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization /media/sf_P1/de_LANASE/filter2/alpha-rarefaction.qzv

NOTE: to calculate and explore diversity metrics

qiime diversity core-metrics-phylogenetic --i-phylogeny /media/sf_P1/de_LANASE/filter2/rooted-tree.qza --i-table /media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --p-sampling-depth 800 --m-metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --output-dir /media/sf_P1/de_LANASE/filter2/core-metrics-results

NOTE: to test for relationships between alpha diversity and study metadata and create .qzv files to view these relationships

qiime diversity alpha-group-significance --i-alpha-diversity /media/sf_P1/de_LANASE/filter2/core-metrics-results/faith_pd_vector.qza --m-metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization /media/sf_P1/de_LANASE/filter2/core-metrics-results/faith-pd-group-significance.qzv

#NOTE: metadata columns will be omitted if they didn't contain categorical data, or the column consisted only of missing values

#NOTE: categorical metadata columns will be omitted if the number of groups is equal to the number of samples, there is only a single group, or the column consisted only of missing data

#NOTE: pairwise group comparisons will be omitted if the two groups being compared each have a sample size (n) of 1 and contain the same single value.

#NOTE: Some samples will be filtered from the input alpha diversity data if they have missing metadata values.

qiime diversity alpha-group-significance --i-alpha-diversity /media/sf_P1/de_LANASE/filter2/core-metrics-results/evenness_vector.qza --m-metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --o-visualization /media/sf_P1/de_LANASE/filter2/core-metrics-results/evenness-group-significance.qzv

#BETA DIVERSITY

#NOTE: beta diversity analysis must be done by each comparison column in the metadata file, in this script the analysis is done by subfamily. (--m-metadata-column Subfamily)

qiime diversity beta-group-significance --i-distance-matrix /media/sf_P1/de_LANASE/filter2/core-metrics-

```
results/unweighted_unifrac_distance_matrix.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv  --m-metadata-column Subfamily
--o-visualization /media/sf_P1/de_LANASE/filter2/core-metrics-
results/unweighted-unifrac-Subfamily-significance.qzv --p-pairwise
#NOTE: same for weighted unifac subfamily
```

```
qiime diversity beta-group-significance --i-distance-matrix
/media/sf_P1/de_LANASE/filter2/core-metrics-
results/weighted_unifrac_distance_matrix.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv  --m-metadata-column Subfamily
--o-visualization /media/sf_P1/de_LANASE/filter2/core-metrics-
results/weighted-unifrac-Subfamily-significance.qzv --p-pairwise
```

```
#NOTE: to create a PCoA plot to explore beta diversity metric

#NOTE: this analysis is done only with quantitative data

#NOTE: to use the unweighted unifrac data as input

qiime emperor plot --i-pcoa /media/sf_P1/P2/core-metrics-
results/unweighted_unifrac_pcoa_results.qza --m-metadata-file
/media/sf_P1/P2/MJAM_samples2.tsv  --p-custom-axes Subfamily  --o-
visualization /media/sf_P1/P2/core-metrics-results/unweighted-unifrac-
emperor-Subfamily.qzv

#NOTE: to repeat with bray Curtis

#NOTE: without editing with my data only, the analysis is done by
subfamily. (--m-metadata-column Subfamily )

qiime emperor plot  --i-pcoa /media/sf_P1/de_LANASE/filter2/core-metrics-
results/bray_curtis_pcoa_results.qza  --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-custom-axes Subfamily --o-
visualization /media/sf_P1/de_LANASE/filter2/core-metrics-results/bray-
curtis-emperor-Subfamily.qzv
```

#Filter feature table by metadata (for table with only camponotus, pogonomyrmex and atta/acromyrmex

#Filter for camponotus

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where "Genera='Camponotus'"
--o-filtered-table /media/sf_P1/de_LANASE/filter2/table_Camponotus.qza
```

#Filter for Pogonomyrmex

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where
"Genera='Pogonomyrmex'" --o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Pogonomyrmex.qza
```

#Filter for Atta/acromyrmex

```
qiime feature-table filter-samples --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --m-metadata-file
/media/sf_P1/de_LANASE/MJAM_samples3.tsv --p-where "Genera IN ('Atta',
```

```
'Acromyrmex')" --o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Atta_Acromyrmex.qza
```

#Collapse feature tables by their taxonomy at level 7.

#Collapse feature table all

```
qiime taxa collapse --i-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria.qza --i-taxonomy
/media/sf_P1/de_LANASE/taxonomy.qza  --p-level 7 --o-collapsed-table
/media/sf_P1/de_LANASE/filter2/table_only_bacteria_level7.qza
```

#Collapse feature table Camponotus

```
qiime taxa collapse --i-table
/media/sf_P1/de_LANASE/filter2/table_Camponotus.qza --i-taxonomy
/media/sf_P1/de_LANASE/taxonomy.qza  --p-level 7 --o-collapsed-table
/media/sf_P1/de_LANASE/filter2/table_Camponotus_level7.qza
```

#Remove unique sequences for Camponotus table

```
qiime feature-table filter-features --i-table
/media/sf_P1/de_LANASE/filter2/table_Camponotus_level7.qza --p-min-samples 2 --o-
filtered-table /media/sf_P1/de_LANASE/filter2/table_Camponotus_level7_nounique.qza
```

#Collapse feature table Pogonomyrmex

```
qiime taxa collapse --i-table
/media/sf_P1/de_LANASE/filter2/table_Pogonomyrmex.qza --i-taxonomy
/media/sf_P1/de_LANASE/taxonomy.qza  --p-level 7 --o-collapsed-table
/media/sf_P1/de_LANASE/filter2/table_Pogonomyrmex_level7.qza
```

#Remove unique sequences for Pogonomyrmex table

```
qiime feature-table filter-features --i-table
/media/sf_P1/de_LANASE/filter2/table_Pogonomyrmex_level7.qza --p-min-samples 2 --o-
filtered-table /media/sf_P1/de_LANASE/filter2/table_Pogonomyrmex_level7_nounique.qza
```

#Collapse feature table Atta/Acromyrmex

```
qiime taxa collapse --i-table
/media/sf_P1/de_LANASE/filter2/table_Atta_Acromyrmex.qza --i-taxonomy
/media/sf_P1/de_LANASE/taxonomy.qza  --p-level 7 --o-collapsed-table
/media/sf_P1/de_LANASE/filter2/table_Atta_Acromyrmex_level7.qza
```

#Remove unique sequences for Atta/Acromyrmex table

```
qiime feature-table filter-features --i-table
/media/sf_P1/de_LANASE/filter2/table_Atta_Acromyrmex_level7.qza --p-min-samples 2 --
o-filtered-table
/media/sf_P1/de_LANASE/filter2/table_Atta_Acromyrmex_level7_nounique.qza
```

#Heatmap for camponotus (species listed)

```
qiime feature-table heatmap --i-table
/media/sf_P1/de_LANASE/filter2/table_Camponotus_level7_nounique.qza --m-
metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --m-metadata-
column Species --o-visualization
```

```
/media/sf_P1/de_LANASE/filter2/Heatmap_Camponotus_Species_euclidean_nouni
que.qzv
```

#Heatmap for Pogonomyrmex (species listed)

```
qiime feature-table heatmap --i-table
/media/sf_P1/de_LANASE/filter2/table_Pogonomyrmex_level7_nounique.qza --
m-metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --m-metadata-
column Species --o-visualization
/media/sf_P1/de_LANASE/filter2/Heatmap_Pogonomyrmex_Species_euclidean_nou
nique.qzv
```

#Heatmap for Atta/Acromyrmex (species listed)

```
qiime feature-table heatmap --i-table
/media/sf_P1/de_LANASE/filter2/table_Atta_Acromyrmex_level7_nounique.qza
--m-metadata-file /media/sf_P1/de_LANASE/MJAM_samples3.tsv --m-metadata-
column Species --o-visualization
/media/sf_P1/de_LANASE/filter2/Heatmap_Atta_Acromyrmex_Species_euclidean_
nounique.qzv
```

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