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(BIOLOGIA CELULAR E MOLECULAR)

MICROBIOMA DE FORMIGAS COM ÊNFASE EM CAMPONOTINI (HYMENOPTERA, FORMICIDAE)"

MANUELA DE OLIVEIRA RAMALHO SANCHEZ

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Área: Biologia Celular e Molecular)

Maio - 2017

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Orientador: Prof. Dr. Odair Correa Bueno
Coorientador: Prof. Dr. Corrie Saux Moreau

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TÍTULO DA TESE: COMUNIDADES DE BACTÉRIAS DE DOIS GÊNEROS DE FORMIGAS Polyrhachis (SPINY ANTS) E Camponotus (CARPENTER ANTS)

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"…from so simple a beginning endless forms most beautiful and most wonderful have been, and are being evolved."

Charles Darwin

"Podemos agrupar as formas de vida em três, cinco ou um milhão de categorias, mas a vida em si nos escapará."

Lynn Margulis, uma mulher da ciência

Sumário

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Resumo

A interação simbiótica tem sido uma das responsáveis pela evolução e a biodiversidade de espécies existentes no planeta. Mais estudos abordando diferentes hospedeiros mostram-se necessários para aumentar o conhecimento do significado evolutivo desta associação na natureza. As formigas pertencentes aos gêneros *Polyrhachis* e *Camponotus* estão contidas na tribo Camponotini e são estreitamente relacionadas além de possuírem ampla distribuição, hábitos diversificados, e estão frequentemente associadas à endossimbiontes. Entretanto existem poucos estudos nesta área, permanecendo então muitas questões a respeito destas associações. Desta maneira, por meio da técnica de Sequenciamento de Nova Geração (NGS) Illumina MiSeq2000, o presente estudo teve como objetivo: I. explorar a comunidade microbiana de diversas espécies de *Polyrhachis* distribuídas em toda sua extensão e verificar os fatores que a influenciam. II. caracterizar a comunidade bacteriana associada aos gêneros *Colobopsis* e *Camponotus*, e analisar se há diferenças na composição da comunidade bacteriana quando comparada entre os diferentes gêneros, colônias e em todos os estágios de desenvolvimento; III. averiguar como se dá a distribuição da comunidade bacteriana nas diferentes partes do corpo (cabeça, mesossoma e gáster) de *Camponotus*, e se esta diversidade está associada ao ambiente onde estas *Camponotus* foram coletadas; IV. caracterizar o ovário de *Camponotus textor*, utilizando técnicas de histologia (HE), documentar a localização de *Blochmannia* e *Wolbachia* na ovogênese por hibridação "in situ" de fluorescência (FISH), e sugerir o mecanismo de desenvolvimento que estas bactérias utilizam para atingir o ovo. Estes estudos demonstraram que existem diversos fatores que podem influenciar a comunidade bacteriana associada a formiga, como a filogenia do hospedeiro, o gênero, a colônia, a ontogenia, diferentes partes do corpo e o ambiente que a formiga foi coletada. Adicionalmente, foi possível sugerir mecanismos adaptativos que garantem que as principais bactérias cheguem ao ovo.

Palavras Chaves: *Blochmannia*, endossimbionte, endobactéria, *Wolbachia*.

Microbiome of ants with emphasis on Camponotini (Hymenoptera, Formicidae)

Abstract

Symbiotic interaction has been one of the factors responsible for the evolution and biodiversity of species on the planet. More studies addressing different hosts are necessary to increase the knowledge of the evolutionary meaning of this association in nature. The ants belonging to the genera *Polyrhachis* and *Camponotus* are contained in the Camponotini tribe and they are closely related in addition to having wide distribution, diversified habits, and are often associated with endosymbionts. However, there are few studies in this area, and many questions remain about these associations. In this way, through the New Generation Sequencing technique (NGS) Illumina MiSeq2000, the present study aimed: I. To explore the microbial community of several species of *Polyrhachis* distributed throughout its range and to verify the factors that influence it. II. Characterize the bacterial community associated with the genus *Colobopsis* and *Camponotus*, and analyze if there are differences in the composition of the bacterial community when compared between different genera, colonies and at all stages of development; III. To determine how the distribution of the bacterial community occurs in the different parts of the body (head, mesosome and gaster) of *Camponotus*, and if this diversity is associated with the environment where these *Camponotus* were collected; IV. To characterize the ovary of *Camponotus textor* using histology techniques (HE), to document the location of *Blochmannia* and *Wolbachia* in oogenesis by fluorescence in situ hybridization (FISH), and to suggest the mechanism of development that these bacteria use to reach the egg . These studies have demonstrated that there are several factors that can influence the ant-associated bacterial community, such as host phylogeny, genera, colony, ontogeny, different body parts, and the environment the ant was collected. In addition, it was possible to suggest adaptive mechanisms that guarantee that the main bacteria reach the egg.

Key words: *Blochmannia*, Camponotini, endosymbionte, endobacteria, *Wolbachia*, NGS.

Introdução Geral

Existem cerca de 20.000 espécies de formigas pertencentes à família Formicidae - Ordem Hymenoptera, as quais são amplamente distribuídas pelo globo terrestre, não havendo representantes apenas no Pólo Norte e Antártida. A grande diversidade do grupo se dá devido ao grande sucesso ecológico, implicando nas várias formas de nidificação, preferências alimentares e comportamento social com divisão de trabalho entre as castas (HÖLLDOBLER; WILSON, 1990; WILSON, 1987).

As formigas da tribo Camponotini Forel 1878, pertencentes à subfamília Formicinae, compreendem oito gêneros existentes, como *Calomyrmex*, *Camponotus*, *Colobopsis*, *Dinomyrmex*, *Echinopla*, *Opisthopsis*, *Overbeckia* and *Polyrhachis* e mais dois gêneros extintos: *Chimaeromyrma* e *PseudoCamponotus* (BOLTON, 2016). Dentre eles, os gêneros *Polyrhachis* e *Camponotus* tem se mostrado estreitamente relacionados (SAMESHIMA et al., 1999). Ambos possuem ampla distribuição e são extremamente diversos, podendo apresentar diferentes formas de nidificação, desde ninhos terrestres a arbóreos, sendo que esta última pode resultar em uma dieta com deficiência nutricional, já que a obtenção do nitrogênio se dá pelo exsudato de plantas e predação de insetos fitófagos (COOK; DAVIDSON, 2006; DAVIDSON et al., 2003). Ambos os gêneros também possuem representantes tecelãs e constroem seus ninhos a partir da seda produzida pelas suas próprias larvas para entrelaçar folhas e galhos (HÖLLDOBLER; WILSON, 1990).

Outra característica comum entre os dois gêneros é a ausência da glândula metaplaural em *Polyrhachis* e também na maioria das espécies de *Camponotus.* Esta glândula seria responsável pela produção de secreções com funções antisépticas, defesa química e marcação de território (YEK; MUELLER, 2011). Johnson, Agapow e Crozier (2003) sugerem que a perda desta glândula possa ter facilitado a nidificação arbórea, uma vez que espécies com hábitos epigeicos e hipogeicos, as quais coletam na superfície do solo e nas suas camadas superficiais, respectivamente, estariam mais sujeitas a infecções, necessitando assim de outras estratégias de defesa. Baseado nesta informação, outro trabalho concluiu que o comportamento de autolimpeza, assim como o veneno com propriedades antimicrobianas são fundamentais para a resistência a doenças dentro da colônia de uma espécie tecelã de *Polyrhachis* (GRAYSTOCK; HUGHES, 2011). Ainda, Vieira, Bueno e Camargo-Mathias (2012) abordaram estudo através de evidências comparativas da morfologia desta glândula em formigas cultivadoras e não cultivadoras de fungos, incluindorepresentantes attines basais e

derivadas (formigas cortadeiras). Tais resultados revelaram que a morfologia da glândula metapleural mostrou-se mais desenvolvida nas formigas cortadeiras evidenciando maior proteção contra a proliferação de fungos e bactérias indesejadas no jardim de fungo. Estes estudos comparativos são escassos, porém tendem a revelar aspectos importantes sobre a biologia do inseto. Portanto, os dois gêneros (*Polyrhachis* e *Camponotus*) foram considerados no presente estudo.

O gênero *Polyrhachis* Smith, 1857 é caracterizado pela sua diversidade taxonômica, ecológica e social (VAN ZWEDEN et al., 2007). Este gênero compreende cerca de 700 espécies e possui ampla distribuição pelo Velho Mundo. Muitos autores sugeriram diversas divisões em subgêneros, porém Bolton (2016) separa da seguinte maneira: *Polyrhachis (Myrma), Polyrhachis (Hemioptica), Polyrhachis (Hagiomyrma), Polyrhachis (Hirtomyrma), Polyrhachis (Campomyrma), Polyrhachis (Myrmothrinax), Polyrhachis (Cyrtomyrma), Polyrhachis (Myrmhopla), Polyrhachis (Chariomyrma), Polyrhachis (Hedomyrma), Polyrhachis (Myrmatopa) e Polyrhachis (Aulacomyrma).*

Os ninhos de *Polyrhachis* podem variar drasticamente desde terrestres (presentes na lama) até arbóreos (no dossel das árvores), em zonas áridas ou em florestas tropicais. Os ninhos podem ser monodômicos ou polidômicos, as colônias podem ser monogínicas ou poligínica e com muitos ou poucos indivíduos (DOROW, 1995; DOROW; KOHOUT, 1995; NIELSEN, 1997; ROBSON; KOHOUT, 2005). Adicionalmente, *Polyrhachis* é o único exemplo de Formicinae que possui fundação semiclaustral (LENOIR; DEJEAN, 1994), ou seja, a rainha sai do ninho em construção para forragear, na tentativa de garantir recurso alimentar, apesar do perigo de predação, ao contrário da fundação do ninho claustral (NICKELE et al., 2013).

O gênero *Camponotus* Mayr, 1861, está subdividido em 43 subgêneros, abrangendo mais de 1000 espécies válidas, 465 subespécies e 31 fósseis (BOLTON, 2016), apresentando ampla distribuição geográfica: Américas, África, Ásia, Europa e Oceania (ANTWEB, 2017). De acordo com Robinson (1996), é o gênero mais representativo e heterogêneo das regiões neotropicais e tropicais, possuindo, inclusive, distribuição nas zonas temperadas.

Conhecidas popularmente como formigas-carpinteiras são comuns nos cerrados brasileiros e podem beneficiar as plantas pela predação dos herbívoros (DEL-CLARO; BARTO; RÉU, 1996). Possuem hábitos noturnos e dieta generalista, podendo nidificar em cavidades no solo, árvores vivas ou mortas e até no interior de construções. As principais espécies encontradas no Brasil (incluindo as espécies encontradas em áreas

urbanas) são *Camponotus atriceps* (Smith, 1858) (=*C.abdominalis*), *Camponotus crassus* Mayr, 1862, *Camponotus rufipes* (Fabricius, 1775), *Camponotus renggeri* Emery, 1894, *Camponotus arboreus* (Smith, 1858), *Camponotus fuscocinctus* Emery, 1888 *e Camponotus sericeiventris* (Guérin-Méneville, 1838) (ZORZENON et al., 2011).

A ocorrência de endossimbiontes é comum nos artrópodes. , Análises em 63 espécies revelaram que 76% estavam infectados (JEYAPRAKASH; HOY, 2000). Buchner (1965) foi um dos pioneiros no estudo de insetos como hospedeiro e os consideravam organismos modelos para o estudo dos endossimbiontes, por serem tolerantes à convivência de micro-organismos internamente e externamente ao corpo. Dentre os Hymenoptera, Formicidae é frequentemente relacionada à presença de endossimbiontes (ZIENTZ et al., 2005). A flexibilidade alimentar obtida por seus membros resulta no sucesso evolutivo do grupo, em parte alcançada pela presença dos endossimbiontes que auxiliam os hospedeiros nutricionalmente (ISHIKAWA, 1989).

Existem dois tipos de interação molecular entre as bactérias simbióticas e seus hospedeiros: a primária e a secundária, e ambos já foram relatados em formigas. A interação primária caracteriza-se por associações especializadas, descendentes de um ancestral, cujas árvores filogenéticas dos simbiontes são congruentes com a dos seus hospedeiros em períodos longos na escala evolutiva, indicando coevolução de ambos (BAUMANN, 2005; MUNSON et al., 1991). Também ocorrem alterações no tamanho do genoma do simbionte primário e este geralmente localiza-se em algum órgão especializado no interior do hospedeiro. Um bom exemplo disso em formigas é a *Blochmannia* (DEGNAN; LAZARUS; WERNEGREEN, 2005; GIL et al., 2004). Esta bactéria foi identificada pela primeira vez em *Camponotus ligniperda* (Latreille, 1802) (BLOCHMAN, 1882), ocorrendo no interior de uma célula especializada denominada de bacteriócito (SAMESHIMA et al., 1999).

A presença de um endossimbionte primário contido em um bacteriócito em Camponotini já foi descrita por Sameshima et al., (1999), Degnan et al.,(2004), Feldhaar et al., (2007), e Wernegreen et al., (2009). Em análises filogenéticas, o endossimbionte permaneceu num clado monofilético de Camponotini, revelando que houve coevolução entre hospedeiro e endossimbionte, assim como a aquisição do micro-organismo deve ter ocorrido no ancestral comum da tribo (SAMESHIMA et al., 1999; WERNEGREEN et al., 2009).

Acredita-se que o endossimbionte *Blochmannia* desempenhe papel nutricional

para o hospedeiro, fornecendo alguns aminoácidos essenciais (FELDHAAR et al., 2007), principalmente no início da vida (DEGNAN; LAZARUS; WERNEGREEN, 2005; GIL et al., 2003; WOLSCHIN et al., 2004). A *Blochmannia*, apesar de ter o seu genoma reduzido,mantem os genes para determinadas funções celulares básicas, como por exemplo, a biossíntese de nove aminoácidos essenciais (exceto Arginina), além de cofatores e enzimas de urease, as quais permitem que o simbionte recicle o nitrogênio da ureia (DE SOUZA et al., 2009; FAN et al., 2013; FELDHAAR et al., 2007). Portanto, a detecção desse endossimbionte em *Camponotus* e *Polyrhachis* torna-se importante, uma vez que estes dependem da *Blochmannia* para auxiliar no fornecimento de aminoácidos essenciais, já que ambos possuem uma dieta com deficiência de nutrientes em consequência de apresentarem hábitos arborícolas (SAMESHIMA et al., 1999; WERNEGREEN et al., 2009). Complementarmente, o papel nutricional da *Blochmannia* não é o único aspecto a se destacar da interação com o seu hospedeiro, pois esta endobactéria contribui para o metabolismo de nitrogênio, enxofre e de lipídeos (DEGNAN; LAZARUS; WERNEGREEN, 2005; GIL et al., 2003; WILLIAMS; WERNEGREEN, 2010).

A interação secundária normalmente é facultativa, podendo ser benéfica ou não, como a *Wolbachia*, uma vez que estimativas sugerem que milhões de espécies podem estar infectadas (SHOEMAKER; KELLER; ROSS, 2003). Há relatos deste endossimbionte associado a diversas espécies de Formicidae, como por exemplo, em *Solenopsis* spp. Westwood, 1840 (MARTINS; SOUZA; BUENO, 2012; SHOEMAKER; KELLER; ROSS, 2003), *Atta* spp. Fabricius, 1804 *Acromyrmex* spp. Mayr, 1865 (FROST et al., 2010), *Wasmannia auropunctata* (Roger, 1863) (REY et al., 2013) e inclusive em *Camponotus textor* Forel 1899, em que todas as colônias analisadas apresentaram uma ou mais cepas do endossimbionte (RAMALHO et al., 2017).

Além dos endossimbiontes *Wolbachia* e *Blochmannia*, este último estreitamente relacionado à tribo Camponotini, inclusive nos gêneros *Polyrhachis e Camponotus*, existem diversos trabalhos relatando a presença de outras espécies de hospedeiros e de endossimbiontes, como é o caso de *Arsenophonus* spp., *Cardinium hertigii*, *Hamiltonella defensa, Spiroplasma* spp., e *Wolbachia* spp. descritos por Russell et al. (2012) encontrados em algumas espécies hospedeiras de formigas e borboletas. No entanto, existem poucos estudos sobre identificação, função e interação de coevolução e codivergência de bactérias associadas aos gêneros *Polyrhachis e Camponotus*.

Perguntas sobre a interação da *Blochmanni*a e ainda outros endossimbiontes com Camponotini, principalmente em *Camponotus* e *Polyrhachis*, permanecem em aberto: o que Camponotini possui de diferente dos outros grupos que fez com que esta simbiose ficasse tão estabelecida na tribo? Será que existe algum endossimbionte relacionado à defesa contra patógenos dentro destes gêneros, já que a ausência da glândula metapleural é frequente? Mesmo em gêneros relacionados e que abrigam a *Blochmannia*, como é o caso de *Polyrhachis* e *Camponotus*, como se dá à distribuição e comparação deste e outros endossimbiontes com hospedeiros coletados em diferentes continentes?

Noggi (1981), Cochran (1985) e Lai, Baumann e Baumann (1994) enfatizaram que apesar de já ser bem difundida a ideia de simbiontes micetócitos (nome também dado ao bacteriócito por alguns autores) estarem frequentemente associados à interação nutricional com seus hospedeiros, pouco se conhece sobre a biologia desta relação. E quanto aos outros endossimbiontes associados? Questões feitas há décadas ainda permanecem muito recorrentes e sem resposta.

O estudo de endossimbiontes não cultiváveis tornou-se viável a partir da década de 70 com a técnica da PCR (*Polymerase Chain Reaction*) e o *16S rRNA* mostrou-se uma ferramenta útil para reconstrução filogenética e caracterização sistemática (PALUMBI, 1996). Apesar destes resultados, todos os trabalhos acima citados foram realizados pela amplificação do *16S* e sequenciamento pelo método de Sanger, os quais se baseiam em um número relativamente pequeno de micro-organismos. Existem diversas técnicas de Sequenciamento de Nova Geração (sigla *NGS*, proveniente do inglês *Next-Generation Sequencing*) que podem ser ferramentas úteis a se agregar ao estudo dos endossimbiontes e hospedeiros. Kautz e colaboradores (2013) compararam a técnica de pirosequenciamento (*NGS*) com métodos tradicionais para descobrir a diversidade bacteriana em *Cephalotes varians* (Smith, 1876). Com a técnica de *NGS* foi possível a recuperação de 445 OTUs (Unidade Taxonômica Operacional) raras que não foram detectados com as técnicas tradicionais, confirmando que este método irá proporcionar a descoberta da microbiota associada ao hospedeiro, e que é uma excelente ferramenta para a caracterização de comunidades anteriormente pouco estudadas, como a diversidade microbiana associada aos insetos.

Análise da microbiota de *Formica exsecta* Nylander, 1846 através do Illumina HiSeq 2000 (*NGS*) resultou em sequências que mostraram homologias com *Wolbachia*, *Arsenophonus*, *Entomoplasmatales* e *Microsporidia*, ainda, foram encontradas

sequências com identidade de outros micro-organismos, como fungos e bactérias comuns do solo (JOHANSSON et al., 2013). Na mosca tsétsé, o Sequenciamento de Nova Geração Illumina com plataforma MiSeq2000, revelou que o endossimbionte primário *Wigglesworthia* foi predominantee, porém também foi confirmada a presença inesperada do *Sodalis*, uma vez que apenas com a técnica de PCR tradicional a baixa infecção por este endossimbionte não havia sido detectada (AKSOY et al., 2014). Esses trabalhos reiteram a importância da técnica de Sequenciamento de Nova Geração para estudos com endossimbionte e hospedeiro, uma vez que podem revelar uma vasta quantidade de dados, incluindo os que são omitidos pelas técnicas tradicionais, assim como fornecer informações que auxiliarão a compreensão das interações biológicas das simbioses.

De uma maneira geral, os resultados apresentados por Ramalho (2017) com a técnica de Sanger, corroboram citações da literatura e adicionam novas informações sobre a espécie pertencente ao gênero *Camponotus*, assim como da presença e distribuição do endossimbionte *Wolbachia e Blochmannia* nas colônias analisadas e sua história evolutiva. Entretanto, seria importante a utilização de novas técnicas para avaliar a variação e a interação das múltiplas cepas nos diferentes estágios de vida das operárias das espécies de *Camponotus*, bem como a sua localização nos diferentes órgãos dos hospedeiros, além sua densidade relativa. A utilização da PCR quantitativa, hibridização fluorescente “in situ” (FISH) e o microscópio confocal de varredura a laser seriam necessários para medir a densidade da *Wolbachia* e *Blochmannia*, além de verificar a distribuição das bactérias entre os tecidos do hospedeiro.

Desta maneira, por meio da técnica do sequenciamento de nova geração, qPCR e hibridização fluorescente “in situ” (FISH) com análise em microscopia confocal de varredura a laser, o presente estudo poderá revelou aspectos importantes sobre a biologia dos gêneros *Polyrhachis* e *Camponotus*, a influência dos endossimbiontes nesse grupo tão diverso, além dos tipos de interações simbióticas envolvidas.

Objetivos

Neste estudo, os principais objetivos são:

1- Verificar, identificar e caracterizar a comunidade microbiana associada aos diferentes subgêneros de *Polyrhachis* em sua distribuição total, e verificar os fatores que o influenciam.

2- Caracterizar a comunidade bacteriana associada a uma colônia do recém-reconhecido gênero *Colobopsis* e três colônias de *Camponotu*s (duas espécies distintas) e mostrar quão diferente é a composição da comunidade bacteriana quando comparada entre os diferentes gêneros, colônias e em todos os estágios de desenvolvimento.

3- Verificar como se dá a distribuição da comunidade bacteriana nas diferentes partes do corpo (cabeça, mesossoma e gáster) de *Camponotus*, e se esta diversidade está associada ao ambiente onde estas *Camponotus* foram coletadas.

4- Caracterizar o ovário de *Camponotus textor*, utilizando técnicas de histologia (HE), documentar a localização de *Blochmannia* e *Wolbachia* na ovogênese por hibridação "in situ" de fluorescência (FISH), e sugerir o mecanismo de desenvolvimento que estas bactérias utilizam para atingir os ovos.

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Capítulo 1

Microbial composition of spiny ants (Hymenoptera: Formicidae: *Polyrhachis*) across their geographic range

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Resumo

Contexto: Relações simbióticas entre insetos e bactérias são encontradas em quase todas as ordens de insetos, incluindo Hymenoptera. No entanto, ainda existem muitas questões restantes sobre essas associações, incluindo quais são os fatores que direcionam a composição bacteriana associada ao hospedeiro. Para entender melhor o significado evolutivo dessa associação na natureza, são necessários mais estudos que abordem a diversidade de hospedeiro em toda sua distribuição e história evolutiva. As formigas do gênero *Polyrhachis* (formigas espinhosas) são distribuídas pelo Velho Mundo e exibem dietas e hábitos generalistas. Este estudo explora a comunidade microbiana de > 80 espécies de *Polyrhachis* distribuídas pelo Velho Mundo e compara a microbiota de amostras e hospedeiros relacionados em diferentes locais biogeográficos e no contexto de sua história filogenética.

Resultados: As bactérias predominantes nas amostras foram Enterobacteriaceae (*Blochmannia* - com provavelmente muitas cepas novas), seguida por *Wolbachia* (com múltiplas cepas), *Lactobacillus*, Thiotrichaceae, *Acinetobacter*, *Nocardia*, *Sodalis* e outros. Recuperamos algumas cepas exclusivas de Enterobacteriaceae como específicas de alguns subgêneros de *Polyrhachis*, corroborando a ideia de coevolução entre hospedeiro e bactérias para este grupo bacteriano. Nossos resultados de correlação (Teste de Mantel e Teste de Mantel Parcial) revelaram que a filogenia do hospedeiro pode influenciar a comunidade bacteriana global, mas a localização geográfica não teve efeito.

Conclusões: Este estudo revela aspectos importantes da biologia dos hospedeiros na estruturação da diversidade e abundância dessas comunidades bacterianas associadas ao hospedeiro, incluindo o papel da filogenia do hospedeiro e história evolutiva compartilhada.

Palavras-chave: *Blochmannia*, *Wolbachia*, *Lactobacillus*, NGS, micróbios, sequenciamento de amplicons.

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Microbial composition of spiny ants (Hymenoptera: Formicidae: *Polyrhachis*) across their geographic range

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Abstract

Background: Symbiotic relationships between insects and bacteria are found across almost all insect orders, including Hymenoptera. However there are still many remaining questions about these associations including what factors drive host-associated bacterial composition. To better understand the evolutionary significance of this association in nature, further studies addressing a diversity of hosts across locations and evolutionary history are necessary. Ants of the genus *Polyrhachis* (spiny ants) are distributed across the Old World and exhibit generalist diets and habits. Using Next Generation Sequencing (NGS) and bioinformatics tools, this study explores the microbial community of >80 species of *Polyrhachis* distributed across the Old World and compares the microbiota of samples and related hosts across different biogeographic locations and in the context of their phylogenetic history.

Results: The predominant bacteria across samples were Enterobacteriaceae (*Blochmannia* - with likely many new strains), followed by *Wolbachia* (with multiple strains), *Lactobacillus,* Thiotrichaceae, *Acinetobacter, Nocardia, Sodalis,* and others. We recovered some exclusive strains of Enterobacteriaceae as specific to some subgenera of *Polyrhachis*, corroborating the idea of coevolution between host and bacteria for this bacterial group. Our correlation results (partial mantel and mantel tests) found that host phylogeny can influence the overall bacterial community, but that geographic location had no effect.

Conclusions: Our work is revealing important aspects of the biology of hosts in structuring the diversity and abundance of these host-associated bacterial communities including the role of host phylogeny and shared evolutionary history.

Keywords: *Blochmannia*, *Wolbachia*, *Lactobacillus*, NGS, microbes, amplicon sequencing

Background

There are over 13,000 described species of ants belonging to the family Formicidae (Hymenoptera), which are widely distributed across the globe. The great diversity of the group is likely due to their ecological variability, including variation in nesting, feeding preferences and social behavior, and division of labor between castes [1, 2]. The genus *Polyrhachis*, Smith, 1857, is the fourth most species rich genus of ants and is characterized by its taxonomic, ecological and social diversity [3–5]. This genus contains more than 700 extant valid species [6], subdivided in the following recognized 13 subgenera: *Aulacomyrma* Emery, *Campomyrma* Wheeler, *Chariomyrma* Forel, *Cyrtomyrma* Forel, *Hagiomyrma* Wheeler, *Hedomyrma* Forel, *Hemioptica* Roger, *Hirtomyrma* Kohout, *Myrma* Billberg, *Myrmatopa* Forel, *Myrmhopla* Forel, *Myrmothrinax* Forel and *Polyrhachis* [5–7]. The genus *Polyrhachis* has a wide distribution across the tropical latitudes in the Old World, from Africa and Asia to Australia and a few Pacific islands, but being absent from Madagascar [7–9]. A possible reason for this restriction to the Old Word could be their late arrival to Africa, which potentially did not permit further dispersal to the New World as the continents had already drifted apart [10].

Nests of *Polyrhachis* can vary dramatically from terrestrial (present in the soil) to arboreal (in the canopy), in arid or tropical forests. Nests can be monodomous or

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polydomous, and colonies may be monogynous or polygynous (single or multiple breeding queens per nest). In addition colonies may vary in size from few to thousands of individuals [7, 11–13] with many species using larval silk to weave nests among plant leaves, a behavior that has been lost several times in the genus [4]. Additionally, *Polyrhachis* is one of the few examples from the subfamily Formicinae known to have semi-claustral colony foundation [14], where the queen will exit the nest during early colony foundation to forage in an attempt to obtain food resources, despite the danger of predation, unlike claustral nest foundation [15]. Recently Mezger and Moreau [10] in a large study (209 taxa) covering almost the entire distribution of the genus inferred the phylogeny and biogeography of the genus. Their molecular data support the monophyly of the genus, although some subgenera are not inferred as monophyletic. The authors were also able to estimate that the likely origin of the genus is South-East Asia, and that there were several dispersals into Australia, but only one to Africa.

In addition to the diversity of life history traits found across the ants, they also exhibit a range of associations with bacterial symbionts as seen in many other insect groups. For instance in an analysis across insect groups representing 63 species 76% were infected with associated bacteria [16]. In fact, Buchner [17] considered insects the model organismal group for the study of endosymbionts, since they coexist with microorganisms internally and externally to the body. Among the Hymenoptera, ants are well known for their associations with bacterial symbionts [18–20]. Diet flexibility exhibited by many species may explain much of the evolutionary success of the group, which is achieved in part due to the presence of endosymbionts that help improve host nutrition [21].

One well-studied example among the ants is the association of *Blochmannia* in the Camponotini ants, which circumscribes eight extant genera (*Calomyrmex*, *Camponotus*, *Echinopla*, *Forelophilus*, *Opisthopsis*, *Overbeckia*, *Phasmomyrmex*, and *Polyrhachis*) including *Polyrhachis,* the focal genus in this study. *Blochmannia* is a Proteobacteria specific to the Camponotini, which has been demonstrated to assist in providing essential amino acids to their host since their diets are defficient in nutrients as a consequence of their arboreal habitats [22, 23]. The nutritional role of *Blochmannia* is not the only beneficial aspect to the host, as it has been shown that *Blochmannia* also has the necessary genes to contribute to the metabolism of nitrogen, sulfur and lipids [24–26]. In addition to *Blochmannia* endosymbionts, among members of the Camponotini tribe, there are other species of endosymbionts that have been documented from these hosts, including *Arsenophonus* spp., *Cardinium hertigii*, *Hamiltonella defense*, and *Spiroplasma* spp. [27, 28]. However, little work has been done on the identification, diversity, and potential co-evolution of bacteria associated with *Polyrhachis*, leaving many remaining questions about these associations including what factors drive host-associated bacterial composition.

To better understand the evolutionary significance of this association in nature, further studies addressing a diversity of hosts across locations are necessary. Therefore to address this question, we focus our study on the bacterial community of a host that exhibits high species diversity and a wide geographic distribution, to reveal more about the factors that influence bacterial communities. Leveraging next-generation sequencing, we document the diversity of bacteria associated with *Polyrhachis* (in 12 of the 13 subgenera), to identify the factors that structure the diversity of bacterial communities found across a diverse and widely distributed group of animals.

Methods

DNA extraction and bacterial DNA sequencing

For this study we included 142 samples of *Polyrhachis* representing 12 of the 13 subgenera from the study of Mezger and Moreau [10]. A complete list of samples used for this study can be found in Additional file 1: Table S1. The taxonomic identifications were determined by Mezger and Moreau [10] and vouchers were deposited in the collection of the Field Museum of Natural History, Chicago, USA during that study. Samples used for analyses were collected immediately into 95% ethanol in the field and and stored in 95% ethanol and kept at −20 °C until extraction of total DNA was performed. Total DNA was extracted from whole ant workers with Qiagen DNeasy Tissue kit following the manufacturer's recommendations with slight modifications following Moreau [29] and we did not use the modification of the Quigen DNeasy kit for gram-positive bacteria. In addition, filtered pipette tips and sterile measurements were applied to avoid contamination of the samples, following recommendations of Moreau [29]. Amplicon sequencing of the microbial community was completed using the V4 region of 16S rRNA using primers described in Caporaso et al. [30], following the Earth Microbiome Project (EMP) protocol (515f primer and 806r; http://www.earthmicrobiome.org/emp-standard-protocols/16s/). PCR was performed in triplicate, each 25 μl PCR reaction contained 12 μl of MO BIO PCR Water (Certified DNA-free), 10 μl of 5 Prime HotMasterMix (1×), 1 μl of forward primer (5 mM concentration, 200 final pM), 1 μl Golay barcode tagged reverse primer (5 mM concentration, 200 pM final) and 1 μL of template DNA, under the following conditions

94 °C for 3 min to denature the DNA with 35 cycles at 94 °C for 45 s, 50 °C is 60 s, and 72 °C for 90 s, with a final extension of 10 min at 72 °C. After amplification, the triplicate reactions were combined (still maintaining the individuality of samples), and to confirm the efficiency of the reaction samples were visualized using gel electrophoresis (1%). The samples were quantified via qPCR and Qubit (Thermo Fisher Scientific) (see bacterial quantification section below), and only then pooled with different samples after controlling for volume (multiplex). For purification, only 100 μL of each pool was cleaned using the UltraClean PCR Clean-Up Kit (MO BIO), following the manufacturer's recommendations. After quantification, the molarity of the pool is determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.1 pM with a 10% PhiX for sequencing on the Illumina MiSeq. A 151 bp × 12 bp × 151 bp MiSeq run was performed using the custom sequencing primers and procedures described in the supplementary methods in Caporaso et al. [30] on the Illumina MiSeq at the Field Museum of Natural History. All raw sequence data is available publicly in Figshare [https://figshare.com/s/290531bea3dee984444e] [31] and also available in the NCBI Sequence Read Archive (SRA) under accession number SRR5136256 and study SRP095836 [32].

Bacterial quantification

To optimize Illumina sequencing efficiency, we measured the amount of bacterial DNA present with quantitative PCR (qPCR) of the bacterial 16S rRNA gene using 515f (5′ - GTGCCAGCMG CCGCGGTAA) and 806r (5′ - GGACTACHVGGGTWT CTAAT) universal bacterial primers of the EMP (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). All samples and each standard dilution were analyzed in triplicate in qPCR reactions. All qPCRs were performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using SsoAdvanced 2X SYBR green supermix (Bio-Rad) and 2 μL of DNA. Standard curves were created from serial dilutions of linearized plasmid containing inserts of the *E. coli* 16S rRNA gene and melt curves were used to confirm the absence of qPCR primer dimers. The resulting triplicate amounts were averaged before calculating the number of bacterial 16S rRNA gene copies per microliter of DNA solution (see Additional file 2: Table S5).

Bioinformatic analysis

The sequences were analyzed in QIIME 1.9.1 [33]. First, the forward and reverse sequences were merged using SeqPrep. Demultiplexing was completed with the *split_libraries_fastq.py* command, commonly used for samples in fastq format. QIIME defaults were used for quality filtering of raw Illumina data. For calling the OTUs, we chose the *pick_open_reference_otus.py* command against the references of Silva 128 [34, 35] 97% identity with UCLUST to create the OTU table (biom format). Sequences with less similarity were discarded. Chimera checking was performed [36] and PyNAST (v1.2.2) was used for sequence alignment [37].

To test whether bacterial community composition is associated with taxonomic or geographic information, and if the taxonomic and geographic hierarchies can influence the bacterial community, we binned our data into different categories: "Subgenera" & "Species" to test taxonomic levels, and "Biogeography" & "Country", to test the effect of geographic collection location. The *summarize_taxa_through_plots.py* command was used to create a folder containing taxonomy summary files (at different levels). Through this analysis it is possible to verify the total percentage of bacteria in each sample and subgenus. Additionally it is also possible to have a summary idea of the bacteria that constitute the bacterial community of *Polyrhachis*. In order to standardize sequencing effort all samples were rarefied to 400 reads. All samples that obtained fewer than 400 bacterial sequences were excluded from further analysis.

We used Analysis of Similarity (ANOSIM) to test whether two or more predefined groups of samples are significantly different, a redundancy analysis (RDA) to test the relationships between samples, and Adonis [38] to determine sample grouping. All these analyses were calculated using the compare_categories.py command in QIIME. The G test of independence (P, FDR_P and Bonferroni_P) was carried out to determine whether OTU presence/absence is associated with a host category through *group_significance.py* command. All these statistical tests serve to test whether the bacterial community is being influenced by any of the categories described above.

Alpha diversity was quantified using observed species richness, Shannon diversity, the Chao1 nonparametric richness estimator and whole-tree phylogenetic diversity and Simpson as implemented in equitability metric. We also compared alpha diversity based on a two-sample t-test using non-parametric (Monte Carlo) methods to test differences in OTU richness among subgenera. Unweighted and weighted UniFrac distance matrices [39], which uses phylogenetic information to calculate community similarity, were produced through the QIIME pipeline. The rarefaction curve was also created in QIIME and it is important to confirm if the sequencing was enough to cover the entire bacterial community associated with *Polyrhachis*. These beta diversity metrics were used to compare community level differences between categories. Jaccard dissimilarity metrics were calculated by beta_diversity.py command in QIIME. A matrix of community pairwise distances was generated

by UniFrac and used to cluster samples by (i) the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method and (ii) principal coordinates analysis (PCoA). The UPGMA and PCoA analyzes that use the UniFrac beta diversity matrices show us which categories are influencing the bacterial community. As these analyzes have different methodologies and they will generate more robustness to the data of the study.

At a sequencing depth of 400, 64 samples passed this cutoff and were included in downstream analyses. To illustrate the relationship between ecological communities [40, 41], we implemented the analysis of multidimensional nonmetric scaling (NMDS) and related statistics in the PAST3 software package [42]. Sorensen (Dice coefficient) and Bray-Curtis similarity indices [40] were used to test the variation and the structure of the bacterial community, respectively. The samples were grouped according to the host subgenera, and after viewing the plots, analyzes of similarity (ANOSIM) with Bonferroni correction was used to determine statistical significance [40, 41, 43]. As this analysis requires at least two representatives from each group, the subgenera that had only one representative were grouped into a category "Mixed".

Networks were visualized using Cytoscape3.2.1 [44] edge-weighted spring embedded algorithm to display the OTUs and sample nodes [45]. Each host-bacterial network was constructed as a graph, in which each node represented a host sample. Connections were drawn between samples representing the shared significant OTUs (each color represents a different OTU). Through the network it is possible to visualize the complexity that surrounds the bacterial community associated with *Polyrhachis* and to look for which category may best explain the pattern found. A heatmap was constructed with all OTUs that had 400 reads represented in the main dataset using heatmap.2 and the vegan package [46] in R [47]. The dendrogram of the samples shown in the heatmap was created with Bray-Curtis dissimilarity hierarchical clustering of bacterial communities in hclust. We also added a column dendrogram to cluster the genera that occur more often together. In this analysis we restrict only the most well represented OTUs and check if there is any OTU specificity within any of the categories described above. With this analysis it is also possible to verify the samples that have multiple infections as can happen with specimens infected with *Wolbachia* and *Blochmannia* [48].

We did analyses of correlation and coevolution: 1) compared the bacterial community following the host phylogeny of Mezger and Moreau [10] (coevolution/vertical transfer); 2) and similarity of bacterial community from hosts based on their locality (horizontal transfer). For this, geographic distances were calculated from sample locality information using geographical collection coordinates (latitude/longitude) of each included sample. They were transformed to UTM distance metric using the "rgdal" package [49] in R [47] and geographic distance matrix was constructed. The weighted distance of all sample were calculated through beta diversity in QIIME. The correlation between the bacterial community and geographic distances of *Polyrhachis,* and bacterial community and host phylogeny were calculated using the Mantel test (999 permutations) using the "vegan" package [46] in R. We also tested for significant associations between bacterial community dissimilarities and host genetic and geographic distances, we used partial Mantel tests, as implemented in the vegan package in R [46].

Results

Bacterial 16S rRNA diversity

Illumina 16S rRNA sequencing of *Polyrhachis* ant hosts reveals a relatively simple microbiota that is remarkably conserved. Our analyses obtained 5443 observed OTUs from a total of 61,225 reads from 132 specimens from 12 of the 13 subgenera of *Polyrhachis* collected from across the Old World, which permitted analyses comparing different host categories: species, subgenera, biogeography and countries.

The diversity and the total number of bacteria found in *Polyrhachis* are represented in Fig. 1. Our analyses recovered variation from 1 to a maximum of 1384 OTUs of bacteria per sample, a lower absolute diversity compared to other herbivorous ants such as *Cephalotes* [20, 50–52]. The predominant bacteria across samples were Enterobacteriaceae (44.40%), *Candidatus Blochmannia* (15.70%), Enterobacteriaceae - other (11.90%), *Wolbachia* (8.80% - multiple strains) and *Lactobacillus* (2.90%), followed by Thiotrichaceae (2.0%), *Acinetobacter* (1.60%), *Nocardia* (1.20%), *Sodalis* (0.80%) and Entomoplasmatales (0.80%) [Additional file 3: Table S2].

Statistical analyses of bacterial community diversity

We performed statistical tests (weighted and unweighted) to examine potential patterns that influence the bacterial community of *Polyrhachis*. From these we found subgeneric taxonomic affliation of the host (Adonis, unweight R^2 = 0.23602 and P = 0.002; Anosim, unweight R^2 = 0.11400 and P = 0.029; RDA, unweight Pseudo F = 1.47656 and significance = 0.001) had more influence on bacterial community composition than broader biogeographic origin, country or species, although not statistically significant.

Through the results of the G test (P, FDR_P and Bonferroni_P), we found bacteria community presence/absence is significantly different across multiple categories (species, subgenera, biogeography and country) [see in Additional file 4: Table S3]. Within the species category more bacteria were significant across samples than the

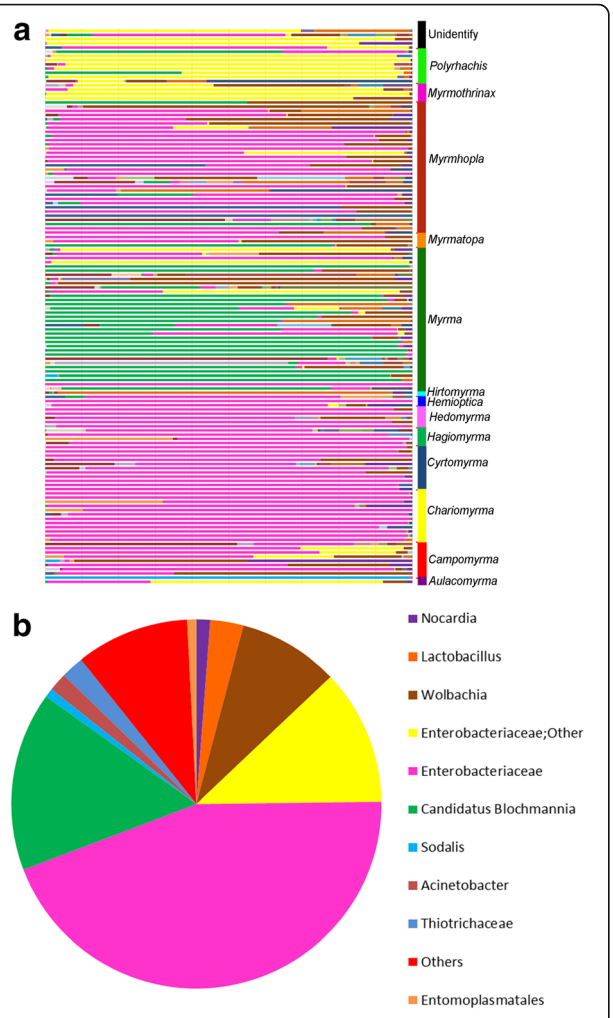

Fig. 1 Summary table of bacterial OTUs found in *Polyrhachis* samples with 16S rRNA amplicon sequencing. **a** *Polyrhachis* subgenera used in this study and their bacterial communities. Bar graphs for each library (one column = community from a single worker) show the percentage of sequence reads classified to selected 97% OTUs. Each color represents a distinct bacterium. The samples were grouped according to the subgenera which they belong. **b** Summary of all OTUs found in this study with legend ordered in proportion of reads found across all 132 samples. The relative abundance of reads at the taxonomic level of bacteria is displayed. Orders that accounted for less than 0.8% in a sample are summarized in a category termed "Other"

other host categories. However, the bacteria Enterobacteriaceae (multiple strains, including *Candidatus Blochmannia*), *Wolbachia* (multiple strains), *Nocardia*, *Sodalis*, Thiotrichaceae and *Lactobacillus* were significant across all categories [Additional file 4: Table S3].

Alpha diversity

Alpha diversity (Chao1, PD whole tree, observed OTUs, Simpson and Shannon) observed across *Polyrhachis* individuals was not high. For the remaining samples at sequencing depth of 400, we recovered high variation of diversity [Additional file 5: Table S4]. Likely due to the small amount of sequence for these samples, we did not obtain significant results when comparing differences in OTU richness among host subgenera. Through the rarefaction curve analysis of observed OTUs, our sequencing coverage of the bacterial communities appears satisfactorily for most samples, but even with the thousands of Illumina sequence reads, sampling was not sufficient to achieve a plateau for all specimens (Fig. 2).

Beta diversity

Through analysis of beta diversity (matrices UniFrac weighted distance, depth 400 (50% of samples)) we find similarity of the bacterial communities from these samples. The UPGMA tree (Weighted UniFrac method) of the entire bacterial community of *Polyrhachis* grouped samples of different subgenera and biogeography, but we realized that the samples were grouped according to high infection of different bacteria (Figs. 3a and 4). Variation among samples in their bacterial taxonomic composition was visualized using constrained principal coordinates analyses (Fig. 3b). The average Jaccard dissimilarity metric was 0.91, which suggests only a few bacterial community members were shared among all individuals of *Polyrhachis*. Also, we found no significant changes in the composition (Soresen index) of the bacterial community of *Polyrhachis* (R = 0 and P = 1). That is, different subgenera do not have significantly different bacteria. But there was an effect of the structure of the bacterial community (Bray-Curtis index, stress 0.044, R = 0.2205 and P = 0.0003) when all subgenera were compared. In the analysis of the subgenera in pairs, it was not possible to identify significant results.

Network analysis

To examine the connection between samples with shared significant OTUs, we used Cytoscape to construct a network graph in which each node represented a host sample. Network analyzes were performed using default parameters using the spring-embedded edge-weighted algorithm (Fig. 5a), and the spring-embedded edge-weighted algorithm manually edited (Fig. 5b), which approaches the samples according to the number of OTUs shared. OTUs with less than 400 reads were hidden for easy viewing. In this analysis, only the edges of Enterobacteriaceae (pink), Enterobacteriacea, other (yellow), *Candidatus Blochmannia* (green), *Wolbachia* (brown), *Lactobacillus* (orange), *Nocardia* (purple), *Sodalis* (light blue), and Thiotrichaceae (dark blue), Others (red) were colored. Note how complex these associations are (Fig. 5).

HeatMap

Through heatmap analysis (bacterial genera and family levels), we investigated the entire bacterial community

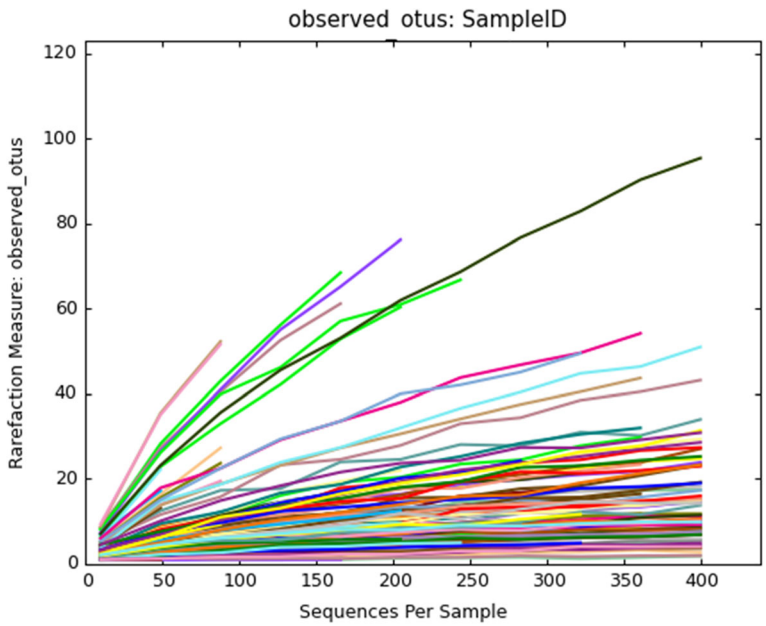

Fig. 2 Rarefaction curves were used to estimate richness in the observed OTUs. The vertical axis shows the bacterial OTUs observed and the number of sequences per sample is shown on the horizontal axis. Note that although sequencing covers thousands of Illumina reads, some samples have not reached the plateau

found in this study and the abundance of OTUs found in each sample. For easy viewing, we choose to show only OTUs with more than 400 reads. It is interesting to note that more than 50% of the bacterial community consisted of Enterobacteriaceae (multiple strains). Several strains of Enterobacteriaceae were restricted to specific subgenera of *Polyrhachis*. This includes *Candidatus Blochmannia*-New.ReferenceOTU70 which was almost exclusively associated with the host subgenus *Myrma* from the Afrotropics, Enterobacteriaceae-New.ReferenceOTU13 which was almost exclusively with subgenus *Polyrhachis,* and Enterobacteriaceae-New.CleanUp.ReferenceOTU0 is found in samples from subgenus *Myrmhopla.*

Another interesting observation is there are four different highly abundant *Wolbachia* strains found across our samples. We observed an infection rate of 49.24% from across our 132 samples. There are even multiple individuals (n = 25, 38.46%) with the presence of a double infection of *Wolbachia*. Also, the presence of *Lactobacillus* was unexpected and was identified from samples from across the distribution of the genus (Fig. 6).

Correlation and coevolution tests

The Mantel test verified the correlation of the bacterial community and geographic distance when analyzed with phylogenetics information from Merzer and Moreau [10] for *Polyrhachis* hosts. In addition using the Mantel test we found support for correlation between the phylogeny of the host and the bacterial community using the "vegan" package [46]) in R (R = 0.2289 and P = 0.0001). We also tested for the influence of locality on the bacterial community sampling, again using the Mantel test through the R software package to generate the pairwise geographical distances of each *Polyrhachis* sample. Our results showed that there is no correlation between the geographical location and the bacterial community overall (R = 0.08582 and P = 0.0756). Lastly through a partial mantel test of the three matrices (bacterial community, host phylogeny and geographical distances), we were able to demonstrate that the phylogeny of the host explains just part (R = 0.2279 and P = 0.0001) of the entire bacterial community, while geographical distance does not have significant influence on structuring the bacterial community of spiny ants (R = 0.09075 and P = 0.0697). While conducting more specific analysis of correlations of individual OTUs with the phylogeny of the host, we did not obtained significant results.

Discussion

The use of NGS technologies to study the microbiome is relatively recent and these data are providing an unprecedented understanding of microbial diversity and putative function in many habitats and across a diversity of hosts. The bacterial communities associated with hosts can vary from simple to complex and can be influenced by environmental, genetic and other factors of the host or host's environment which can make the task of understanding the elements determining host-association a challenge [53]. The mechanisms that govern the ecology

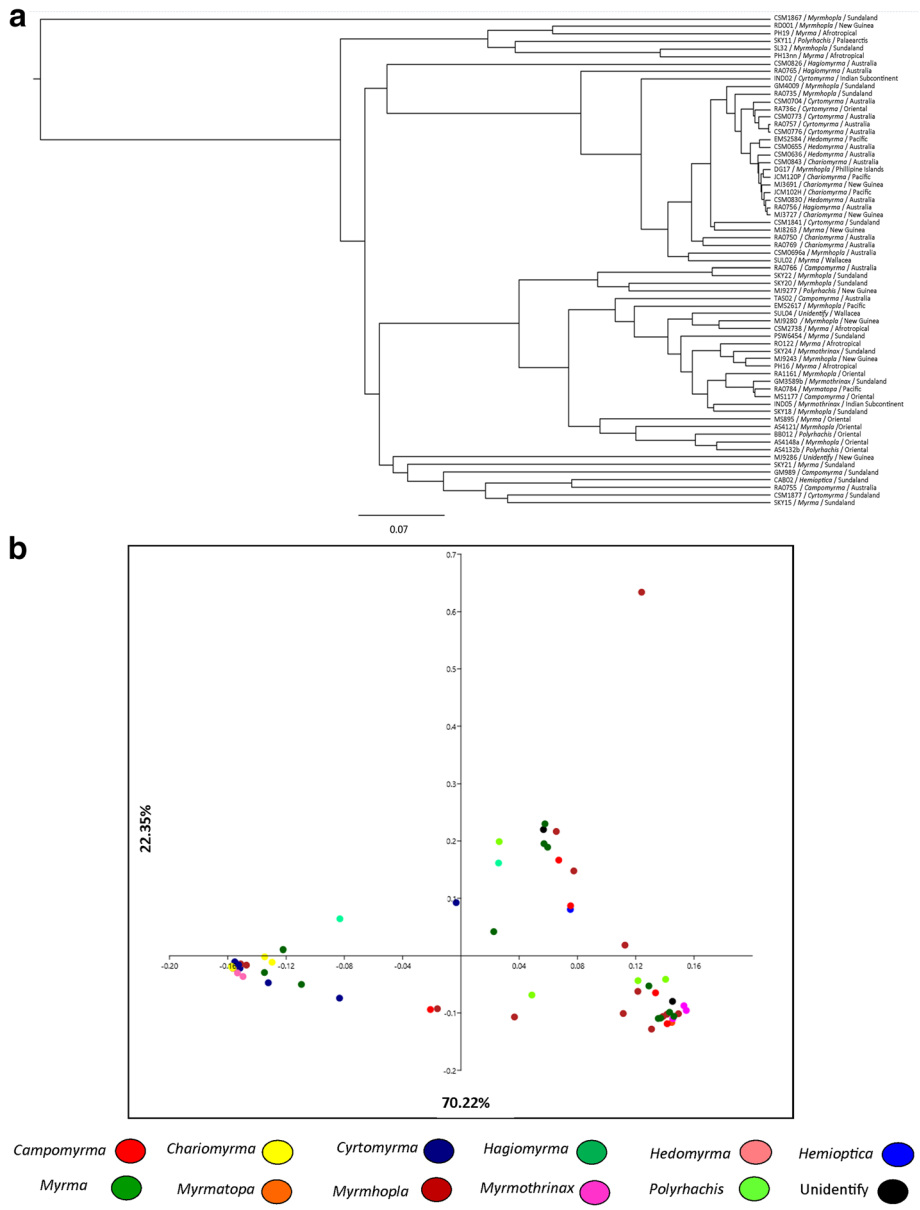

Fig. 3 Beta diversity found in *Polyrhachis* samples rarefied to a read depth of 400 (50% of samples). Note that after this depth only 64 samples remained. **a** UPGMA tree (unweighted UniFrac method) of the entire bacterial community of *Polyrhachis*. Through the tree it is possible to visualize that were grouped samples of several subgenera and distinct localities. **b** PCoA plots (weighted UniFrac method) of bacterial communities associated with *Polyrhachis* at the 97% OTU level. Axis 1 = 70.22% and axis 2 = 22.35%. The dots were colored according to the subgenera they belong to. Note that although not fully clustered, there is a certain ordering of subgenera, indicating that host phylogeny plays an important role in structuring the bacterial community

and evolution of the microbiota inside most hosts are still unknown and detailed studies are limited [27, 45, 51–56]. Besides revealing the bacterial community associated with hosts, studies that attempt to explain changes and what factors influence this bacterial community are still scarce [57]. Many factors may influence the microbiota associated with the host, for example: diet, pH, host phylogeny (coevolution), life stage, and host location [58–62]. Of all these factors, the phylogeny of the host and diet has a strong effect on bacterial communities for many hosts [45]. In a study involving ants, Anderson et al. [63] found similarity of the bacterial communities between species of the same trophic level, and found differences between herbivorous and predatory species. However, geographic location can also be an important mechanism influencing the microbiome [53]. Our results are the first to characterize the bacterial community associated with the diverse spiny

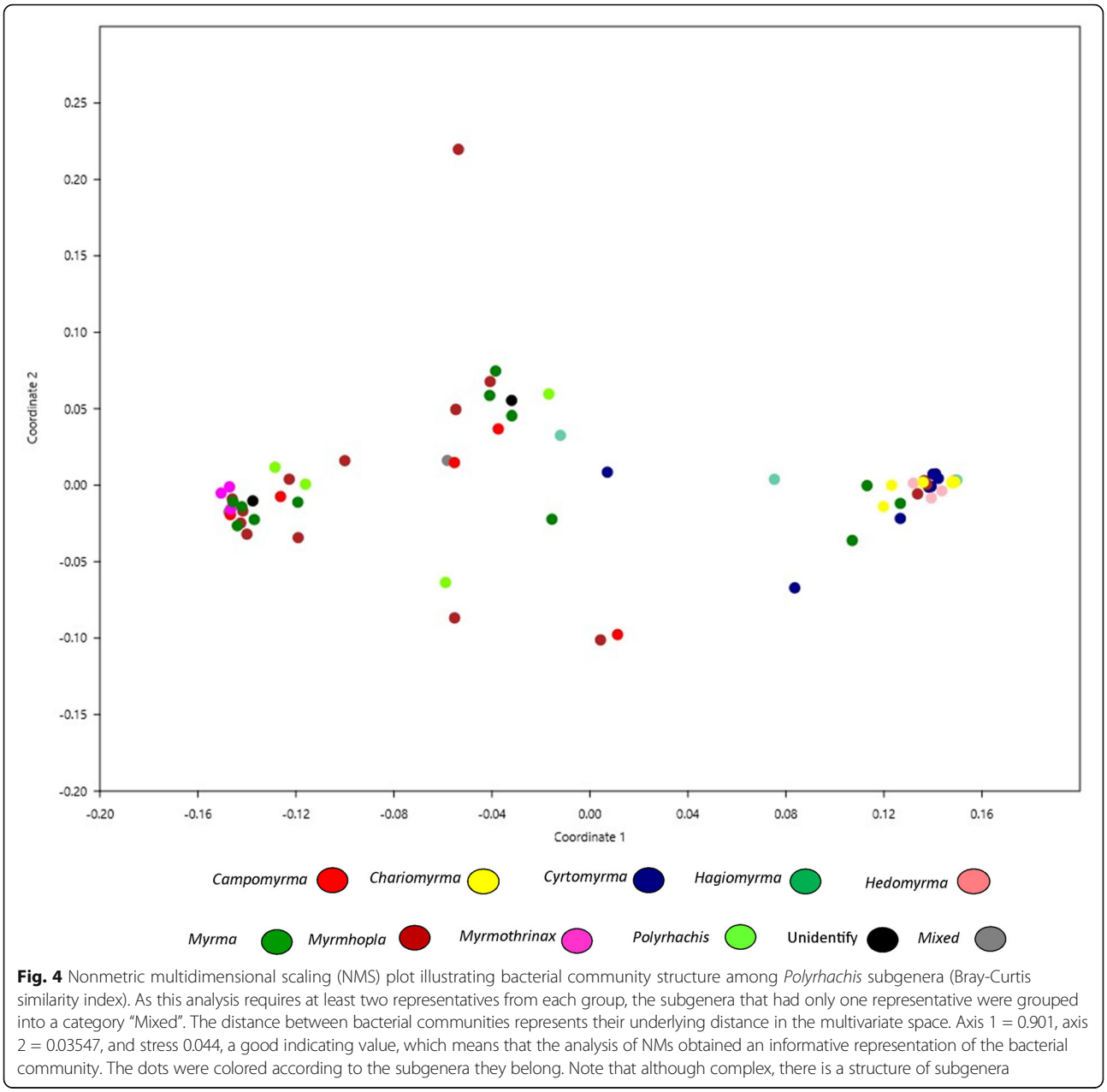

Fig. 4 Nonmetric multidimensional scaling (NMS) plot illustrating bacterial community structure among *Polyrhachis* subgenera (Bray-Curtis similarity index). As this analysis requires at least two representatives from each group, the subgenera that had only one representative were grouped into a category "Mixed". The distance between bacterial communities represents their underlying distance in the multivariate space. Axis 1 = 0.901, axis 2 = 0.03547, and stress 0.044, a good indicating value, which means that the analysis of NMs obtained an informative representation of the bacterial community. The dots were colored according to the subgenera they belong. Note that although complex, there is a structure of subgenera

ant genus *Polyrhachis* from across their distributional range. Additionally, we were able to test whether the host phylogeny or biogeography could be influencing the diversity of bacterial communities found associated with this animal group.

Our results highlight how complex associations of different bacteria associated with *Polyrhachis* can be. This suggests that the evolutionary history of the host can influence the bacterial community in *Polyrhachis*. Ley et al. [45] who analyzed mammalian bacterial communities found correlations between diet and host microbiota, which they related to the gut physiology of the host. Compared to vertebrates, insects have a lower diversity of gut bacterial communities and these can be more variable [64, 65], which makes the understanding of the mechanisms that may influence communities difficult.

In a study analyzing various insects Jones et al. [55] also recovered low bacterial richness, as has been found in other studies [66–68]. One possible explanation is that the host has a mechanisms to prevent the establishment of new bacteria, as a way to defend against pathogens [64]. Although the high infection with *Wolbachia* found in this study could also be an explanation for the low richness found in *Polyrhachis*, since this bacteria can reduce the diversity of bacterial communities [69].

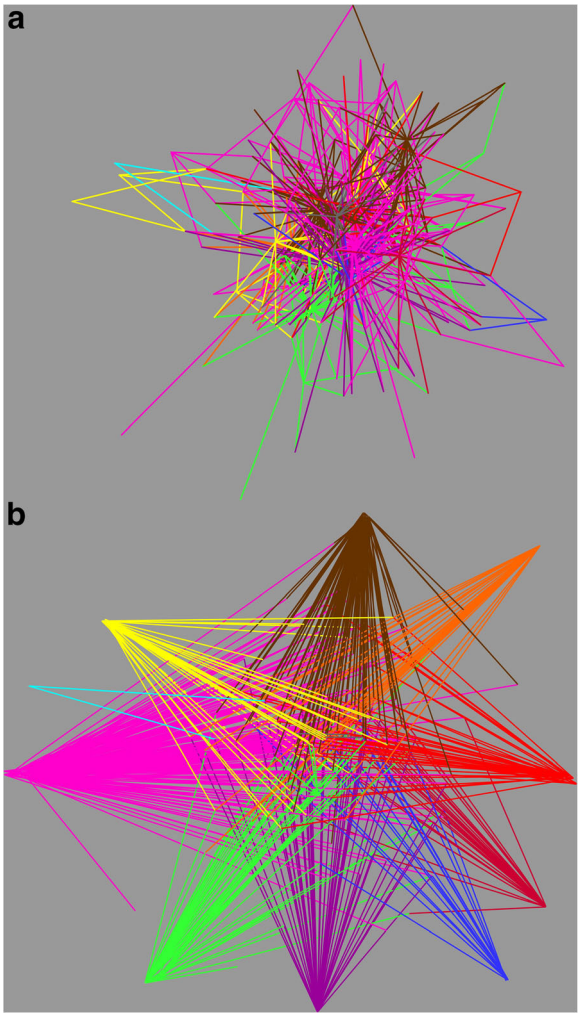

Fig. 5 Network analysis of Polyrhachis with edges representing the main community bacterial members. The edges were colored according to the different shared bacteria: Nocardia (purple), *Lactobacillus* (orange), *Wolbachia* (brown), Enterobacteriaceae (pink), Enterobacteriacea - other (yellow), *Candidatus Blochmannia* (green), *Sodalis* (light blue), Thiotrichaceae (dark blue), and Others (red) **a** Default parameters of Spring-embedded edge-weighted algorithm. The host nodes are not visible for easy viewing, but can still be represented by each vertice. In this analyze the vertices (host) that share more OTUs appear close together. Note how complex the bacterial community network is. **b** Default parameters manually edited. After this edition, it is possible to visualize which bacteria are better represented in this network. As in this case, the bacterium Enterobacteriaceae in pink is highly represented in the bacterial community of *Polyrhachis*

Composition of the bacterial community

The bacteria most commonly found in our study were Enterobacteriacea (multiple strains). It was found present in all sampled individuals (at least one strain) across different subgenera of *Polyrhachis* ants, sampled from across their known geographical range (Fig. 1). *Blochmannia*, a member of the Enterobacteriacea, is known to possess primary interactions in Camponotini ants, which includes *Polyrhachis*. For symbionts of *Polyrhachis* the phylogenetic trees are congruent with those of their hosts across long periods of evolutionary time, indicating the coevolution of host and symbiont in previous studies [70–72] and the current study. In fact in previous studies this endosymbiont was recovered as a monophyletic group associated with Camponotini ants, showing coevolution of host and endosymbiont and suggests the acquisition of this microorganism must have occurred in the common ancestor of this ant tribe [22, 23].

The *Blochmannia* endosymbiont is known to play a nutritional role for the host, providing several essential amino acids [73], especially in early life [24, 25, 74]. *Blochmannia* also maintains certain genes for basic cellular functions, such as biosynthesis of the nine essential amino acids (excluding Arginine), and urease cofactors and enzymes, which allows the symbiont to recycle urea nitrogen provided by the host's excretory system [73, 75, 76]. In addition, the nutritional role of *Blochmannia* is not the only potential interaction with its host, as it has also maintained genes needed to contribute to the metabolism of nitrogen, sulfur and lipids [24–26].

Overall we detected low *Candidatus Blochmannia* abundance, contrary to what we expected based on previous studies from this ant genus [22, 23]. But *Blochmannia* are known to have high mutational rates [77], suggesting that many if not most of the bacteria only identified as "Enterobacteriaceae" or "Enterobacteriaceae - other" may in fact be *Blochmannia*. This high mutation rate and the relatively short fragment of 16S rRNA that can be sequenced using NGS methods is likely responsible for our inability to assign most Enterobacteriaceae to lower taxonomic categories.

When we restricted our analysis to the bacterial genus level, 15.70% of samples included *Candidatus Blochmannia*. When we reduced the hierarchical level to Family, we recovered Enterobacteriaceae in more than 70% of all bacterial communities across geographical localities and host subgenera, with all individuals having at least one OTUs from this family. We also found some strains of Enterobacteriacea associated with specific host subgenera. This is potentially indicative of co-evolution and specificity of the strain to the host. For example we found *Candidatus Blochmannia*-New.ReferenceOTU70 associated with subgenus *Myrma* from the Afrotropics, Enterobacteriaceae-New.ReferenceOTU13 associated with *Polyrhachis*, and Enterobacteriaceae-New.CleanUp.ReferenceOTU0 associated with *Myrmhopla*.

This may suggest *Blochmannia* has undergone rapid change since its mutational rate is known to be high [77], which could prevent the identification of these OTUs as *Blochmannia*. Previous studies from the tribe Camponotini using traditional molecular techniques, i.e. Sanger sequencing of the entire 16S rRNA, showed a

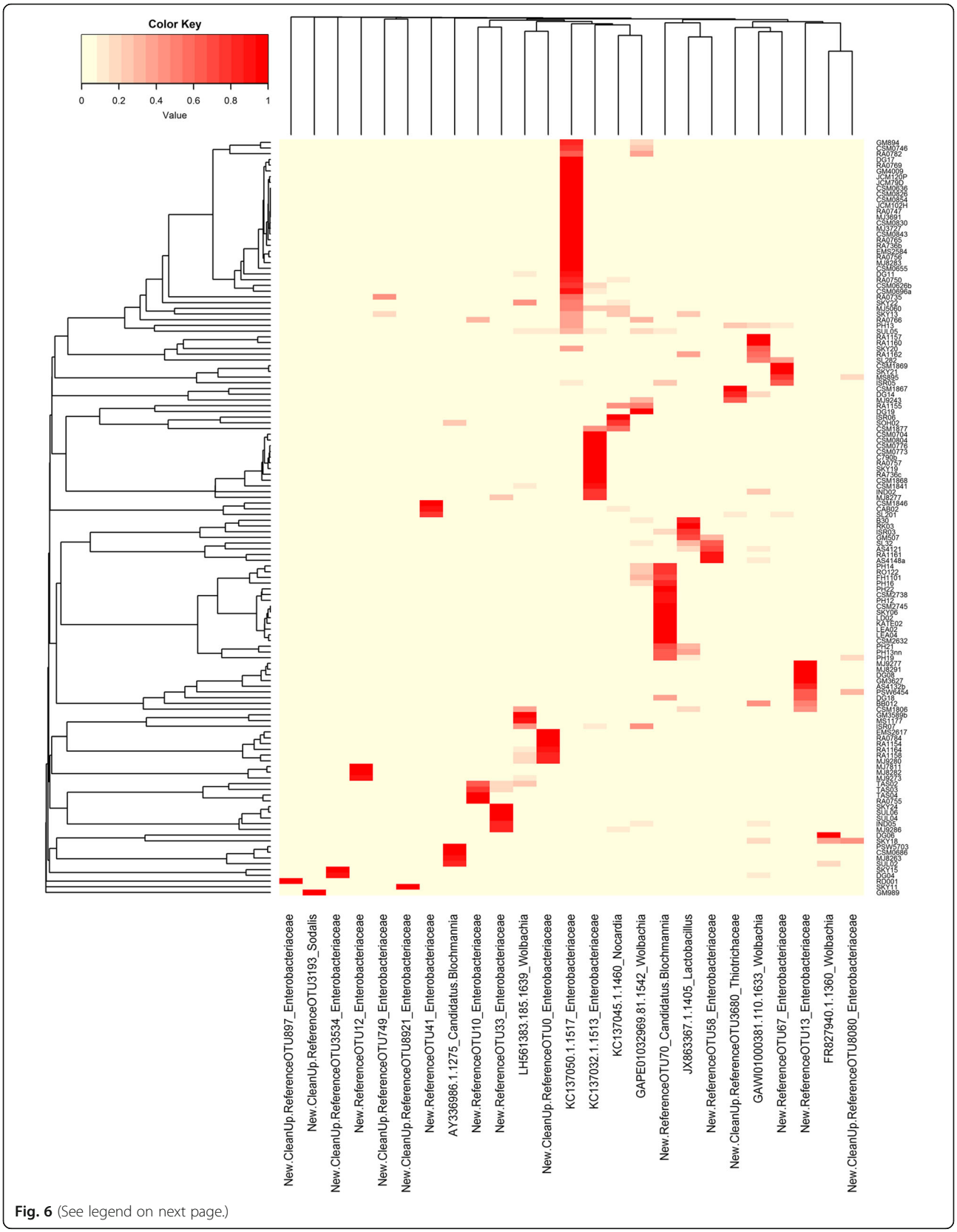

Fig. 6 (See legend on next page.)

(See figure on previous page.)
Fig. 6 The colors in the heatmap indicate variation in the relative abundance of different bacteria in *Polyrhachis*, ranging from 0% (light yellow) to 100% (red). Dendrograms were generated from Bray–Curtis distance matrices. For easy viewing, we choose to show only OTUs with more than 400 reads Note there are strains of Enterobacteriaceae restricted to specific subgenera of *Polyrhachis*, such as *Candidatus Blochmannia*-New.ReferenceOTU70 with *Myrma* from the Afrotropics, Enterobacteriaceae-New.ReferenceOTU13 with *Polyrhachis,* and Enterobacteriaceae-New.CleanUp.ReferenceOTU0 with *Myrmhopla*. In this analysis the presence of multiple *Wolbachia* infections in some Polyrhachis samples is also evident

strong relationship of this bacterium with the host tribe [22, 23]. Even assuming that all Enterobacteriaceae found in this study belong to the bacterial genus *Blochmannia*, our data is still without precedent, since Brown and Wernegreen [78] using NGS in a study involving *Camponotus* found that *Blochmannia* typically constituted 95–98% of reads, and in our study of *Polyrhachis* only 70% (*Blochmannia* and all OTUs of Enterobacteriaceae combine). This lack of sequence conservation suggests that this bacterium may not be preforming these same fundamental roles suggested by previous studies, at least for the genus *Polyrhachis*. More studies are needed to reveal the function of these bacteria in the genus.

Although our results suggest that even without the modification of the Qiagen DNeasy kit for gram-positive bacteria, our DNA extraction method was able to obtain some DNA from gram-positive bacteria, but this could still influence the diversity of bacteria we are able to detect and our method may be omitting some gram positive bacteria. One interesting finding we uncovered is regarding selection of reference options for calling OTUs in Silva 128 [34, 35]. Initially we chose the pick_closed_reference_otus.py command instead of pick_open_reference_otus.py command, but this greatly reduced the number of bacteria sampled in our study. Through this command the aligned sequences are compared to the reference database, and if it does not match with any reference, the sequence was excluded from the analysis. In other words, the use of this command is not able to identify novel diversity, being restricted to already-known taxa [79]. As it is known that *Polyrhachis* have *Blochmannia* [22, 23], and this bacterium has a high mutational rate [77], the pick_open_reference_otus.py command enabled the detection of unknown OTUs (i.e., those that are not represented in the reference database) compared with the closed reference of Silva 128 [34, 35]. The open reference option was able to find 429 additional OTUs (New.Reference and New.CleanUp.Reference). And when we limited our search to only OTUs with over 400 reads of 25 OTUs that met these criteria, 16 were new (Fig. 6). With that in mind, we strongly suggest that in cases where high bacterial mutational rate is known, the use of open reference instead of closed reference to insure detection of bacterial diversity is advised.

Other studies have shown *Wolbachia* as a major player within the bacterial community of invertebrates [19, 27, 28, 55, 78]. For example, in the screening of 24 *Polyrhachis* species, five (20.8%) were infected with *Wolbachia* [27]. Kautz et al. [28] found *Wolbachia* in 25% of *Polyrhachis* analyzed from Australia. In our analysis we found *Wolbachia* in 65 samples of *Polyrhachis* (49.24%), and of these samples 25 showed multiples strain infections (38.46%). All strains have a wide distribution across our samples of *Polyrhachis*. Although *Wolbachia* is known for manipulating the reproduction of the host, its function in ants is still unclear.

The next most common bacteria associated with *Polyrhachis* is *Lactobacillus* found in 31 samples (23.48%). This bacterium was found widely distributed across host subgenera and across host locations. Recent work in the ants has shown the presence of *Lactobacillus*, but its function in this group is not yet fully understood. Kellner et al. [80], also through NGS techniques, found 56% of their samples of *Mycocepurus smithii* (a fungus-farming ant) contained Lactobacillales. *Lactobacillus* have antimicrobial properties and are widely used in the food industry and fermentation of milk products [81]. *Lactobacillus* expresses antimicrobial properties through lactic acid secretion to acidify environmental conditions that some other bacteria and fungi cannot tolerate. Therefore Kellner et al. [80] believe that *Lactobacillus* may serve an important role as defense pathogens in the *M. smithii* system. In another study involving termites, *Lactobacillus* was found in the insect feces where, in addition to this protection function, it can also serve as a substrate or fertilizer [82, 83].

Polyrhachis, along with a few other ant genera, is known for the absence of a metapleural gland [84]. Four possible functions are assigned to this gland: antimicrobials, chemical defense, recognition odor and territorial marking. The first two functions are well accepted and supported by several studies, while the last two require further investigation [85]. This gland is essential for ground nesting ants, since they are more susceptible to infections due to the dark and sometimes damp conditions of their nesting habitat. Although many species of *Camponotus* and *Polyrhachis* nest arboreally, those with terrestrial habits should have evolved alternative antimicrobial defenses [86]. Based on this hypothesis, another study suggested that the behavior of self-cleaning, as well as the use of venom with antimicrobial properties, are the key to disease resistance within the colony of a weaver ant species of *Polyrhachis dives,*

Smith [87]. With this in mind, *Lactobacillus* could be assisting in the defense of the colony potentially replacing the role of the metapleural gland for this genus.

In our findings Entomoplasmatales is present in only 0.80% of the bacterial community found in *Polyrhachis*. This result is different than those previously reported in the literature, as Kautz et al. [28] observed 46% infection rate by *Spiroplasm* (Entomoplasmatales) and Russell et al. [27] found 20% infection by *Spiroplasm* for this genus. Russell et al. [27] also suggested that *Spiroplasm* enrichment could be a feature specific to *Polyrhachis* and their close relatives. This may not be a genus-wide attribute, because four of the six *Polyrhachis* included in their study were from the Australian Wet Tropics and came from species in the subgenus *Chariomyrma* (4/6 species infected). Our findings do not support this as we did not find *Spiroplasm* strongly associated with *Polyrhachis*, even within the subgenus *Chariomyrma*.

The correlation (partial mantel and mantel tests) found in this study indicates that host phylogeny (vertical transfer) could influence the bacterial community to some extent. Our statistical tests also gave similar results to those observed for the mantel tests, suggesting that the phylogeny of the host (subgenera) explains part of the bacterial community, and host location (country or biogeography) none. This result corroborates Meirelles et al. [88] that also did not find any geographic signature in the bacterial community from the fungus-growing ant, *Atta texana* (Buckley). Certainly the specificity found in some strains of Enterobacteriaceae within subgenera of *Polyrhachis* contributed to our findings of correlation between bacterial community and phylogeny of the host (vertical transfer). All these data provide support for the coevolution of *Polyrhachis* and their microbiome, since geography can be seen as an approximation to the sum of environmental effects, such as local weather patterns and availability of food sources, which select for and influence local community assemblages. But we cannot assume that horizontal transfer does not also contribute to the diversity of bacterial communities found. Our findings of what drives the bacterial community of *Polyrhachis* corroborates the findings of Sanders et al. [52] and Ley et al. [45]. The microbiota found in these studies also demonstrated that there is a significant effect of phylogeny of the host. Therefore, although there is a difference (both in abundance and diversity) between bacterial communities of different ants we still understand very little about the mechanisms that influence the microbiome.

Conclusions

These results of varing infection rates of *Polyrhachis* by a diversity of bacteria demonstrate the power of next--generation sequencing to uncover host-associated bacteria. In addition, our data uncovered novel bacteria, showing that with this technique it is possible to explore and discover bacterial diversity never before studied from hosts. We also recovered some species or groups of bacteria associated with only one host subgenus suggesting host-specificity and host-phylogeny could be a determining factor in the distribution of bacterial community in these associations. Furthermore, we did not recover any patterns of bacterial diversity correlated with a specific host geographic region, suggesting these microbes are not just being picked up in the environment. In the general context, we observed the complexity of an entire bacterial community associated with *Polyrhachis* throughout their geographic range. We focused our discussion on the most commonly recovered bacteria because we believe that these bacteria described above have an important role and may be able to influence the evolution and ecology of the host. General knowledge about the host united with information on the host's microbiome are important tools to understand more about the evolutionary complexity of these associations in nature.

Additional files

Additional file 1: Table S1. Specimens of *Polyrhachis* used in this study. (XLSX 18 kb)

Additional file 2: Table S5. Bacterial Quantification through 16S rRNA gene (qPCR) of all Polyrhachis samples. Each sample was analyzed in triplicate therefore follows the values of average and standard deviation for each sample. (XLSX 15 kb)

Additional file 3: Table S2. Percentage of the most common bacteria found in *Polyrhachis* samples. (XLSX 11 kb)

Additional file 4: Table S3. Analysis of G test. G test of independence (P, FDR_P and Bonferroni_P) across *Polyrhachis* samples to determine whether OTU presence/absence is associated with different host categories. (XLSX 18 kb)

Additional file 5: Table S4. Alpha diversity estimation. Chao1, PD whole tree, Observed OTUs, Simpson and Shannon observed in *Polyrhachis* individuals. (XLSX 21 kb)

Abbreviations

EMP: Earth microbiome project; NGS: Next generation sequencing; NMDS: Multidimensional nonmetric scaling; OTU: Operational taxonomic unit; PD: Phylogenetic diversity; RDA: Redundancy analysis; UTM: Universal transverse mercator

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Availability of data and materials
All raw sequence data is available publicly in https://figshare.com/s/290531bea3dee984444e and NCBI SRA accession number SRR5136256 and study SRP095836.

Authors' contributions
MOR and CSM designed the experiments, analyzed the data and wrote the manuscript. MOR performed the experiments. OCB assists in data analysis and discussions. All authors read and approved the final manuscript.

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

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Additional files

Additional File 1: Additional Table 1.xlsx. Specimens of *Polyrhachis* used in this study.

Specimen	Voucher	Country	*Species*	*Subgenus*	Biogeography
AS4121	FMNH-INS2842201	Cambodia	*Polyrhachis sp. near furcata*	*Myrmhopla*	Oriental
AS4132a	FMNH-INS2842202	Cambodia	*Polyrhachis sp.*	*Polyrhachis*	Oriental
AS4132b	FMNH-INS2842202	Cambodia	*Polyrhachis sp.*	*Polyrhachis*	Oriental
AS4148a	FMNH-INS2842204	Cambodia	*Polyrhachis sp. near furcata*	*Myrmhopla*	Oriental
AS4148b	FMNH-INS2842204	Cambodia	*Polyrhachis sp. near furcata*	*Myrmhopla*	Oriental
B30	FMNH-INS2842220	Borneo, Malaysia	*Polyrhachis abdominalis*	*Myrmhopla*	Sundaland
BB012	FMNH-INS2842178	China	*Polyrhachis bihamata*	*Polyrhachis*	oriental
C790b	FMNH-INS2842170	Australia	*Polyrhachis cf. yorkana*	*Cyrtomyrma*	Australia
CAB02	FMNH-INS2842183	Borneo, Malaysia	*Polyrhachis boltoni*	*Hemioptica*	Sundaland
CB01	FMNH-INS2842007	South Korea	*Polyrhachis lamellidens*	*Polyrhachis*	Palaearctis
CSM1813	CSM1813	Borneo, Malaysia	*Polyrhachis sp.*	*Polyrhachis*	Sundaland
RA736a	RA736a	Laos	*Polyrhachis dives*	*Myrmhopla*	Oriental
CSM0626b	CSM0626b	Australia	*Polyrhachis schoopae*	*Chariomyrma*	Australia
CSM0636	CSM0636	Australia	*Polyrhachis argentosa*	*Hedomyrma*	Australia
CSM0655	CSM0655	Australia	*Polyrhachis rufifemur*	*Hedomyrma*	Australia
CSM0686	CSM0686	Australia	*Polyrhachis foreli*	*Myrma*	Australia
CSM0696a	CSM0696a	Australia	*Polyrhachis mucronata*	*Myrmhopla*	Australia
CSM0704	CSM0704	Australia	*Polyrhachis robsoni*	*Cyrtomyrma*	Australia
CSM0746	CSM0746	Australia	*Polyrhachis thais*	*Hedomyrma*	Australia
CSM0773	CSM0773	Australia	*Polyrhachis brevinoda*	*Cyrtomyrma*	Australia
CSM0776	CSM0776	Australia	*Polyrhachis abbreviata*	*Cyrtomyrma*	Australia
CSM0797	CSM0797	Australia	*Polyrhachis ornata*	*Hedomyrma*	Australia
CSM0804	CSM0804	Australia	*Polyrhachis mackayi*	*Cyrtomyrma*	Australia
CSM0826	CSM0826	Australia	*Polyrhachis thusnelda*	*Hagiomyrma*	Australia
CSM0830	CSM0830	Australia	*Polyrhachis cupreata*	*Hedomyrma*	Australia
CSM0843	CSM0843	Australia	*Polyrhachis senilis*	*Chariomyrma*	Australia
CSM0854	CSM0854	Australia	*Polyrhachis sokolova*	*Hagiomyrma*	Australia
CSM1806	CSM1806	Borneo, Malaysia	*Polyrhachis bihamata*	*Polyrhachis*	Sundaland
CSM1841	CSM1841	Borneo, Malaysia	*Polyrhachis danum*	*Cyrtomyrma*	Sundaland
CSM1846	CSM1846	Borneo, Malaysia	*Polyrhachis boltoni*	*Hemioptica*	Sundaland
CSM1854	CSM1854	Borneo, Malaysia	*Polyrhachis cephalotes*	*Myrmhopla*	Sundaland
CSM1866	CSM1866	Borneo, Malaysia	*Polyrhachis inermis*	*Myrma*	Sundaland
CSM1867	CSM1867	Borneo, Malaysia	*Polyrhachis armata*	*Myrmhopla*	Sundaland
CSM1868	CSM1868	Borneo, Malaysia	*Polyrhachis lepida*	*Cyrtomyrma*	Sundaland
CSM1869	CSM1869	Borneo, Malaysia	*Polyrhachis nigropilosa*	*Myrma*	Sundaland
CSM1877	CSM1877	Borneo, Malaysia	*Polyrhachis lepida*	*Cyrtomyrma*	Sundaland
CSM2632	CSM2632	Uganda	*Polyrhachis sp.*	*Myrma*	Afrotropical
CSM2738	CSM2738	Uganda	*Polyrhachis sp.*	*Myrma*	Afrotropical
CSM2745	CSM2745	Uganda	*Polyrhachis sp.*	*Myrma*	Afrotropical
DG02	FMNH-INS2842099	Phillipines	*Polyrhachis armata*	*Myrmhopla*	Phillipine Islands
DG03	FMNH-INS2842100	Phillipines	*Polyrhachis illaudata*	*Myrma*	Phillipine Islands
DG04	FMNH-INS2842101	Phillipines	*Polyrhachis carbonaria*	*Aulacomyrma*	Phillipine Islands
DG06	FMNH-INS2842103	Phillipines	*(Polyrhachis (Myrmatopa) sp.*	*Myrmatopa*	Phillipine Islands

DG08	FMNH-INS2842105	Phillipines	*Polyrhachis bihamata*	*Polyrhachis*	Phillipine Islands
DG09	FMNH-INS2842106	Phillipines	*Polyrhachis noesaensis*	*Myrma*	Phillipine Islands
DG11	FMNH-INS2842108	Phillipines	*Polyrhachis saevissima*	*Myrmhopla*	Phillipine Islands
DG14	FMNH-INS2842110	Phillipines	*Polyrhachis armata*	*Myrmhopla*	Phillipine Islands
DG17	FMNH-INS2842115	Phillipines	*Polyrhachis saevissima*	*Myrmhopla*	Phillipine Islands
DG18	FMNH-INS2842116	Phillipines	*Polyrhachis bihamata*	*Polyrhachis*	Phillipine Islands
DG19	FMNH-INS2842117	Phillipines	*Polyrhachis (Myrmothrinax)*	*Myrmothrinax*	Phillipine Islands
EMS2584	FMNH-INS2842021	Solomon Islands	*Polyrhachis campbelli*	*Hedomyrma*	Pacific
EMS2617	FMNH-INS2842022	Solomon Islands	*Polyrhachis cf. bismarckensis*	*Myrmhopla*	Pacific
FH1101	FMNH-INS2842191	Uganda	*Polyrhachis sp.*	*Myrma*	Afrotropical
GM3589b	ABNC00467	Borneo, Malaysia	*Polyrhachis (Myrmothrinax) sp.*	*Myrmothrinax*	Sundaland
GM3627	FMNH-INS2842167	Borneo, Malaysia	*Polyrhachis bihamata*	*Polyrhachis*	Sundaland
GM4009	FMNH-INS2842209	Borneo, Malaysia	*Polyrachis (Myrmhopla) sp. 3*	*Myrmhopla*	Sundaland
GM507	FMNH-INS2842211	Borneo, Malaysia	*Polyrhachis furcata*	*Myrmhopla*	Sundaland
GM894	FMNH-INS2842208	Borneo, Malaysia	*Polyrhachis (Myrmhopla) sp. 2*	*Myrmhopla*	Sundaland
GM989	ABNC01058	Borneo, Malaysia	*Polyrhachis equina*	*Campomyrma*	Sundaland
IND02	FMNH-INS2841999	India	*Polyrhachis rastellata*	*Cyrtomyrma*	Indian Subcontinent
IND05	FMNH-INS2842002	India	*Polyrhachis thrinax*	*Myrmothrinax*	Indian Subcontinent
ISR03	FMNH-INS2842137	Israel	*Polyrhachis lacteipennis*	*Myrmhopla*	Palaearctis
ISR05	FMNH-INS2842139	Vietnam	*Polyrhachis (Myrma) sp.*	*Myrma*	Oriental
ISR06	FMNH-INS2842140	Thailand	*Polyrhachis (Myrma) sp.*	*Myrma*	Oriental
ISR07	FMNH-INS2842141	Vietnam	*Polyrhachis (Myrmhopla) sp.*	*Myrmhopla*	Oriental
JCM102H	FMNH-INS52507	Palau	*Polyrhachis sp.*	*Chariomyrma*	Pacific
JCM120P	FMNH-INS52602	Palau	*Polyrhachis sp.*	*Chariomyrma*	Pacific
JCM79D	FMNH-INS52422	Palau	*Polyrhachis sp.*	*Chariomyrma*	Pacific
K34	FMNH-INS2842219	Borneo, Malaysia	*Polyrhachis armata*	*Myrmhopla*	Sundaland
KATE02	FMNH-INS2842181	South Africa	*Polyrhachis schistacea*	*Myrma*	Afrotropical
LD02	FMNH-INS2842154	Ghana	*Polyrhachis sp.*	*Myrma*	Afrotropical
LEA01	FMNH-INS2842071	Mozambique	*Polyrachis schistaceae*	*Myrma*	Afrotropical
LEA02	FMNH-INS2842072	Mozambique	*Polyrachis schistaceae*	*Myrma*	Afrotropical
LEA04	FMNH-INS2842068	Mozambique	*Polyrachis schistaceae*	*Myrma*	Afrotropical
LEA05	FMNH-INS2842067	Mozambique	*Polyrachis schistaceae*	*Myrma*	Afrotropical
MJ3691	FMNH-INS2842074	Papua New Guinea	*Polyrhachis sp.*	*Chariomyrma*	New Guinea
MJ3727	FMNH-INS2842075	Papua New Guinea	*Polyrhachis sp.*	*Chariomyrma*	New Guinea
MJ5060	FMNH-INS2842078	Papua New Guinea	*Polyrhachis sp.*	*Unidentify*	New Guinea
MJ7811	FMNH-INS2842095	Papua New Guinea	*Polyrhachis sexspinosa group*	*Myrmhopla*	New Guinea
MJ8263	FMNH-INS2842082	Papua New Guinea	*Polyrhachis sp.*	*Myrma*	New Guinea
MJ8277	FMNH-	Papua New Guinea	*Polyrhachis sp.*	*Unidentify*	New Guinea

	INS2842083				
MJ8282	FMNH-INS2842086	Papua New Guinea	*Polyrhachis sexspinosa group*	*Myrmhopla*	New Guinea
MJ8283	FMNH-INS2842087	Papua New Guinea	*Polyrhachis sp.*	*Chariomyrma*	New Guinea
MJ8291	FMNH-INS2842088	Papua New Guinea	*Polyrhachis sp.*	*Polyrhachis*	New Guinea
MJ9243	FMNH-INS2842199	Papua New Guinea	*Polyrhachis sp. near bicolor*	*Myrmhopla*	New Guinea
MJ9273	FMNH-INS2842089	Papua New Guinea	*Polyrhachis sexspinosa group*	*Myrmhopla*	New Guinea
MJ9277	FMNH-INS2842091	Papua New Guinea	*Polyrhachis sp.*	*Polyrhachis*	New Guinea
MJ9280	FMNH-INS2842092	Papua New Guinea	*Polyrhachis mucronata-group sp.*	*Myrmhopla*	New Guinea
MJ9286	FMNH-INS2842093	Papua New Guinea	*Polyrhachis sp.*	*Unidentify*	New Guinea
MJ9287	FMNH-INS2842094	Papua New Guinea	*Polyrhachis sp.*	*Unidentify*	New Guinea
MS1177	FMNH-INS2842018	China	*Polyrhachis shixingensis*	*Campomyrma*	Oriental
MS895	FMNH-INS2842222	China	*Polyrhachis illaudata*	*Myrma*	Oriental
PH12	FMNH-INS2842057	Gabon	*Polyrhachis revoili*	*Myrma*	Afrotropical
PH13	FMNH-INS2842058	Zimbabwe	*Polyrhachis schistacea*	*Myrma*	Afrotropical
PH13nn	FMNH-INS2842059	South Africa	*Polyrhachis schistocea*	*Myrma*	Afrotropical
PH14	FMNH-INS2842060	South Africa	*Polyrhachis gagates*	*Myrma*	Afrotropical
PH16	FMNH-INS2842062	Republic of the Congo	*Polyrhachis latharis*	*Myrma*	Afrotropical
PH19	FMNH-INS2842063	Republic of the Congo	*Polyrhachis decemdentata*	*Myrma*	Afrotropical
PH20	FMNH-INS2842064	South Africa	*Polyrhachis viscosa*	*Myrma*	Afrotropical
PH21	FMNH-INS2842215	Mozambique	*Polyrhachis schistacea*	*Myrma*	Afrotropical
PH22	FMNH-INS2842065	Tanzania	*Polyrhachis schistacea*	*Myrma*	Afrotropical
PSW5703	FMNH-INS2842051	Australia	*Polyrhachis andromache*	*Myrma*	Australia
PSW6454	FMNH-INS2842054	Borneo, Malaysia	*Polyrhachis obesior*	*Myrma*	Sundaland
RA0735	RA0735	Singapore	*Polyrhachis abdominalis*	*Myrmhopla*	Sundaland
RA0747	RA0747	Australia	*Polyrhachis hookeri*	*Chariomyrma*	Australia
RA0750	RA0750	Australia	*Polyrhachis aurea*	*Chariomyrma*	Australia
RA0752	RA0752	Australia	*Polyrhachis monteithi*	*Cyrtomyrma*	Australia
RA0755	RA0755	Australia	*Polyrhachis "BATH3"*	*Campomyrma*	Australia
RA0756	RA0756	Australia	*Polyrhachis trapezoidea*	*Hagiomyrma*	Australia
RA0757	RA0757	Australia	*Polyrhachis australis*	*Cyrtomyrma*	Australia
RA0765	RA0765	Australia	*Polyrhachis ammon*	*Hagiomyrma*	Australia
RA0766	RA0766	Australia	*Polyrhachis flavibasis*	*Campomyrma*	Australia
RA0769	RA0769	Australia	*Polyrhachis "charlo5"*	*Chariomyrma*	Australia
RA0782	RA0782	Myanmar	*Polyrhachis illaudata*	*Myrma*	Oriental
RA0783	RA0783	Solomon Islands	*Polyrhachis sp.*	*Hedomyrma*	Pacific
RA0784	RA0784	Solomon Islands	*Polyrhachis sp.*	*Myrmatopa*	Pacific
RA1154	RA1154	Laos	*Polyrhachis mucronata-group sp.*	*Myrmhopla*	Oriental
RA1155	RA1155	Laos	*Polyrhachis sp.*	*Campomyrma*	Oriental
RA1157	RA1157	Laos	*Polyrhachis illaudata*	*Myrma*	Oriental
RA1158	RA1158	Laos	*Polyrhachis mucronata-group sp.*	*Myrmhopla*	Oriental
RA1160	RA1160	Laos	*Polyrhachis illaudata*	*Myrma*	Oriental
RA1161	RA1161	Laos	*Polyrhachis sp. near furcata*	*Myrmhopla*	Oriental
RA1162	RA1162	Laos	*Polyrhachis illaudata*	*Myrma*	Oriental
RA1163	RA1163	Laos	*Polyrhachis illaudata*	*Myrma*	Oriental
RA1164	RA1164	Laos	*Polyrhachis mucronata-group sp.*	*Myrmhopla*	Oriental

RA736b	RA736b	Thailand	*Polyrhachis sp.*	*Chariomyrma*	Oriental
RA736c	RA736c	Thailand	*Polyrhachis cf. laevissima*	*Cyrtomyrma*	Oriental
RD001	FMNH-INS2842216	Papua New Guinea	*Polyrhachis sp.*	*Myrmhopla*	New Guinea
RK03	FMNH-INS2842173	Australia	*Polyrhachis loweryi*	*Hirtomyrma*	Australia
RO122	FMNH-INS2842193	Tansania	*Polyrhachis sp.*	*Myrma*	Afrotropical
SKY06	FMNH-INS2842030	Laos	*Polyrhachis sp.*	*Myrma*	Oriental
SKY08	FMNH-INS2842032	Singapore	*Polyrhachis sp.*	*Myrmatopa*	Sundaland
SKY11	FMNH-INS2842035	Japan	*Polyrhachis lamellidens*	*Polyrhachis*	Palaearctis
SKY13	FMNH-INS2842037	Singapore	*Polyrhachis abdominalis*	*Myrmhopla*	Sundaland
SKY15	FMNH-INS2842039	Singapore	*Polyrhachis sp.*	*Myrma*	Sundaland
SKY18	FMNH-INS2842042	Singapore	*Polyrhachis proxima*	*Myrmhopla*	Sundaland
SKY19	FMNH-INS2842043	Singapore	*Polyrhachis sp.*	*Cyrtomyrma*	Sundaland
SKY20	FMNH-INS2842044	Singapore	*Polyrhachis sp.*	*Myrmhopla*	Sundaland
SKY21	FMNH-INS2842045	Singapore	*Polyrhachis nigropilosa*	*Myrma*	Sundaland
SKY22	FMNH-INS2842046	Singapore	*Polyrhachis bicolor group*	*Myrmhopla*	Sundaland
SKY24	FMNH-INS2842048	Singapore	*Polyrhachis sp.*	*Myrmothrinax*	Sundaland
SL32	FMNH-INS2842217	Borneo, Malaysia	*Polyrhachis furcata*	*Myrmhopla*	Sundaland
SL201	FMNH-INS2842163	Peninsula Malaysia	*Polyrhachis boltoni*	*Hemioptica*	Sundaland
SL202	FMNH-INS2842164	Peninsula Malaysia	*Polyrhachis armata*	*Myrmhopla*	Sundaland
SL282	FMNH-INS2842165	Peninsula Malaysia	*Polyrhachis illaudata*	*Myrma*	Sundaland
SOH02	FMNH-INS2842133	Singapore	*Polyrhachis beccari*	*Myrma*	Sundaland
SUL02	CAG1a	Indonesia	*Polyrhachis (Myrma) sp. 1*	*Myrma*	Wallacea
SUL04	CAW_0003	Indonesia	*Polyrhachis sp. 8*	*Unidentify*	Wallacea
SUL05	CAW_0002	Indonesia	*Polyrhachis sp. 7*	*Unidentify*	Wallacea
SUL06	CAW_0004	Indonesia	*Polyrhachis sp. 9*	*Unidentify*	Wallacea
TAS01	FMNH-INS2842005	Australia	*Polyrhachis hexacantha*	*Campomyrma*	Australia
TAS02	FMNH-INS2842006	Australia	*Polyrhachis hexacantha*	*Campomyrma*	Australia
TAS03	FMNH-INS2842206	Australia	*Polyrhachis phryne*	*Campomyrma*	Australia
TAS04	FMNH-INS2842207	Australia	*Polyrhachis semipolita*	*Campomyrma*	Australia

Additional File 2: Additional Table 2.xlsx. Percentage of the most common bacteria found in *Polyrhachis* samples.

Bacteria	**Total**
Enterobacteriaceae	44.4%
Candidatus Blochmannia	15.7%
Enterobacteriaceae;Other	11.9%
Wolbachia	8.8%
Lactobacillus	2.9%
Thiotrichaceae	2,00%
Acinetobacter	1.6%
Nocardia	1.2%
Sodalis	0.8%
Entomoplasmatales	0.8%
Others	9.9%

Additional File 3: Additional Table 3.xlsx. Analysis of G test. G test of independence (P, FDR_P and Bonferroni_P) across *Polyrhachis* samples to determine whether OTU presence/absence is associated with different host categories.

Subgenera				
OTU	**Test-Statistic**	**P**	**FDR_P**	**Bonferroni_P**
Enterobacteriaceae_KC137032.1.1513	1534.69257175	0.0	0.0	0.0
*Wolbachia*_GAWI01000381.110.1633	188.303368265	0.0	0.0	0.0
*Wolbachia*_LH561383.185.1639	365.081936629	0.0	0.0	0.0
Enterobacteriaceae_KC137050.1.1517	2336.399022	0.0	0.0	0.0
Candidatus *Blochmannia*_AY336986.1.1275	380.065489064	0.0	0.0	0.0
Nocardia_KC137045.1.1460	147.103973829	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU13	1029.32056729	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU10	616.482995925	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU33	2070.49243815	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU70	483.272646665	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU58	382.465846419	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU41	1323.35359568	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU32	357.263449283	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU67	225.645022738	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU0	1739.4795952	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU897	117.772506256	0.0	0.0	0.0
*Sodalis*_New.CleanUp.ReferenceOTU3193	383.12801756	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU3534	150.186434234	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU35	539.4315126	0.0	0.0	0.0

48	86			
Thiotrichaceae_New.CleanUp.ReferenceOTU3680	175.832727429	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8080	105.985820992	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8921	452.91167329	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU49	86.7282349349	7.27196081129e-14	1.71681509588e-12	3.94867472053e-11
*Wolbachia*_GAPE01032969.81.1542	80.9419962491	9.69890834313e-13	2.19437801263e-11	5.26650723032e-10
Lactobacillus_JX863367.1.1405	76.2702395272	7.72937269744e-12	1.67881974988e-10	4.19704937471e-09
*Wolbachia*_ GAUE02014372.1.1238	68.2022592707	2.67953881306e-10	5.59611375188e-09	1.45498957549e-07
Enterobacteriaceae_New.CleanUp.ReferenceOTU749	47.4203723746	1.81069984373e-06	3.5114643398e-05	0.000983210015145
Haemophilus_HM267665.1.1361	47.284808019	1.91403311012e-06	3.58386199584e-05	0.00103931997879
Species				
OTU	**Test-Statistic**	**P**	**FDR_P**	**Bonferroni_P**
Enterococcus_HG798451.1.1400	185.972356063	0.0	0.0	0.0
Enterobacteriaceae_KC137032.1.1513	9707.01191165	0.0	0.0	0.0
*Wolbachia*_FR827940.1.1360	1236.16447085	0.0	0.0	0.0
*Wolbachia*_GAWI01000381.110.1633	1548.65779505	0.0	0.0	0.0
Serratia_FM179752.1.1686	182.416021659	0.0	0.0	0.0
*Wolbachia*_LH561383.185.1639	2980.66621894	0.0	0.0	0.0
Enterobacteriaceae_KC137050.1.1517	10274.7683019	0.0	0.0	0.0
Prevotella_GQ398426.1.1515	183.208025487	0.0	0.0	0.0
Candidatus *Blochmannia*_AY336986.1.1275	3753.4986914	0.0	0.0	0.0
Candidatus Rhabdochlamydia_AY223862.1.1366	218.058656017	0.0	0.0	0.0
Nocardia_KC137045.1.1460	592.313282921	0.0	0.0	0.0
*Wolbachia*_GAPE01032969.81.1542	1304.11533936	0.0	0.0	0.0
Acinetobacter_JN904060.1.1395	332.213663733	0.0	0.0	0.0
Enterobacteriaceae_FJ913065.1.1309	599.846928588	0.0	0.0	0.0
Entomoplasmatales_GU293217.1.1487	243.08501901	0.0	0.0	0.0
Haemophilus_HM267665.1.1361	212.548547935	0.0	0.0	0.0
Lactobacillus_JX863367.1.1405	1482.6984328	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU13	2461.00271209	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU10	3620.58928248	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU33	4534.47207849	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU70	3560.4693998	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU58	3514.13285574	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU48	332.871775734	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU41	2020.14563835	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU32	1668.63931686	0.0	0.0	0.0

Enterobacteriaceae_New.ReferenceOTU67	3612.74492075	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU66	825.856472528	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU49	695.28108579	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU0	4351.88484667	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU187	793.036785737	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU749	1280.70468026	0.0	0.0	0.0
*Sodalis*_New.CleanUp.ReferenceOTU3193	3018.79484848	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU3548	2507.61917472	0.0	0.0	0.0
Thiotrichaceae_New.CleanUp.ReferenceOTU3680	3697.65766106	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU4597	638.80223185	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU4609	669.91155766	0.0	0.0	0.0
Thiotrichaceae_New.CleanUp.ReferenceOTU5225	494.525130588	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU6148	280.362690826	0.0	0.0	0.0
Entomoplasmatales_New.CleanUp.ReferenceOTU7765	371.771254793	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU7912	720.376463652	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8080	2004.47116429	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8921	2839.8517461	0.0	0.0	0.0
EntomoplasmatalesNew.CleanUp.ReferenceOTU9551	205.073821192	0.0	0.0	0.0
Acinetobacter_HM248444.1.1359	177.395658894	1.11022302463e-16	1.37011614175e-15	6.02851102371e-14
Enterobacteriaceae_New.CleanUp.ReferenceOTU2541	160.592660226	4.64073224293e-14	5.59981690647e-13	2.51991760791e-11
Lactobacillales_FN185731.1.1513	160.368615615	5.02931030155e-14	5.93677281248e-13	2.73091549374e-11
Enterobacteriaceae_New.CleanUp.ReferenceOTU897	153.091453812	6.70241639966e-13	7.74343001067e-12	3.63941210502e-10
Enterobacteriaceae_New.CleanUp.ReferenceOTU3534	151.79011308	1.0593748101e-12	1.19841775392e-11	5.75240521883e-10
Streptophyta_GACN01029607.149.2074	125.296757618	7.96894084054e-09	8.83088750289e-08	4.32713487641e-06
Gluconobacter_New.ReferenceOTU11	125.121296087	8.43009650975e-09	9.15508480959e-08	4.57754240479e-06
Mycoplasma_New.CleanUp.ReferenceOTU4766	117.154233083	1.03486528236e-07	1.10182715357e-06	5.6193184832e-05
Stramenopiles_LN563239.1.1318	109.88669077	9.34718905388e-07	9.76062241587e-06	0.000507552365625
Biogeography				
OTU	**Test-Statistic**	**P**	**FDR_P**	**Bonferroni_P**
Enterococcus_HG798451.1.1400	133.639598232	0.0	0.0	0.0
Enterobacteriaceae_KC137032.1.1513	721.038993359	0.0	0.0	0.0
*Wolbachia*_FR827940.1.1360	126.732538491	0.0	0.0	0.0
*Wolbachia*_GAWI01000381.110.1633	615.767525002	0.0	0.0	0.0
*Wolbachia*_LH561383.185.1639	157.689308267	0.0	0.0	0.0
Enterobacteriaceae_KC137050.1.1517	1700.85969375	0.0	0.0	0.0
Candidatus *Blochmannia*_AY336986.1.1275	713.520714606	0.0	0.0	0.0
Lactobacillales_FN185731.1.1513	119.6679759	0.0	0.0	0.0

	07			
Lactobacillus_JX863367.1.1405	135.181597307	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU13	605.290336067	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU10	165.14755451	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU33	1039.20584882	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU70	1133.79115531	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU58	613.676887015	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU67	173.699112521	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU0	691.073909482	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU897	222.194093526	0.0	0.0	0.0
*Sodalis*_New.CleanUp.ReferenceOTU3193	109.821362471	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU3534	107.139133983	0.0	0.0	0.0
Thiotrichaceae_New.CleanUp.ReferenceOTU3680	149.110615158	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU4609	110.446977556	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8921	1779.28944752	0.0	0.0	0.0
*Wolbachia*_GAPE01032969.81.1542	97.9849441753	1.11022302463e-16	2.51187959321e-15	6.02851102371e-14
Enterobacteriaceae_New.CleanUp.ReferenceOTU8080	98.0388998329	1.11022302463e-16	2.51187959321e-15	6.02851102371e-14
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU7912	88.1681645843	1.23234755733e-14	2.67665889453e-13	6.69164723632e-12
Enterobacteriaceae_New.CleanUp.ReferenceOTU3548	84.9584084053	5.31796828795e-14	1.11063722322e-12	2.88765678036e-11
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU1864	77.6918068387	1.42053036001e-12	2.85684439068e-11	7.71347985484e-10
Enterobacteriaceae_New.ReferenceOTU41	70.8424448322	3.04788416727e-11	5.91071822439e-10	1.65500110283e-08
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU187	67.5302117855	1.3261702847e-10	2.48313953307e-09	7.20110464592e-08
Leuconostoc_GQ267986.1.1426	58.7484341836	6.24729068477e-09	1.13075961394e-07	3.39227884183e-06
Enterobacteriaceae_New.ReferenceOTU49	58.0741225785	8.37131597375e-09	1.46633050766e-07	4.54562457375e-06
Candidatus *Blochmannia*_New.ReferenceOTU32	57.7304217507	9.71617708512e-09	1.64871379913e-07	5.27588415722e-06
Enterobacteriaceae_New.CleanUp.ReferenceOTU4597	45.4591388351	1.79677677892e-06	2.95651451804e-05	0.000975649790952
Serratia_FM179752.1.1686	45.2209012775	1.98396896445e-06	3.16851514028e-05	0.001077295147 69
Nocardia_KC137045.1.1460	42.8063969728	5.38541784101e-06	8.35509110762e-05	0.002924281887 67
Enterobacteriaceae_New.CleanUp.ReferenceOTU749	42.5883797941	5.89037526488e-06	8.88464935787e-05	0.003198473768 83
Country				
OTU	**Test-Statistic**	**P**	**FDR_P**	**Bonferroni_P**
Enterobacteriaceae_KC137032.1.1513	2199.23814332	0.0	0.0	0.0
*Wolbachia*_FR827940.1.1360	187.144632541	0.0	0.0	0.0
*Wolbachia*_GAWI01000381.110.1633	481.769790905	0.0	0.0	0.0
*Wolbachia*_LH561383.185.1639	417.7391361	0.0	0.0	0.0
Enterobacteriaceae_KC137050.1.1517	2758.24771843	0.0	0.0	0.0
Candidatus *Blochmannia*_AY336986.1.1275	897.709576607	0.0	0.0	0.0

*Wolbachia*_GAPE01032969.81.1542	347.762375123	0.0	0.0	0.0
Lactobacillus_JX863367.1.1405	310.770538107	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU13	737.913643221	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU10	191.690873669	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU33	1429.77802552	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU70	2645.7778925	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU58	2309.08204145	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU41	150.601416753	0.0	0.0	0.0
Candidatus *Blochmannia*_New.ReferenceOTU32	123.939799041	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU67	515.036157545	0.0	0.0	0.0
Enterobacteriaceae_New.ReferenceOTU49	167.658517951	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU0	1368.5586135	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU187	193.933453401	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU749	122.913514455	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU897	255.235685736	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU1864	493.527485739	0.0	0.0	0.0
*Sodalis*_New.CleanUp.ReferenceOTU3193	233.408554642	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU3534	297.14812978	0.0	0.0	0.0
Thiotrichaceae_New.CleanUp.ReferenceOTU3680	272.702210184	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU4609	138.783367903	0.0	0.0	0.0
Candidatus *Blochmannia*_New.CleanUp.ReferenceOTU7912	171.073629474	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8080	203.33645532	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU8921	2096.71350716	0.0	0.0	0.0
Enterobacteriaceae_New.CleanUp.ReferenceOTU3548	95.926311668	1.99840144433e-13	3.61710661423e--12	1.08513198427e-10
Enterobacteriaceae_New.ReferenceOTU66	76.078505284 9	8.40140623737e-10	1.47160115706e--08	4.56196358689e-07
Enterobacteriaceae_New.CleanUp.ReferenceOTU6148	67.161685553 3	3.11765705474e-08	5.29027431476e--07	1.69288778072e-05
Leuconostoc_GQ267986.1.1426	65.330566521 8	6.46192847009e-08	1.06328095735e--06	3.50882715926e-05
Nocardia_KC137045.1.1460	57.562541988 6	1.33997934049e-06	2.14002582907e--05	0.000727608781884

Additional File 4: Additional Table 4.xlsx. Alpha diversity estimation. Chao1, PD whole tree, Observed OTUs, Simpson and Shannon observed in *Polyrhachis* individuals.

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Sample	**Reads**	**Shannon**	**PD_whole_tree**	**Chao1**	**Observed_otus**	**Simpson**
AS4121	1065.0	2.1037554058	3.0679	63.5	38.0	0.587650598426
AS4132b	514.0	0.915715422044	2.19579	22.5	12.0	0.264152371724
AS4148a	605.0	1.03359805968	1.98931	28.3333333333	19.0	0.274565944949
B30	153.0	0.651681473342	1.36337	5.5	5.0	0.212824127472

BB012	670.0	2.80279885691	3.13918	81.75	39.0	0.752911561595
C790b	376.0	0.212379277664	3.45822	37.0	9.0	0.0420439112721
CAB02	570.0	2.56141010007	7.39751	176.875	64.0	0.560480147738
CSM0626b	122.0	5.50686867232	10.016	94.0	64.0	0.962125718818
CSM0636	745.0	0.567248239042	3.30396	27.0	18.0	0.128797801901
CSM0655	493.0	0.371873082578	0.98071	4.0	4.0	0.115178420812
CSM0686	793.0	1.662490724	3.74573	37.0	26.0	0.469559464991
CSM0696a	172.0	5.00298572492	10.34107	129.125	70.0	0.915021633315
CSM0704	495.0	0.264550865806	0.9348	4.0	4.0	0.0742291602898
CSM0746	341.0	3.22623040805	10.51778	105.0	51.0	0.720908832913
CSM0773	1384.0	0.515442352324	2.68216	43.5	26.0	0.119125680778
CSM0776	603.0	0.0177073172577	0.9348	2.0	2.0	0.0033112491715
CSM0804	355.0	0.265549617902	1.8917	6.0	5.0	0.0659869073597
CSM0826	408.0	1.32900374386	6.0031	72.0	30.0	0.313425124952
CSM0830	675.0	0.262352965699	1.35637	16.5	9.0	0.0608614540466
CSM0843	896.0	0.460924328515	2.21086	33.0	15.0	0.103767239318
CSM0854	254.0	1.4845035219	1.70546	9.0	8.0	0.541571083142
CSM1806	374.0	2.96987885585	6.62468	80.1	45.0	0.721839343418
CSM1841	598.0	0.91671813265	1.40886	16.5	14.0	0.257340521918
CSM1846	185.0	-0.0	0.0	1.0	1.0	0.0
CSM1867	401.0	0.0251579877769	0.64222	2.0	2.0	0.00497509343847
CSM1868	390.0	1.82534809635	5.00679	61.1428571429	34.0	0.43798816568
CSM1869	86.0	-0.0	0.0	1.0	1.0	0.0
CSM1877	404.0	4.8606312152	10.9771	210.066666667	96.0	0.918378100186
CSM2632	359.0	0.313728250189	1.2531	10.0	7.0	0.0759149913486
CSM2738	1190.0	1.95427628096	1.82638	45.5	43.0	0.533186921828
CSM2745	141.0	-0.0	0.0	1.0	1.0	0.0
DG04	162.0	1.93987287733	1.85788	19.2	15.0	0.593583295229
DG06	279.0	1.90351769164	1.4892	23.3333333333	14.0	0.653665805938
DG08	374.0	0.525931796513	0.93267	16.0	10.0	0.123537990792
DG11	340.0	0.969131944227	1.57506	40.0	12.0	0.294584775087
DG14	232.0	1.05475155824	1.18341	31.0	10.0	0.361177170036
DG17	495.0	0.828219138682	1.78994	26.0	11.0	0.230180593817
DG18	305.0	1.28459360556	1.80613	17.5	10.0	0.515001343725
DG19	82.0	2.37355776255	3.48874	25.2	18.0	0.587745389649
EMS2584	684.0	0.615899671787	0.96789	6.0	6.0	0.180115249136
EMS2617	783.0	0.418576507032	1.43322	32.0	11.0	0.107795116207
FH1101	188.0	1.75477750638	1.09932	21.0	14.0	0.556473517429
GM3589b	717.0	1.28142153126	0.86719	15.0	10.0	0.531577217174
GM3627	336.0	0.704031192636	3.13183	41.0	13.0	0.169288548753
GM4009	581.0	1.92467489217	3.25037	28.0	19.0	0.63624648582
GM507	226.0	1.82656144528	2.40857	32.0	11.0	0.664969848853
GM894	192.0	1.14225886472	1.77695	17.5	10.0	0.377332899306
GM989	443.0	0.115414057618	1.86781	16.0	6.0	0.0224204964102
IND02	507.0	1.20919602137	1.94877	20.0	13.0	0.435403366673
IND05	877.0	0.972065396663	1.15318	35.0	14.0	0.293563238416
ISR03	114.0	5.55614549643	10.69196	112.214285714	62.0	0.969221298861
ISR05	90.0	2.91140645366	4.44414	37.0	24.0	0.689382716049
ISR06	222.0	4.85004656022	10.08744	165.176470588	81.0	0.897897897898
ISR07	169.0	5.09490707244	10.247	125.909090909	62.0	0.948636252232
JCM102H	413.0	0.205936375701	1.93675	7.75	7.0	0.0430207130252
JCM120P	419.0	0.414356273664	2.14322	48.0	12.0	0.0927199093193
JCM79D	317.0	0.846894317962	2.54186	41.0	13.0	0.221755615042

KATE02	185.0	0.327702808019	1.89038	12.0	7.0	0.0739810080351
LD02	315.0	0.660529736815	2.80479	24.5	17.0	0.140307382212
LEA02	300.0	1.05805261545	3.98843	45.0	25.0	0.224822222222
LEA04	233.0	0.291837313988	0.91388	8.0	5.0	0.0750428263552
MJ3691	491.0	0.271690710578	1.7004	7.0	7.0	0.0599798408004
MJ3727	504.0	0.103301537263	1.57633	16.0	6.0	0.0197231670446
MJ5060	100.0	1.58765465373	1.79612	15.0	10.0	0.5486
MJ7811	249.0	0.23662927776	1.37826	5.5	5.0	0.0551926581829
MJ8263	960.0	0.808673598794	1.7712	14.5	13.0	0.2190234375
MJ8277	224.0	1.12124015503	0.9758	5.0	5.0	0.431481186224
MJ8282	383.0	1.19913241248	2.2033	53.0	17.0	0.313656784081
MJ8283	219.0	1.14747144502	2.50856	65.5	20.0	0.261462438231
MJ8291	368.0	1.31030590984	2.73524	47.0	30.0	0.284277528355
MJ9243	804.0	2.13171133929	1.5856	50.0	24.0	0.698757456499
MJ9273	339.0	0.631098706054	1.05595	6.5	6.0	0.207812323248
MJ9277	711.0	0.070044575172 7	1.0144	5.0	4.0	0.0139934839502
MJ9280	886.0	1.2051214708	3.18201	35.428571428 6	26.0	0.348419609781
MJ9286	603.0	0.962474422466	1.7844	29.25	18.0	0.295988273118
MS1177	915.0	1.71810850915	2.34646	27.5	24.0	0.548294663919
MS895	409.0	1.88482912913	1.85377	43.0	15.0	0.628798249652
PH12	338.0	1.41069908492	1.50342	21.0	11.0	0.5146353419
PH13	76.0	3.80053383413	3.67915	40.333333333 3	22.0	0.902354570637
PH13nn	431.0	1.19863994149	1.38497	12.0	10.0	0.487002115622
PH14	393.0	1.08286478976	1.07743	35.5	13.0	0.409390802142
PH16	947.0	2.11111960297	1.46137	38.428571428 6	32.0	0.640972603977
PH19	425.0	2.99025020486	4.77314	81.111111111 1	45.0	0.74491349481
PH21	147.0	1.54128982121	0.98725	20.0	10.0	0.52755796196
PH22	208.0	0.571982159992	1.53408	11.0	8.0	0.146403476331
PSW5703	353.0	1.42364695446	2.85628	29.0	22.0	0.33578633967
PSW6454	1271. 0	1.28081889458	1.86557	16.333333333 3	13.0	0.493251068903
RA0735	1064. 0	1.45379313323	4.39119	55.428571428 6	31.0	0.545409576573
RA0747	225.0	0.741080098057	1.11785	6.0	6.0	0.226409876543
RA0750	416.0	1.51570762226	4.44551	40.375	29.0	0.384788738905
RA0755	445.0	1.23922266692	5.16021	142.33333333 3	34.0	0.27252114632
RA0756	603.0	0.859806313084	1.26363	11.5	11.0	0.234543149372
RA0757	527.0	0.079535978473 7	1.35634	11.0	5.0	0.0151082530092
RA0765	1356. 0	2.74550849239	7.82596	89.545454545 5	60.0	0.65165200442
RA0766	532.0	2.2361999218	4.66418	155.5	29.0	0.723648877834
RA0769	416.0	1.89672549816	1.63767	20.0	15.0	0.568763868343
RA0782	218.0	4.24836759564	11.44134	134.75	63.0	0.871854221025
RA0784	722.0	0.527644123375	2.09048	26.0	12.0	0.152657668373
RA1154	96.0	0.25015240246	1.34386	7.0	4.0	0.0611979166667
RA1155	97.0	3.05152490359	5.28051	381.0	30.0	0.757572536933
RA1157	151.0	0.741872454323	2.54187	92.0	14.0	0.164203324416
RA1158	243.0	1.23130528695	2.37657	23.333333333 3	14.0	0.377686328304
RA1160	206.0	1.97895646656	4.0375	74.5	22.0	0.549297766048
RA1161	1290. 0	0.875321954024	2.39251	33.857142857 1	26.0	0.220441079262
RA1162	374.0	3.61994194722	6.60961	96.333333333 3	55.0	0.806986187766
RA1164	218.0	1.03159186736	1.91558	27.0	13.0	0.280742361754
RA736b	351.0	0.196728317748	1.006	5.5	5.0	0.044885999302
RA736c	755.0	0.450182334439	2.03597	28.0	16.0	0.100058769352
RD001	588.0	0.291754041037	3.10775	48.0	12.0	0.0601427645888

RK03	212.0	0.914766306083	2.59798	21.0	14.0	0.220140619438
RO122	943.0	1.27282881831	3.55259	40.5	24.0	0.424155663937
SKY06	267.0	0.319510044425	2.89697	46.0	10.0	0.0661532634768
SKY11	439.0	0.536939738809	2.07648	10.75	10.0	0.134744008178
SKY13	340.0	2.38295340553	3.68083	97.0	19.0	0.764584775087
SKY15	494.0	0.291958691768	2.16903	20.5	10.0	0.0634824370175
SKY18	866.0	2.20922735867	3.27678	72.625	41.0	0.680786606148
SKY19	370.0	0.459827900347	3.02502	33.3333333333	15.0	0.0947260774288
SKY20	414.0	1.95586818108	2.50645	21.1428571429	19.0	0.626257322225
SKY21	690.0	0.838061403645	5.965	59.1428571429	32.0	0.173593782819
SKY22	893.0	2.53209055904	5.21382	55.5454545455	40.0	0.753673275658
SKY24	921.0	0.107719083792	1.59951	7.5	6.0	0.02155991045
SL201	76.0	3.5868822049	4.65922	87.75	30.0	0.794667590028
SL282	115.0	3.02899800245	3.86354	38.6	23.0	0.791379962193
SL32	1076.0	2.11538551014	3.87579	72.1111111111	44.0	0.611180401045
SOH02	194.0	1.52434060059	2.60369	16.5	16.0	0.374056754172
SUL02	775.0	1.19583584628	4.06477	38.5	28.0	0.375215816857
SUL04	542.0	0.443235903166	1.79607	10.5	9.0	0.119510899906
SUL05	257.0	4.98957240814	8.30025	112.153846154	69.0	0.931005768445
SUL06	383.0	1.86553752618	3.83408	35.1428571429	24.0	0.540667671059
TAS02	626.0	2.24272769432	2.23196	35.75	22.0	0.684221539467
TAS03	158.0	1.31572502212	0.95239	12.0	9.0	0.434946322705
TAS04	129.0	1.06384382201	0.93845	4.0	4.0	0.44552611021

Additional File 5: Additional Table 5.xlsx. Bacterial quantification through 16S rRNA gene (qPCR) of all *Polyrhachis* samples. Each sample was analyzed in triplicate therefore follows the values of average and standard deviation for each sample.

Samples	Average	Stand Deviation	Samples	Average	Stand Deviation	Samples	Average	Stand Deviation
B30	14285,43608	1459,3092	EMS2617	124737,98	1608,566	SKY22	46795,435	2524,7755
CSM636	277442,4457	159998,34	IND05	103518,39	11214,761	SUL04	37735,684	2053,2716
BB012	77246,7959	79755,418	LEA04	39397,456	7176,2721	SKY11	87411,654	4762,3453
CSM0655	105817,9332	49838,206	FH1101	1167,1389	155,84207	SKY24	1865201	20450,074
C790B	15707,7435	1492,3984	JCM102H	21697,738	2470,0787	SUL05	621505,94	1074759,9
CSM0686	22663,99421	8990,3964	MJ3691	1812414	275750,64	SKY13	1312,9258	28,6435
AS4121	500148,3551	230325,89	MJ924	88304,875	1290,6434	SL32	48890,479	2342,3648
CAB02	7890,596614	1095,8442	PH12	93972,869	3317,9529	SUL06	7395,2222	266,30049
CSM0696A	305,6088804	48,326714	MJ3727	1186942,2	51429,608	SKY15	57000,711	2667,6549
AS4132A	153368,7282	14485,968	MJ7293	77835,524	9490,5593	SL_20_1	415,80483	25,781942
CB_01	16282,36467	2259,5663	PH13	196033,94	11306,491	TAS01	62738,608	31208,242
CSM0704	143368,3363	14073,851	MJ5060	140461,32	5849,4605	SKY18	201432,01	4659,7367
AS4132B	920889,3232	346567,07	MJ9277	8007692,3	492329,87	SL_20_2	2019,964	67,150639
CSM1813	2723,556068	339,49461	PH19	10786,968	542,06845	TAS02	200722,24	9118,5706
CSM0746	1278,469533	400,39349	MJ7811	2082,5166	1519,8754			
AS4148A	1134812,191	58229,079	MJ9280	746487,51	130652,35			
RA736A	5,882250092	4,3874282	PH20	304382,28	22733,993			
CSM0773	352699,7531	78840,965	MJ8263	199298,35	132196,95			
AS4148B	903603,3203	35684,657	MJ9286	541324,09	7104,2376			
CSM0626B	226,2195716	60,483023	PH21	16,797524	10,376417			
CSM0776	1015184,766	54404,125	MJ8282	3111044,5	60669,396			
CSM0797	121,8140419	14,190769	MJ9287	135083,35	14938,621			
CSM1846	307190,8979	70826,041	PH22	26678,132	736,10604			
CSM2632	699607,5781	93577,796	MJ8283	15953,001	1391,6396			
CSM0804	114497,9723	38817,031	MS1177	105187,52	17236,414			
CSM1854	6090,874285	727,9305	PSW5703	1849,4353	63,44052			
CSM2738	1910409,126	434910,3	MJ8291	207577,5	21589,748			
CSM0826	1069,324009	212,72922	MS895	3782,5221	224,50895			
CSM1863	2,156491552	0,1139426	PSW6454	659438,63	15642,695			
CSM2745	428294,3499	153235,75	RA0735	217956,59	29544,887			
CSM0830	109340,6953	9102,7702	RA0766	8908,3238	1386,757			
CSM1866	42624,71954	19604,775	RA1158	28660,726	9572,0671			
DG03	167061,589	57396,79	RA0747	33346,896	28988,388			
CSM0843	63767,11045	6663,9025	RA0769	13345,403	1264,3103			
CSM1867	199411,2734	176680,67	RA1160	3195,039	457,45528			

				7	
DG04	54472,49325	8958,254	RA0750	17473,816	324,02404
CSM0854	1,807397994	0,1885984	RA0782	119,96222	12,781646
CSM1868	45100,59112	3431,6898	RA1161	154418,66	37189,924
DG06	5834,92888	365,98091	RA0752	38617,211	1701,4746
CSM1806	2558,9709	1930,6993	RA0783	277802,38	12427,21
CSM1869	275538,119	87639,696	RA1162	21325,894	896,63197
DG08	27347,17117	698,91926	RA0755	7704,8836	1661,0869
CSM1841	394345,9909	161905,47	RA0784	146785,95	29912,552
CSM1877	22836,02351	7548,8792	RA1163	57831,629	0
DG09	47008,31146	5841,1743	RA0756	1090492,1	38445,761
DG11	123871,8079	18672,04	RA1154	573265,13	106055,64
GM6327	986,1161918	0	RA1164	38433,093	7184,9107
JCM120P	4895,757874	2619,4363	RA0757	1092628,3	148178,15
DG14	48567,46854	5727,6072	RA1155	3762,4885	339,04734
GM4009	12136,05968	2148,961	RA736B	522993	81944,225
JCM79D	8592,794302	5969,3552	RA0765	24479,596	936,82826
DG17	646378,7309	48034,126	RA1157	3707,3087	254,28854
GM507	2299,54803	62,983413	RA736C	224204,16	187415,48
KATE02	10099,41909	910,80333	RD001	82839,328	2572,4557
DG20	208,8266102	18,487416	SKY19	188954,24	8426,5379
GM894	34799,47425	2983,0216	SL_28_2	947,52311	19,236482
LD02	2170,294598	82,179516	RK03	951,73041	45,904865
DG23	46,12275618	12,198254	SKY20	61134,449	824,73012
GM989	12302,75889	1996,1371	SOH02	48608,999	2336,8535
LEA01	84225,60062	4103,2945	RO122	51647,326	9613,9351
EMS2584	1175512,262	437447,93	SKY21	65758,695	4277,8317
IND02	18846,92247	354,47723	SUL02	94537,687	1673,7398
LEA02	2852,875116	1360,0333	SKY06	28516,052	206,06161

Capítulo 2

Species-specific signatures of the microbiome from *Camponotus* and *Colobopsis* ants across developmental stages

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Resumo

As relações simbióticas entre hospedeiros e bactérias são comuns na natureza, e estas podem ser responsáveis pelo sucesso evolutivo de vários grupos de animais. Entre as formigas, essas associações têm sido bem estudadas em alguns gêneros de Camponotini, mas ainda há várias questões quanto à generalidade dos achados anteriores em todos os membros dessa tribo de formigas e se as comunidades bacterianas mudam através do desenvolvimento nesses hospedeiros. Este estudo é o primeiro a caracterizar a comunidade bacteriana associada a uma colônia do gênero recentemente reconhecido *Colobopsis* e três colônias de *Camponotus* (duas espécies distintas) e mostrar como é diferente a composição da comunidade bacteriana quando comparada entre os diferentes gêneros. Nossos dados revelam que *Colobopsis* (espécies: *Co. riehlii*) e *Camponotus* (espécies: *Ca. floridanus e Ca. planatus*) possuem microbiota distinta, e conseguimos verificar que a identidade da espécie contribui mais para a diversidade bacteriana. Também demonstramos que não houve diferenças significativas entre colônias da mesma espécie (*Camponotus planatus*) e entre estádios de desenvolvimento de diferentes colônias. Achamos que alguns estágios de desenvolvimento possuem bactérias distintas, confirmando que cada estágio de desenvolvimento poderia ter uma microbiota específica. Nossos resultados mostram que as espécies são um dos fatores que moldam a comunidade bacteriana nestas formigas de Camponotini. Estudos adicionais do microbioma intracolonial de outros hospedeiros e em todo o desenvolvimento podem revelar pistas adicionais sobre a função e importância das bactérias no reconhecimento de colônias, saúde individual e colônia e melhoramento nutricional.

Palavras-chave: Next-Generation Sequencing; *Blochmannia*; *Wolbachia*; Formicidae

Abstract

Symbiotic relationships between hosts and bacteria are common in nature, and these may be responsible for the evolutionary success of various groups of animals. Among ants, these associations have been well studied in some genera of the Camponotini, but several questions remain regarding the generality of the previous findings across all the members of this ant tribe and if bacterial communities change across development in these hosts. This study is the first to characterize the bacterial community associated with a colony of the recently recognized genus *Colobopsis* and three colonies of *Camponotus* (two distinct species) and show how different the composition of the bacterial community is when compared across the different genera. Our data reveal that *Colobopsis* (species: *Co. riehlii*) and *Camponotus* (species: *Ca. floridanus* and *Ca. planatus*) have distinct microbiota, and we were able to verify that the identity of the species contributes more to the bacterial diversity. We also demonstrated that there were no significant differences between colonies of the same species (*Camponotus planatus*), and between stages of development from different colonies. We did find that some developmental stages have distinct bacteria, confirming that each stage of development could have a specific microbiota. Our results show species are one of the factors that shape the bacterial community in these Camponotini ants. Additional studies of the intra-colonial microbiome of other hosts and across development may reveal additional clues about the function and importance of bacteria in colony recognition, individual and colony health, and nutritional upgrading.

Keywords: Next-Generation Sequencing; *Blochmannia*; *Wolbachia*; *Formicidae*

Introduction

Symbiotic interactions are thought to be one of the factors responsible for the ecological success of many groups of animals and plants [1–4]. Symbiotic microbes can influence the host through the manipulation of the host's reproduction or provide direct benefits to the host through nutrition, defense, or even environmental tolerance [5–8]. Social animals often interact intimately with other members of their group and offspring through grooming and trophallaxis. These activities facilitate the transmission and sharing of bacteria, often making their microbiota extremely specialized [9,10]. As these behaviors may facilitate symbiont transfer, social insects are considered models to evaluate evolutionary aspects of microbial community diversity and acquisition [11].

Among the Hymenoptera (bees, wasps, and ants), many species of ants (Formicidae) are known to possess diverse and stable microbial communities [12–18]. The importance of bacterial symbionts related to nutrition has been shown to be fundamental in ant species feeding low on the trophic scale [17,19,20] as is the case for the ant tribe Camponotini. One of the most well-known ant genera recognized for having symbiotic relationships with bacteria is *Camponotus* Mayr, 1861 [21–24]. The genus is currently subdivided into 43 subgenera, covering more than 1000 valid species and 31 fossils [25], with an almost world wide geographic distribution. They are popularly known as carpenter ants, have diurnal and nocturnal activity with a generalist diet, and have diverse nesting habits. Arboreal nesting species may specialize on a diet that is nutritional deficient, since their diet is largely derived from the exudate of plants and phytophagous insects [20,26,27].

The phylogeny of Camponotini, especially within *Camponotus*, has always been complex, and several studies using different approaches have indicated that

Camponotus is not monophyletic [28–31]. Ward et al. [32] in a recent phylogenetic analysis of the group elevated *Colobopsis* Mayr 1861, to the genus level, but still belonging to the tribe Camponotini. Prior to this it was considered a subgenus of *Camponotus*. Completely understanding the phylogeny and evolution of *Camponotus* remains a difficult task, which will require the efforts of researchers from around the world, due to their wide distribution and high species diversity. *Colobopsis*, now considered a distinct ant genus, has 94 valid species, with a distribution across the Australasia, Indomalaya, Neartic, Neotropical, Oceania and Paleartic regions [33]. This genus has strictly arboreal habits, and nests inside dead branches. As in the distantly related turtle ant genus, *Cephalotes*, they can employ phragmotic major workers to block the nest entrance with their heads as a line of passive defense [34–36].

With the recognition of this new genus, *Colobopsis*, it now raises the intriguing question whether it also hosts *Blochmannia* or related endosymbionts, as has been noted in several studies for *Camponotus* [21,22,37]. In a recent study Brown and Wernergreen [37], using next-generation sequencing (NGS) techniques, found that 95 - 98% of the reads of *Camponotus chromaiodes* were dominated by the intracellular bacteria *Blochmannia* and *Wolbachia*. However the variation across the different stages of development and for additional species and genera remains unclear.

It is known that diet [38,39], parasitic infection [40], host age [41], phylogeny of host [15,42] may contribute to changes in the bacterial community. Thus, the natural variation found across insect microbiota may indicate important influences of host biology. To this end, our understanding of factors that determine the bacterial communities of *Camponotus floridanus, Camponotus planatus* and *Colobopsis riehlii* remain unclear. In addition detailed comparative surveys of the microbiota present in different castes and across development within a colony are still lacking [15].

This study focuses on the bacterial community of different colonies of *Camponotus* and *Colobopsis* across their stages of development, to reveal more about the factors that influence bacterial communities. Therefore, the present work raises the following questions: 1) What exerts greater influence in these microbiomes, the colony/species or developmental stage? 2) Are *Camponotus floridanus, Camponotus planatus* and *Colobopsis riehlii* bacterial communities different? 3) Do individuals from different colonies, but the same species, have similar microbiota? 4) Are there differences between the stages of development within the same colony? And finally 5) Does the same stage of development from different colonies/species have similar bacteria? Leveraging next generation amplicon sequencing, we address these questions and document the diversity of bacteria to help identify the factors that structure the bacterial communities found across a diverse and widely distributed group of animals.

Materials and Methods

Sample collection and determination of the different stages of development

All specimens used in this study were collected by authors MOR and CSM in April 2015 from the Florida Keys, USA - Watson Creek bridge, Monroe County (24.69786N, 081.3405W). These specimens were collected under the permissions of the Florida Department of Environmental Protection - Division of Recreation and Parks (permit number 0127201515 to CSM). Three *Camponotus* colonies, representing two species (*Ca. floridanus and Ca. planatus*), and one *Colobopsis riehlii* colony were obtained from hollow twigs of trees and brought to the lab. After screening in the laboratory, the samples were immediately preserved in 95% ethanol and stored at -20°C

before DNA extraction. In order to determine the castes / stages of development, we selected the eggs, larva with variation of size (L1 = small larvae and L2 = large larvae – last larval instar), pupae classified according to the pigmentation of the eye and body (P1, P2 and P3 - from the white eye to total pigmentation, respectively), small and large workers (W1 and W2 to represent adult worker size polymorphism), queens and males [43,44]. Within each entire colony, the quantity of each caste/subcaste/stage was determined (see Table 1). The taxonomic identification were determined using taxonomic keys for *Camponotus* and *Colobopsis* species in the southeastern US (available in: http://mississippientomologicalmuseum.org.msstate.edu//Researchtaxapages/Formicidaepages/Identification.Keys.htm#.WE7qIH31-3H - **from Creighton 1950, Snelling 1988; Mark Deyrup, pers. comm., William MacKay-***Camponotus* website) and vouchers were deposited in the collection of the USP Zoology Museum in São Paulo, Brazil.

Table 1. Colonies of *Camponotus* and *Colobopsis* collected in the Florida Keys, Florida, USA for the present study, and the number of individuals from each caste available in each colony.

Collection code	Species	Egg	L 1	L 2	P 1	P 2	P 3	W 1	W 2	Male	Queen	Total
MOR#59	*Camponotus floridanus*	0	18	13	5	18	0	29	30	0	0	113
MOR#69	*Camponotus planatus*	125	30	33	2	10	1	70	26	11	7	315
MOR#73	*Camponotus planatus*	10	32	1	0	9	3	31	6	5	0	97
MOR#62	*Colobopsis riehlii*	0	33	5	4	2	1	13	9	0	1	68

L1 and L2 refers to larva; P1, P2 and P3 refers to pupal stage 1, 2 and 3; W1 and W2 - refers to minors W1 and majors W2.

DNA Extraction and Bacterial DNA Sequencing

Total DNA was extracted from 85 samples (three specimens of each caste and

colony, when available) of entire individuals with the Qiagen DNeasy Tissue kit following the manufacturer's recommendations with filtered pipette tips and sterile techniques were applied to avoid contamination following Moreau [45].

Additionally, the samples were sterilized on the surface as described in Moreau [45]. Although we did not use the modification of the Qiagen DNeasy kit for Gram-positive bacteria, we did follow the extraction method recommended by Rubin et al. [46]. This method is able to detect Gram positive bacteria in large quantities, but this could still influence the diversity of bacteria we are able to detect. We amplified the V4 region of 16S rRNA using primers described in Caporaso et al. [47], following the Earth Microbiome Project (EMP) protocol (515f primer and 806r; http://www.earthmicrobiome.org/emp-standard-protocols/16s/). Per sample three PCR reactions were performed (triplicate) when samples were available, each 25 µl PCR reaction contained 12 µl of MO BIO (MO BIO, Solana Beech, USA) PCR Water (Certified DNA-free), 10 µl of 5 Prime HotMasterMix (1x) (5 PRIME, Gaithersburg, USA), 1 µl of forward primer (5 mM concentration, 200 final pM), 1 µl Golay barcode tagged reverse primer (5 mM concentration, 200 pM final) and 1 µL of template DNA (>0.20 ng/ µl), under the following conditions 94˚C for 3 min, with 35 cycles at 94˚C for 45 s, 50˚C for 60 s, and 72˚C for 90 s, with a final of 10 min at 72˚C. After the triplicate reactions were combined we confirmed amplification efficiency using agarose gel electrophoresis (1%). The samples were quantified via qPCR and Qubit (Thermo Fisher Scientific) with High Sensitivity Assay Kit (Life Technologies Corp., Carlsbad, USA), and only then pooled with different samples after controlling for volume to include the same amount of genetic material. For purification, only 100 µL of each pool was cleaned using the UltraClean PCR Clean-Up Kit (MO BIO, Solana Beech, USA), following the manufacturer's recommendations. The molarity of the pool was

determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.1 pM with a 10% PhiX for sequencing on the Illumina MiSeq at the Field Museum of Natural History, Chicago. A 151 bp x 12 bp x 151 bp MiSeq Illumina run was performed using the custom sequencing primers and procedures described in the supplementary methods in Caporaso et al. [47]. All raw sequence data are publicly available in Figshare [https://figshare.com/s/290531bea3dee984444e] and NCBI SRA accession number SRR5136256 and study SRP095836.

Bacterial Quantification

We measured the amount of bacterial DNA present in all samples with quantitative PCR (qPCR) of the bacterial 16S rRNA gene using 515f (5' - GTGCCAGCMGCCGCGGTAA) and 806r (5' - GGACTACHVGGGTWTCTAAT) universal bacterial primers of the EMP (http://www.earthmicrobiome.org/emp-standard-protocols/16s/) to check Illumina sequencing efficiency. All qPCRs were performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, USA) using SsoAdvanced 2X SYBR green supermix (Bio-Rad) and 2 µL of DNA. Standard curves were created from serial dilutions of linearized plasmid containing inserts of the *E. coli* 16S rRNA gene and melt curves were used to confirm the absence of qPCR primer dimers. The protocol for standardization following the recommendations of Rubin et al. [46]. All samples were analyzed in triplicates including a blank. The results were averaged before calculating the number of bacterial 16S rRNA gene copies per microliter of DNA solution (S6 Table).

Bioinformatic Analysis

The sequences were analyzed in QIIME 1.9.1 [48]. We merged the forward and reverse sequences through SeqPrep. Demultiplexing was completed and QIIME defaults were used for quality filtering of raw Illumina data. We implemented the

pick_open_reference_otus.py command using the SILVA 128 reference database with 97% identity [49,50] to call OTUs, and UCLUST to create the OTU table. Sequences with less similarity were discarded. Chimera checking was performed with UCLUST [51] and PyNAST (v1.2.2) was used for sequence alignment [52].

To test whether the composition of the bacterial community is more related to the colony/species itself, or whether it is more related to the different stages of development, we separated our analyses into two categories: different colonies/species (MOR#59, MOR#62, MOR#69, and MOR#73) and sample type (different stages of development available in colonies/species sampled, see Table 1).

The summarize_taxa_through_plots.py command was used to create a folder containing taxonomy summary files. In order to standardize, all samples that obtained less than 400 bacterial sequences after quality filtering were excluded from the subsequent analysis. We started our analysis with 73 samples (triplicate of each caste when available), and after filtering to a sequencing depth of 400, 63 samples passed this cutoff and were included in downstream analyses. Ten samples were excluded (see S9 Table), and they are identified with a yellow star in Figure 1. All analyzes started from the bacterial OTU data. We implemented an analysis of multidimensional nonmetric scaling (NMDS) and related statistics in the PAST3 software package [53] to illustrate the relationship between ecological communities [54,55]. Sorensen (Dice coefficient) and Bray-Curtis, similarity and dissimilarity indices, respectively [54] were used to test the variation and the structure of the bacterial communities, respectively. The samples were grouped according to the host colony/species and sample type. Analyses of similarity (ANOSIM) with Bonferroni correction was used to determine statistical significance [54,55]. The SIMPER analysis was conducted to verify the contribution of each OTU for grouping between colonies/species and different sample type [55].

The G test of independence (P, FDR_P and Bonferroni_P) was carried out to determine whether OTU presence/absence is associated with different colonies/species or different sample types. We also used Analysis of Similarity (ANOSIM) to test predefined groups of samples were significantly different, Adonis [56] to determine sample grouping and a redundancy analysis (RDA) to test the relationships between samples.

Observed species richness, Shannon diversity, the Chao1 nonparametric richness estimator, whole-tree phylogenetic diversity, Simpson, and equitability metrics were calculated to compare alpha diversities based on a two-sample t-test using non-parametric (Monte Carlo) methods to test differences in OTU richness among categories. Unweighted and weighted UniFrac distance matrices [57], which use phylogenetic information to calculate community similarity, were used to calculated beta diversity. These beta diversity metrics were used to compare community level differences between two categories: colonies/species and sample type. Jaccard dissimilarity metrics were calculated and the average was compared. A matrix of community pairwise distances was generated by UniFrac and used to cluster samples by principal coordinates analysis (PCoA).

Cytoscape v3.2.1 [58] edge-weighted spring embedded algorithm was used to visualize networks of bacterial community [59]. Connections were drawn between samples representing the shared significant OTUs. A heatmap was constructed with all OTUs that had 100 reads using heatmap.2 and the vegan package [60] in R [61]. The dendrogram was created with Bray-Curtis dissimilarity hierarchical clustering of bacterial communities in hclust.

Phylogenetic Tree Reconstruction

To investigate the possible relatedness of some of our unassigned bacterial OTUs, we downloaded from GenBank the closet Blast hits for our selected sequences and other

strains of *Blochmannia* available from different Camponotini genera. We were able to include *Blochmannia* from all Camponotini genera except *Dinomyrmex* and *Overbeckia*. Sequences were assembled and edited using Bioedit Sequence Alignment Editor [62] and aligned with the Clustal W tool [63]. We implemented a maximum likelihood analysis using PhyML 3.0 [64] on the CIPRES web portal [65]. The GTR+I+G model of sequence evolution was implemented. Branch lengths and bootstrap support are reported. To facilitate visualization, the clade of *Wolbachia* (brown) and *Blochmannia* (green) were colored in FigTree [66].

Results

From the four colonies analyzed we obtained 1,322 observed OTUs from a total of 152,500 reads from 73 samples from one colony of *Colobopsis riehlii* and three colonies of *Camponotus* from two species (one colony of *Ca. floridanus* and two colonies of *Ca. planatus*), which permitted analyses comparing different colonies/species and developmental stages. To visualize the diversity of OTUs found per sample we used the summarize_taxa_through_plots.py command (Fig 1). For *Colobopsis*, 19 samples across the stages of development were analyzed, resulting in 134 OTUs from a total of 16,591 reads, ranging from 206 to 3008 reads per samples. For analysis of the colonies of *Ca. floridanus* and *Ca. planatus* were recovered 1,188 OTUs resulting from 135,909 reads ranging from 10 to 13,989 reads, with the latter value belonging to one from the queens analyzed.

Fig 1. Summary graph of bacterial OTUs found in *Colobopsis riehlii*, *Camponotus floridanus* and *Camponotus planatus* colonies with 16S rRNA amplicon sequencing. **A.** Different colonies and species used in this study and their bacterial communities. **B.** Summary of all OTUs found in *Colobopsis riehlii*. The main bacterium is Enterobacteriaceae in pink, followed by *Wolbachia* in brown **C.** Summary of all OTUs

found in *Ca. floridanus and Ca. planatus*. The main bacterium is *Candidatus Blochmannia* in green. The yellow stars highlight samples that were excluded after the read depth standardization of 400 reads was implemented.

According to our results, the bacterial communities of *Colobopsis riehlii* and other *Camponotus* colonies (*Ca. floridanus and Ca. planatus*) are distinct. The predominant bacteria found in the samples of *Camponotus* were *Candidatus Blochmannia* (93.9%), *Wolbachia* (1.0%) (multiple strains), Enterobacteriaceae (0.8%), followed by other bacteria in smaller quantities. For the *Colobopsis* samples the predominant bacteria were Enterobacteriaceae (72.8%), *Wolbachia* (multiple strains) (22.2%), Pasteurellales (2.2%) mainly related to a specific stage of development, *Sodalis* (1.7%), Other Enterobacteriaceae (0.4%), followed by additional bacteria at low amounts (S8 Table).

By analyzing the bacteria found across different stages of development within a colony (sample type), we recovered bacteria associated with only a specific stage of development such as the Pasteurellales, which is present in the second larval stage of *Colobopsis riehlii*, and *Wolbachia* present only in the queens and males of *Camponotus planatus*. Our data also reveal that the larval stage exhibits much greater microbial diversity than the other stages of development (S7 Fig).

Patterns that influence the bacterial community

We performed statistical tests (weighted and unweighted, depth 400, 63 samples included) to examine potential patterns that influence the bacterial community of these Camponotini samples, and for this we analyzed the following two variables: differences between colonies/species and sample type (developmental stage). A list of the 10 samples that did not reach the depth of 400 reads and were excluded from the analysis

are included in Table S9. From these we found different colonies/species (Table 2), can influence the bacterial community of these Camponotini samples, although for the sample type we did not obtain significant results for the weighted distance.

Table 2. Patterns that explain bacterial community diversity.

	Colonies/Species	
	Unweight	**Weight**
Adonis	R^2= 0.1658 and *p*= 0.001	R^2= 0.6520and *p*= 0.001
Anosim	R^2= 0.2020 and *p*= 0.001	R^2= 0.4676 and *p*= 0.001
RDA	Pseudo F= 3.8582 and significance=0.001	Pseudo F= 30.8438 and significance=0.001
	Sample Type (developmental stage)	
	Unweight	**Weight**
Adonis	R^2= 0.2084 and *p*= 0.001	R^2= 0.1925 and *p*= 0.167
Anosim	R^2= 0.1381 and *p*= 0.006	R^2= 0.0580 and *p*= 0.1170
RDA	Pseudo F= 1.543 and significance=0.005	Pseudo F=1.3569and significance= 0.193

Colonies/Species have greater influence than Sample Type (developmental stage).

Through analyses of beta diversity (matrices UniFrac weighted distance) we observed bacterial communities among all Camponotini samples. PCoA analysis showed that the bacterial community becomes more distinct when comparing the different species than when comparing the stages of development across all species (Fig 2). The average Jaccard dissimilarity metric was 0.90 for *Camponotus* colonies (one of *Ca. floridanus* and two of *Ca. planatus*), which suggests only few of the bacterial community members are shared among all individuals of different developmental stages of *Camponotus*, but for *Colobopsis riehlii* Jaccard dissimilarity of 0.65 was inferred, which suggests more of bacteria was shared among the colony.

Fig 2. PCoA plots of bacterial communities associated with Camponotini samples (weighted UniFrac method). A. Different colonies/species (axis 1 = 88.8% and axis 2 = 7.12%) and **B.** developmental stage/sample type (axis 1 = 88.8% and axis 2 = 7.12%). Note that the "Colony/Species" category influences the bacterial community more than "Sample Type".

No significant changes in the composition of the bacterial community (Sorensen index)

were observed between the colonies/species and among sample type (S3 Table). However, when we analyzed the bacterial community structure (Bray-Curtis index, stress 0.051, for different colonies, and 0.051 for different sample type), we found significant results such as difference between samples MOR#73 (*Ca. planatus*) vs. MOR#62 (*Co. riehlii*) and MOR#69 (*Ca. planatus*) vs. MOR#62 (*Co. riehlii*) (Fig 3, S3 Table). For these analyses we did not recover significant differences between developmental stages.

Fig 3. Nonmetric multidimensional scaling (NMDS) plot illustrating bacterial community structure among different colonies/species with 95% ellipses. Bray-Curtis, stress 0.081, Axis 1: 0.9817, Axis 2: 7.471E-06 and sample type Bray-Curtis, stress 0.085, Axis 1: 0.9807, Axis 2: 0.0002. Note that species play an important role in structuring the bacterial community.

The SIMPER between-groups analysis revealed that the OTUs recovered in the comparisons between the different colonies/species, are essentially the same OTUs responsible for structuring these bacterial communities within significance groups (S4 Table). But we also observe that there are multiple strains of *Candidatus Blochmannia*, Enterobacteriaceae and *Wolbachia* present across these samples.

To examine the complicated associations between samples with shared significant OTUs, we used Cytoscape to construct a network graph in which each node represented a host sample. Network analyzes were performed using the spring-embedded edge-weighted algorithm (Fig 4), which approaches the samples according to the number of OTUs shared, and we colored the edges according to the different colonies/species (Fig 4A), and in the different stages of development (Fig 4B). OTUs with less than 100 reads were hidden for easy viewing. From this analysis we observe greater structuring between species than across different stages of development.

Fig 4. Network analysis of Camponotini samples with edges representing the main community bacterial members. **A.** The edges were colored according to the different colonies: MOR#59 – *Camponotus floridanus* in red, MOR#69 – *Ca. planatus* in orange, MOR#73 – *Ca. planatus* in green, MOR#62 – *Colobopsis riehlii* in blue. **B.** The edges were colored according to the different stages of development: egg in red, L1 in light green, L2 in green, P1 in blue, P2 in light blue, P3 in aquamarine, W1 in pink, W2 in light pink, queen in yellow and male in brown. Note that it is the same image as in A, but now colored according to the different stages of development.

Bacterial communities of *Camponotus (Ca. floridanus and Ca. planatus)* and *Colobopsis riehlii* are different

Our statistical results confirm that the bacterial community of *Camponotus* (*Ca. floridanus and Ca. planatus*) and *Colobopsis riehlii* are different (Table 3). This can clearly be seen in Figs 2 and 3. This result shows that even in closely related genera, the microbial communities are different, at least for the colonies/species analyzed in this study.

Table 3. Bacterial communities of *Camponotus* (*Ca. floridanus and Ca. planatus*) and *Colobopsis riehlii* are different.

	Camponotus (Ca. floridanus and Ca. planatus)* vs. *Colobopsis riehlii	
	Unweight	**Weight**
Adonis	R^2= 0.11235 and *p*= 0.001	R^2= 0.6525 and *p*= 0.001
Anosim	R^2= 0.1058 and *p*= 0.030	R^2= 0.8546 and *p*= 0.001

In the colonies/species analyzed in this study, the microbial communities are different.

Camponotus planatus from distinct colonies have similar bacterial communities

Of all the colonies analyzed in the present study, the two *Camponotus planatus* colonies (MOR#69 and MOR#73), have the highest similarity, as observed from the statistical

tests that resulted significant differences (Table 4), but a small difference if we compare with the other colonies. This result corroborates S3 Table.

Table 4. *Camponotus planatus* from distinct colonies have similar bacterial communities.

	MOR#69 vs.MOR#73	
	Unweight	**Weight**
Adonis	R^2= 0.0441 and *p*= 0.046	R^2= 0.056 and *p*= 0.046
Anosim	R^2= 0.054 and *p*= 0.085	R^2= -0.050 and *p*= 0.971

The *Camponotus planatus* colonies have the highest similarity if compared with others colonies from this study.

There are microbiota differences in the stage of development between host species

Statistical analyzes show that there are significant differences in the development stage across two of the species, *Camponotus planatus* (MOR#69 and MOR#73) and *Colobopsis riehlii* (MOR#62). This pattern could also be true for *Camponotus floridanus,* but unfortunately after rarefaction only a few individuals from this colony (MOR#59) could be included and therefore we were not able to conduct beta diversity analyses on this species. As the main bacteria across all of these colonies are *Blochmannia* and Enterobacteriaceae (for *Camponotus: Ca. floridanus* and *Ca. planatus,* and *Colobopsis riehlii* respectively; S4 Table), the abundance of OTU (weighted) may not be appropriate to test for significant differences across the developmental stages within each colony. Therefore the results of unweighted distances were presented on Table 5, and there are significant differences in the development stage across two of the species.

Table 5. There are microbiota differences in the stage of development between host species

	Unweight
***Camponotus planatus*: MOR#69**	
Anosim	R^2= 0.222 and *p*= 0.039
***Camponotus planatus*: MOR#73**	
Adonis	R^2= 0.451 and *p*= 0.032
Anosim	R^2= 0.1838 and *p*= 0.050
***Colobopsis riehlii*: MOR#62**	

Anosim	R^2= 0.217 and *p*= 0.042

Note that within each colony analyzed separately there is a difference between the stages of development.

The same stage of development in different *Camponotus* species have similar bacteria

To address this question we binned our *Camponotus* samples (*Ca. floridanus* and *Ca. planatus*) into the following groups: larva (L1 and L2), pupae (P1, P2 and P3), workers (W1 and W2) and finally a mixed group with queens, males and eggs (all directly derived from the queen). The results show that there were no significant differences when we analyzed each of these groups, (Table 6), which reveals that there is similarity in each of these stages of development, even when they were grouped from different colonies (See S1 Fig).

Table 6. The same stage of development in different *Camponotus* colonies have similar bacteria

	Larva	
	Unweight	**Weight**
Adonis	R^2= 0.11564 and *p*= 0.189	R^2= 0.07935 and *p*= 0.544
Anosim	R^2= -0.833 and *p*= 0.616	R^2= 0.0026 and *p*= 0.48599
	Pupae	
	Unweight	**Weight**
Adonis	R^2= 0.32723 and *p*= 0.176	R2= 0.29675 and *p*= 0.290
Anosim	R^2= 0.1230 and *p*= 0.238	R2= 0.0846 and *p*= 0.270
	Worker	
	Unweight	**Weight**
Adonis	R^2= 0.06758 and *p*= 0.622	R^2= 0.07672 and *p*= 0.639
Anosim	R^2= -0.0611 and *p*= 0.7219	R^2= -0.040 and *p*= 0.675
	Males, Queens and Eggs	
	Unweight	**Weight**
Adonis	R^2= 0.11767 and *p*= 0.928	R^2= 0.16173 and *p*= 0.359
Anosim	R^2= -0.1019 and *p*= 0.821	R^2= 0.02450 and *p*= 0.329

Note that there were no significant differences when we binned the same stage of development.

Bacteria responsible for differences between colonies/species and development stages

Through the results of the G test (P, FDR_P and Bonferroni_P), we found bacterial community presence/absence is significantly different across sample type and

colonies/species (S5 Table). Between colonies/species more OTUs were significantly different across samples than the other sample type category (different stages of development within a colony). However, the bacteria Enterobacteriaceae (multiple strains, including *Candidatus Blochmannia*), *Wolbachia* (multiple strains) and Pasteurellales were present across all categories (S5 Table). Separate G-test analyses of the different developmental stages within each *Colobopsis riehlii* and *Camponotus* colony *(Ca. floridanus* and *Ca. planatus)* recovered the different OTUs, except for Enterobacteriaceae (S5 Table).

According to our results of measures of Alpha diversity (Chao1, PD whole tree, observed OTUs, Simpson and Shannon), we found that the samples of *Camponotus floridanus, Ca. planatus* and *Colobopsis riehlii* are not very diverse, showing few different OTUs. Likely due to this low diversity, we did not obtain significant results when comparing alpha diversities based on a two-sample t-test using non-parametric (Monte Carlo) methods. Through the rarefaction curve analysis of OTUs observed, our sequencing coverage of the bacterial communities appears satisfactory for most samples. However for some samples, it was not possible to reach a plateau (S2 Fig).

For easy viewing on our HeatMap, we are presenting only OTUs with more than 100 reads. We grouped the samples according to the quantity and type of associated bacteria. Our results revealed that there are some OTUs specific to a particular colony, such as OTU AJ245591.1.1215 - *Candidatus Blochmannia* was restricted to the *Ca. floridanus* colony MOR#59. The *Colobopsis* colony was also distinct from the others, having specific OTUs, such as OTU EU348326.1.1455-Pasteurellales, KF249887.1.1350–*Wolbachia* and CP010049.668121.669704-Enterobacteriaceae.

For the colonies of *Ca. planatus* (MOR#69 and MOR#73) we also observed several samples from different development stage with two distinct strains of

Candidatus Blochmannia: AF495758.1.1401, and the new strain New.ReferenceOTU1, suggesting possible multiple infection by this endosymbiont. For *Wolbachia* we found one strain with high abundance, mainly in *Colobopsis* (KF249887.1.1350), and we observed an infection rate of 94.73% from across our *Colobopsis* colony (n = 19). The OTU GAUE02014372.1.1238 - *Wolbachia* was found only in males and queens of *Camponotus* present in colony MOR#69 (*Ca. planatus*). Lastly the CP010049.668121.669704-Enterobacteriaceae strain was recovered from larva (L2) of *Camponotus planatus*, colony MOR#69 (Fig 5). In less quantity the strain New.ReferenceOTU71 - *Wolbachia* (difficult visualization in Fig 5) was found in both colony MOR#69 and colony MOR#62, at different stages of development.

Fig 5. The colours in the heatmap indicate variation in the relative abundance of different bacteria in different colonies/species and sample type of Camponotini samples. These range from 0% (light yellow) to 100% (red). We choose to show only OTUs with more than 100 reads, for easy viewing. Dendrograms were generated from Bray–Curtis distance matrices. Note there are OTUs restricted to specific colonies/species.

Phylogenetic Tree: *Blochmannia* and Enterobacteriaceae OTUs are related.

The inferred maximum likelihood phylogeny received high boostrap support across the major nodes placing our samples with their closest relatives. All the sequences of *Blochmannia* are grouped in a single clade with high bootstrap support (99%). In addition, the OTUs identified as Enterobacteriaceae in the present study are closely related to *Blochmannia*, corroborating the hypothesis that all Enterobacteriaceae are actually *Blochmannia* (Fig 6).

Fig 6. Phylogenetic tree of the main OTUs, their closest relatives, and *Blochmannia*

from Camponotini genera sequences available in GenBank. The maximum likelihood phylogeny of the 16S rRNA region of the main bacterial symbionts of this study along with the closests matches on GenBank. Bootstrap support is shown on branches. The labels are given with GenBank accession number (GenBank sequences) or collection code (sequences generated in the present study - colored in red). The branch color refers to bacteria with *Wolbachia* in brown and *Blochmannia* in green.

Discussion

In Camponotini ants the presence of bacteria such as *Blochmannia*, considered a primary endosymbiont, and *Wolbachia*, as secondary, is already well known [21,22,37,67], but the diversity of the entire bacterial community has not been fully documented and differences across developmental stage have not been adequately explored. Although our study included a modest number of colonies (85 individual samples from four colonies) our results are the first to characterize the bacterial community associated with a colony of the recently recognized genus *Colobopsis* (species: *Colobopsis riehlii*) and three colonies of *Camponotus* (two distinct species: *Ca. floridanus and Ca. planatus*) and show how different the composition of the bacterial community is when compared across the different colonies/species (different genera and different species - collected in the same location), and how they are conserved when comparing across the different stages of development within a colony.

In general, our data reveal that *Colobopsis riehlii* and *Camponotus (Ca. floridanus* and *Ca. planatus)* have distinct microbiota, although they are closely related ant genera. The OTUs from these two host genera are distinct. We were able to verify that the identity of the species contributes more to the bacterial diversity than the stage of development. A significant difference between species is likely due to the different bacterial communities between *Camponotus* and *Colobopsis* ant species. We also

demonstrated that there were no significant differences between colonies of the same species (*Camponotus planatus*), and between stages of development from different colonies, confirming that each stage of development may have a specific microbiota. Our results show different host species likely shape the bacterial community in Camponotini ants. Clear visual and statistical evidence also separates *Colobopsis riehlii* from the others *Camponotus* colonies, corroborating the studies by Blaimer et al. [31] and Ward et al. [32] in elevating *Colobopsis* as a separate genus from *Camponotus*.

In this study, bacterial community structure and composition in ants of the same colony were most similar to each other, a pattern recovered in other ant species [13,40,46,68]. This is likely because social insects live in densely populated colonies with highly related individuals [69], which may facilitate the sharing of the microbiota. In addition, it is often observed that Camponotini ants exhibits mouth-to-mouth (stomodeal) trophallaxis, i.e. the sharing of liquid nutrients through mutual feeding [70,71]. Nutrients stored in the crop or 'social stomach' are shared throughout the colony during trophallaxis [72], which is thought to be a primary means for horizontal bacterial transfer within a colony [26,73–75]. This intense interaction and exchange of microbiota may reinforce colony-specific signatures [40,76], and also appears to occur with Camponotini ants.

Blochmannia, a member of the Enterobacteriaceae, is known to provide important functions in Camponotini ants, which includes, *Camponotus*, *Colobopsis*, *Polyrhachis* and others, whose phylogenetic trees of symbionts are congruent with those of their hosts across long periods of evolutionary time, indicating the coevolution of host and symbiont [77,78]. In addition to its nutritional role [23], especially in early life [79], it has also maintained genes needed to contribute to the metabolism of nitrogen, sulfur and lipids [80–82].

The high mutational rate of *Blochmannia* [83] may influence and disrupt the identification of OTUs at the bacterial genus level for the short sequences generated by most amplicon methods, therefore Enterobacteriaceae or Other Enterobacteriaceae – may in fact be *Blochmannia.* For *Camponotus (Ca. floridanus* and *Ca. planatus)* we detected high *Candidatus Blochmannia* abundance. We also expected this bacterium in high abundance for *Colobopsis riehlii* but our results did not reveal this at first. Our phylogenetic analysis of the main OTUs found in our study grouped in the same clade as *Blochmannia* and Enterobacteriaceae with high statistical support. All the individuals of *Camponotus* (*Ca. floridanus and Ca. planatus*) and *Colobopsis riehlii* analyzed in the present study have some type of Enterobacteriaceae as the main bacterium in their microbiota and based on our phylogenetic analysis is likely *Blochmannia*. Our study also found 44 samples of *Camponotus planatus*, from two colonies (MOR#69 and MOR#73), with two strains of *Blochmannia* (double infection). This result corroborates Ramalho et al. [67] finding of double infections of *Blochmannia* in *Camponotus textor* Forel, an exclusively Neotropical species.

Wolbachia, a major invertebrate endosymbiont [16,80–82] famous for manipulating the reproduction of the host [87], was the second most common endosymbiont found in all *Colobopsis riehlii* samples, occurring in all individuals of the colony, across all stages of development. There are many studies associating this bacterium with Formicidae, but its function remains unclear. Interestingly OTU GAUE02014372.1.1238 of *Wolbachia* was found only in the reproductive castes (queen and males) in the colony MOR#69 of *Camponotus planatus* but was not found in the other stages of development across the colony. Another OTU of *Wolbachia* (New.ReferenceOTU71) although in a lower concentration was found in *Colobopsis riehlii* (MOR#62) and in *Camponotus planatus* (MOR#69). This low infection rate by

Wolbachia (1%) has also been found in another North American *Camponotus* [14], although is not true across the genus as *Ca. textor* was found to be highly associated with *Wolbachia* [67].

The next most common bacterium associated was Pasteurellales - EU348326.1.1455 found specifically in the larval stage (L2) in *Colobopsis riehlii* (2.20%). Pasteurellales are one of the major orders within the class Gammaproteobacteria [88,89]. This bacterium is often present in the respiratory, alimentary and reproductive tract of various birds and mammals, including humans [89,90]. This group of bacteria has been identified from another arboreal ant, *Pseudomyrmex ferrugineus* [91], but their function in ants is not clear.

Previous studies have reported the presence of other symbionts in *Camponotus*, including *Spiroplasma* which has been reported in other species of *Camponotus* [14], but was not found in our results. Acetobacteraceae was also recently found in *Camponotus*, and is believed to be strongly host specific [37]. We also recovered this bacterium (multiple strains) in 11 individuals of *Camponotus* (20.37%), but with few copies per individual, ranging from 1 - 4 reads.

Studies that try to understand the patterns that explain the association of microbiota and host inform us about the potential impacts and roles of these symbioses. In the present study we show that the *Colobopsis riehlii* microbiota is distinct from *Ca. floridanus* and *Ca. planatus*, a closely related genus. In general, the microbiota presented here appears as a species-specific signature, whereas most developmental stages do not have distinct microbiota. Although we present some differences across development, especially in the larval stage, the intense social interaction between individuals of a colony likely homogenizes the microbiota among colony members. Additional studies of the intra-colonial microbiome of other hosts and across

development may reveal additional clues about the function and importance of bacteria in colony recognition, individual and colony health, and nutritional upgrading.

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List of Figures

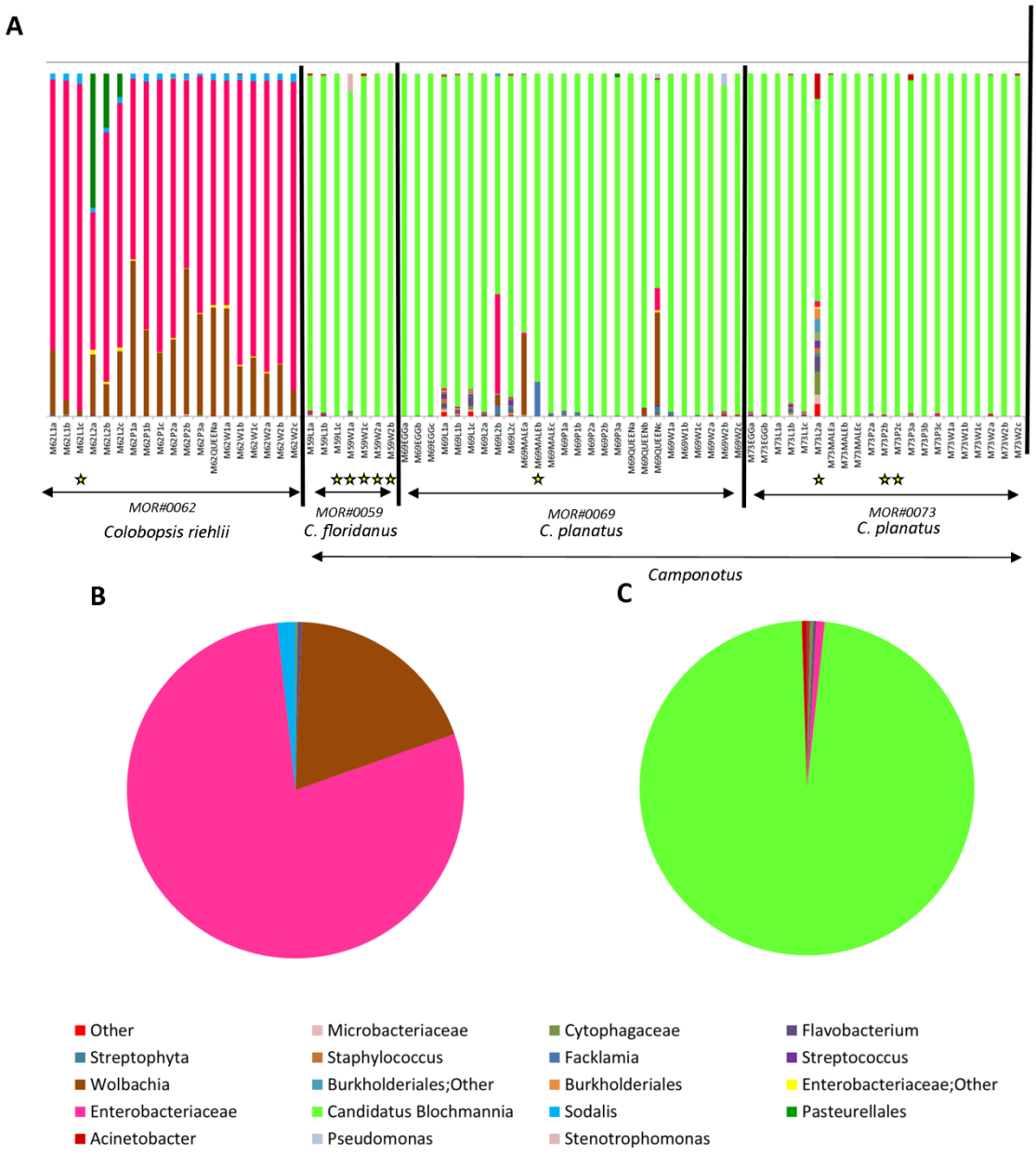


Fig 1. Summary graph of bacterial OTUs found in *Colobopsis riehlii*, *Camponotus floridanus* and *Camponotus planatus* colonies with 16S rRNA amplicon sequencing. **A.** Different colonies and species used in this study and their bacterial communities. **B.** Summary of all OTUs found in *Colobopsis riehlii*. The main bacterium is Enterobacteriaceae in pink, followed by *Wolbachia* in brown **C.** Summary of all OTUs found in *Ca. floridanus and Ca. planatus*. The main bacterium is *Candidatus Blochmannia* in green. The yellow stars highlight samples that were excluded after the read depth standardization of 400 reads was implemented.

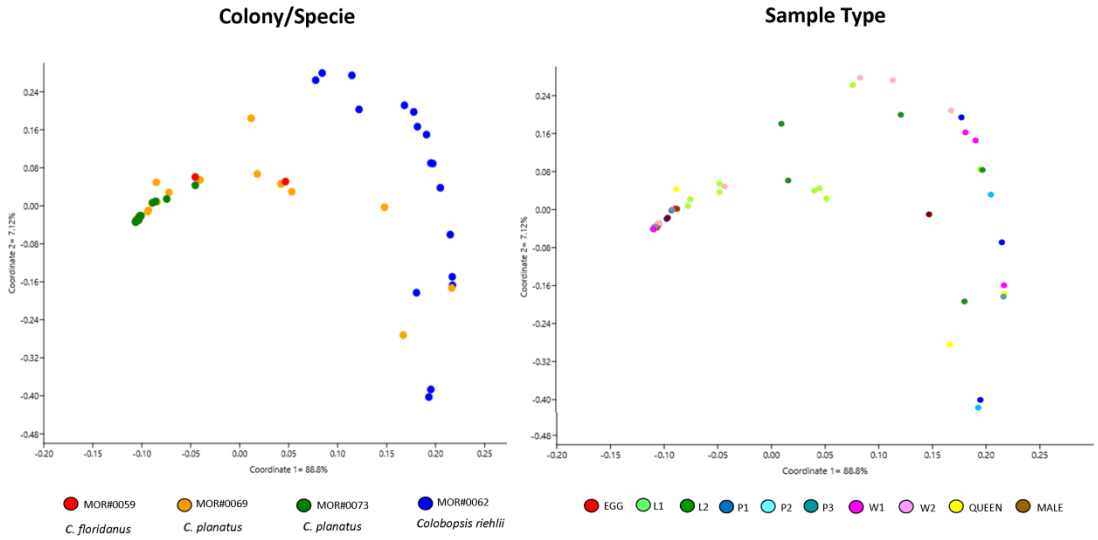


Fig 2. PCoA plots of bacterial communities associated with Camponotini samples (weighted UniFrac method). A. Different colonies/species (axis 1 = 88.8% and axis 2 = 7.12%) and **B.** developmental stage/sample type (axis 1 = 88.8% and axis 2 = 7.12%). Note that the "Colony/Species" category influences the bacterial community more than "Sample Type".

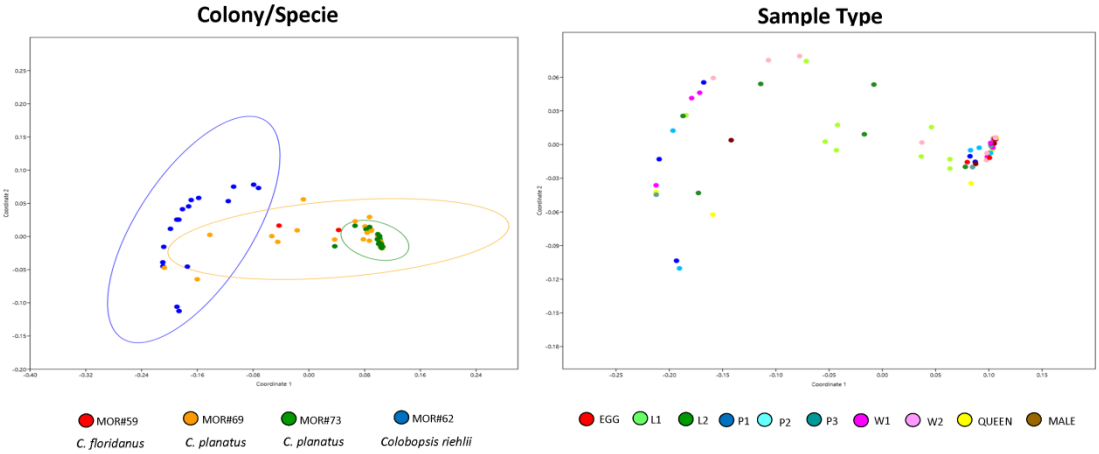


Fig 3. Nonmetric multidimensional scaling (NMDS) plot illustrating bacterial community structure among different colonies/species with 95% ellipses. Bray-Curtis, stress 0.081, Axis 1: 0.9817, Axis 2: 7.471E-06 and sample type Bray-Curtis, stress 0.085, Axis 1: 0.9807, Axis 2: 0.0002. Note that species play an important role in structuring the bacterial community.

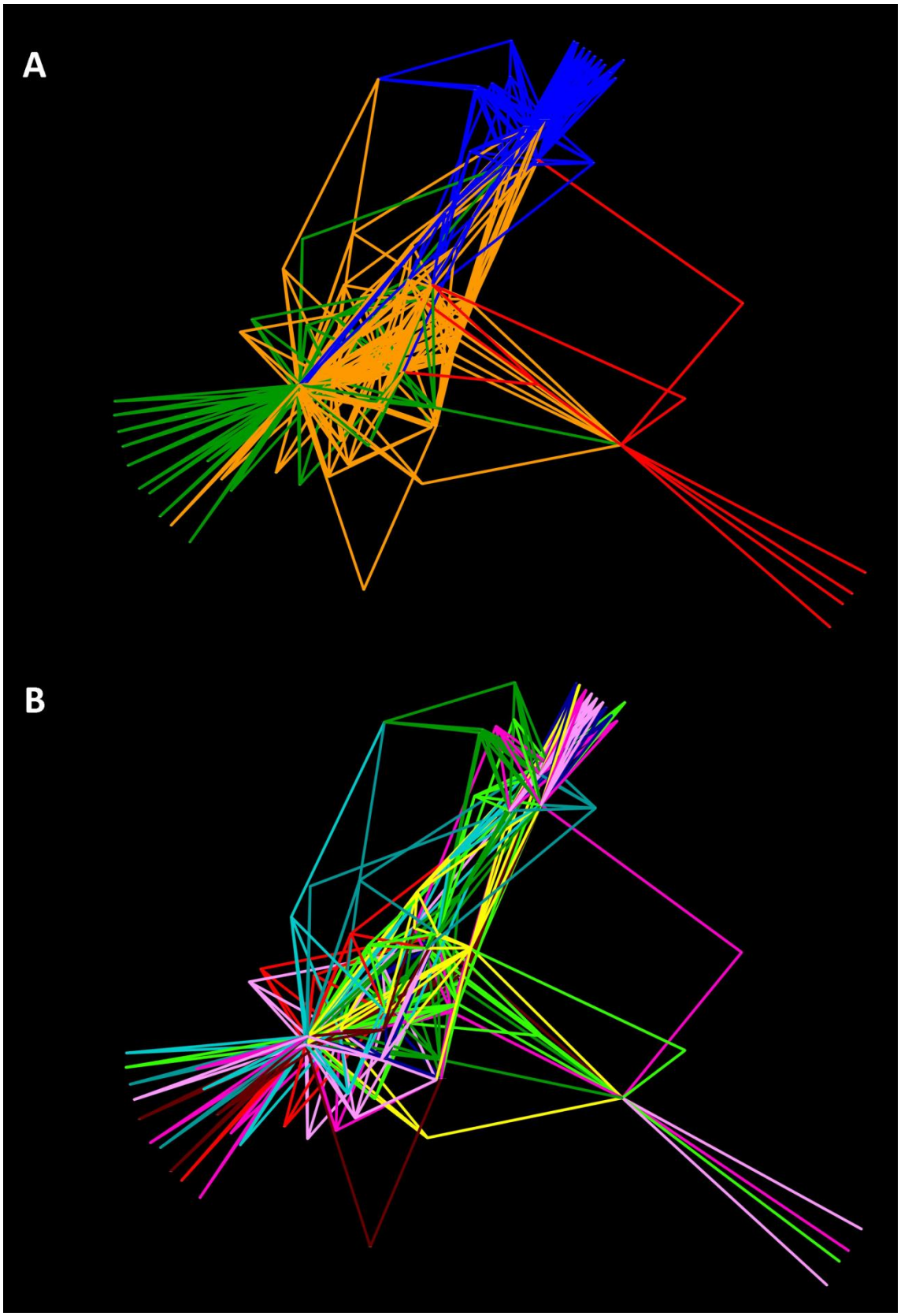


Fig 4. Network analysis of Camponotini samples with edges representing the main community bacterial members. **A.** The edges were colored according to the different colonies: MOR#59 – *Camponotus floridanus* in red, MOR#69 – *Ca. planatus* in orange, MOR#73 – *Ca. planatus* in green, MOR#62 – *Colobopsis riehlii* in blue. **B.** The edges

were colored according to the different stages of development: egg in red, L1 in light green, L2 in green, P1 in blue, P2 in light blue, P3 in aquamarine, W1 in pink, W2 in light pink, queen in yellow and male in brown. Note that it is the same image as in A, but now colored according to the different stages of development.

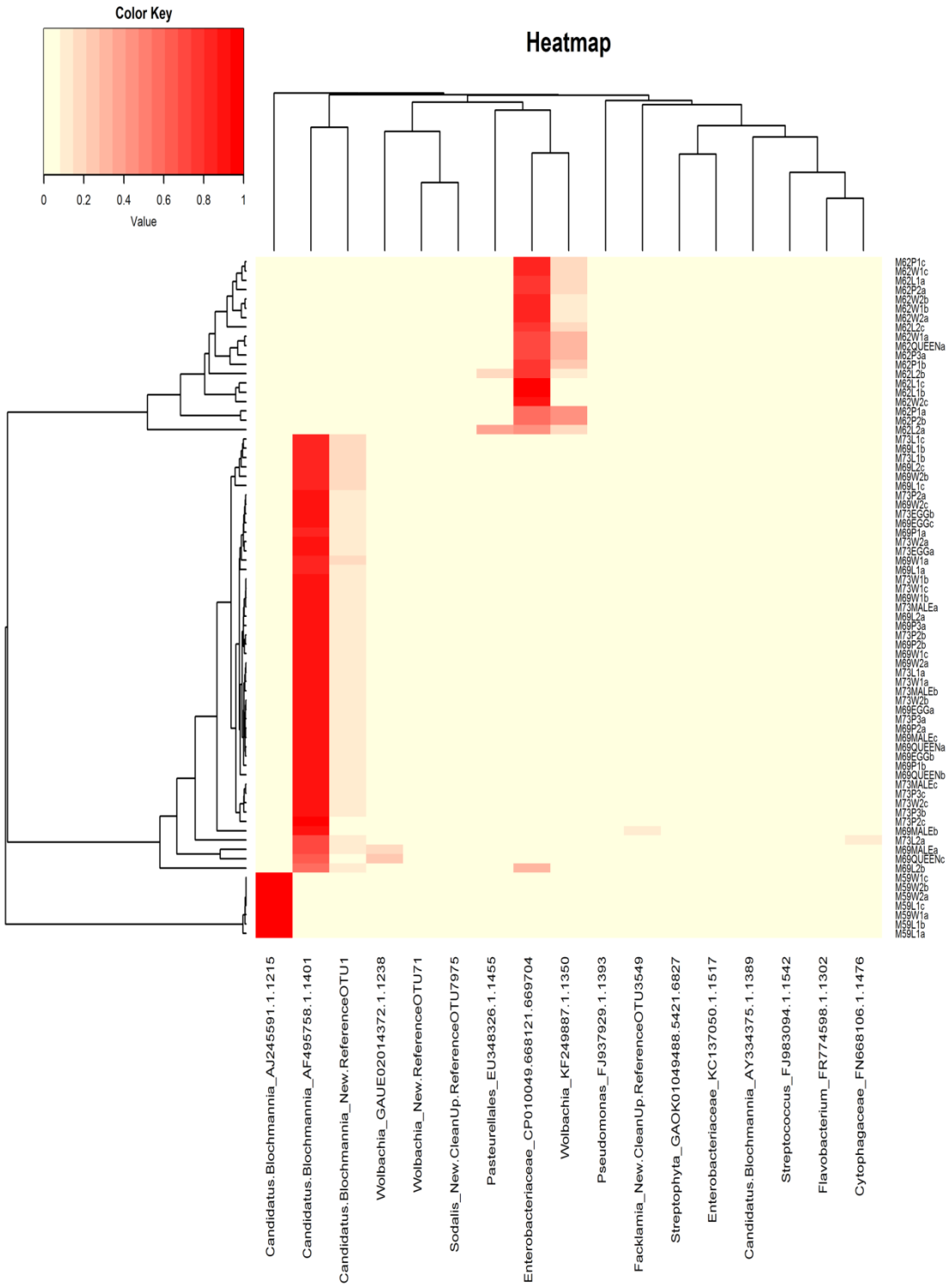


Fig 5. The colours in the heatmap indicate variation in the relative abundance of different bacteria in different colonies/species and sample type of Camponotini

samples. These range from 0% (light yellow) to 100% (red). We choose to show only OTUs with more than 100 reads, for easy viewing. Dendrograms were generated from Bray–Curtis distance matrices. Note there are OTUs restricted to specific colonies/species.

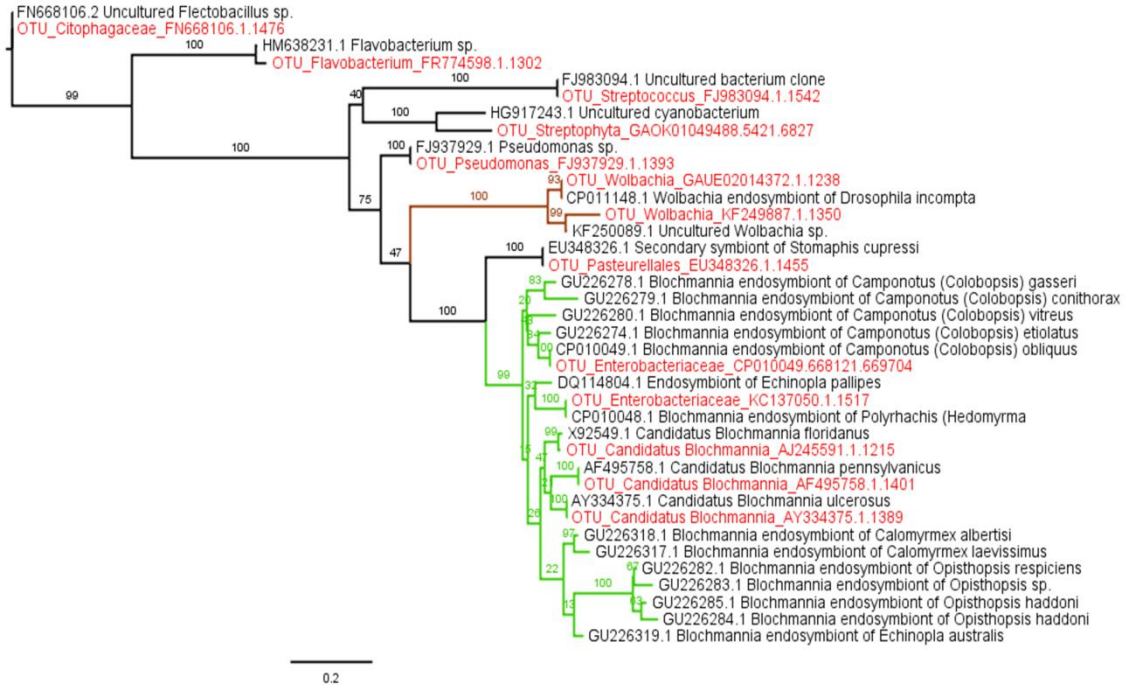
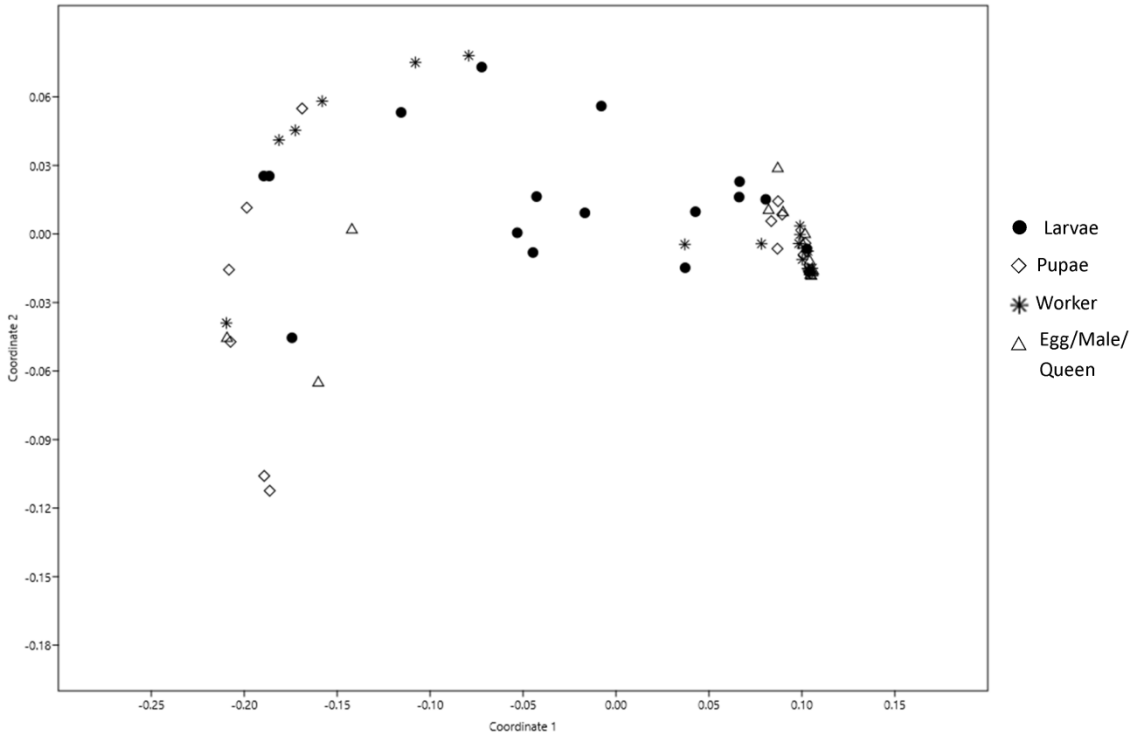
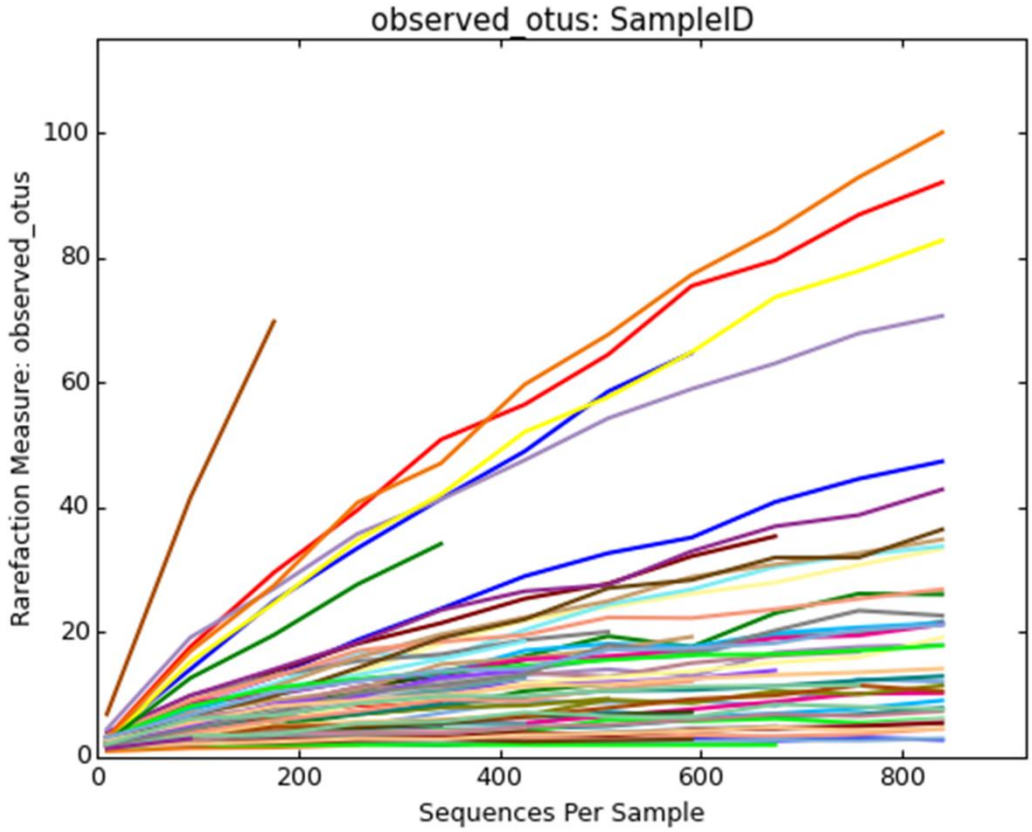


Fig 6. Phylogenetic tree of the main OTUs, their closest relatives, and *Blochmannia* from Camponotini genera sequences available in GenBank. The maximum likelihood phylogeny of the 16S rRNA region of the main bacterial symbionts of this study along with the closests matches on GenBank. Bootstrap support is shown on branches. The labels are given with GenBank accession number (GenBank sequences) or collection code (sequences generated in the present study - colored in red). The branch color refers to bacteria with *Wolbachia* in brown and *Blochmannia* in green.

Supporting information



S1 Fig. **Nonmetric multidimensional scaling (NMDS) plot illustrating bacterial community structure among different development stages**. Bray-Curtis, stress 0.029, Axis 1: 0.9683, Axis 2:0.0527.



S2 Fig. Rarefaction curves with OTUs by sequences per samples. Rarefaction curves analyzed across the different stages of development. The queen was more diverse than the others and when compared between the colonies of *Camponotus (Ca. floridanus* and *Ca. planatus)* and *Colobopsis riehlii.*

.

S3 Table. Analyses of similarity (ANOSIM). These results are evaluating variation in the composition and structure of bacterial communities (global effect), and the colonies/species and sample type that showed significant differences.

Between Colonies	**Sorensen Index**		**Bray-Curtis Index**	
Global effect	R^2= 0.03284	*p*= 0.1012	**R^2= 0.4617**	***p*= 0.0001**
MOR#73 vs. MOR#62	R^2= 0.077	*p*= 0.256	**R^2=0.9931**	***p*= 0.0006**
MOR#62 vs. MOR#69	R^2=-0.0252	*p*= 1	**R^2= 0.7261**	***p*= 0.0006**

Between Sample Type	**Sorensen Index**		**Bray-Curtis Index**	
Global effect	R^2= 0.0194	*p*= 0.241	R^2= 0.0434	*p*= 0.1587

S4 Table. SIMPER analysis reveals contribution of specific operational taxonomic units (OTUs). This test indicates the contribution of specific operational taxonomic units (OTUs) to the observed differences in community structure among different colonies/species of Camponotini.

	Overall Average Dissimilarity	Most Influential OTUs / Taxonomy	Percent Contribuition to Difference
MOR#*62* vs. MOR#*73*	**99.95**	CP010049.668121.669704 / Enterobacteriaceae	42.09
		AF495758.1.1401 / *Candidatus Blochmannia*	38.99
		KF249887.1.1350 / *Wolbachia*	10.75
		New.ReferenceOTU1 / *Candidatus Blochmannia*	4.708
		EU348326.1.1455 / Pasteurellales	1.558
		New.CleanUp.ReferenceOTU7975 / *Sodalis*	0.8577
		New.ReferenceOTU71 / *Wolbachia*	0.3812
		GAUE02014372.1.1238 / *Wolbachia*	0.3471
MOR#62 vs. MOR#69	**98.59**	AF495758.1.1401 / *Candidatus Blochmannia*	66.93
		CP010049.668121.669704 / Enterobacteriaceae	16.46
		New.ReferenceOTU1 / *Candidatus Blochmannia*	8.841
		KF249887.1.1350 / *Wolbachia*	4.137
		GAUE02014372.1.1238 / *Wolbachia*	1.705
		EU348326.1.1455 / Pasteurellales	0.5837
		New.CleanUp.ReferenceOTU7975 / *Sodalis*	0.3307
		New.ReferenceOTU71 / *Wolbachia*	0.1582
		FR774598.1.1302 / *Flavobacterium*	0.1303
		GAOK01049488.5421.6827 / Streptophyta	0.13
		New.CleanUp.ReferenceOTU3549 / *Facklamia*	0.1238
		FJ983094.1.1542 / *Streptococcus*	0.1143

S5 Table. Analysis of G test of independence (P, FDR_P and Bonferroni_P) across Camponotini samples. Hence, it determines whether OTU presence/absence is associated with different colonies and sample type.

G-test	Sample Type Test-			Bonferroni	
OTU	Statistic	P	FDR_P	_P	taxonomy
GAUE0201437	108.080				k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales;
2.1.1238	21043	0.0	0.0	0.0	f__Rickettsiaceae; g__Wolbachia; s__
CP010049.668	462.673				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
121.669704	293633	0.0	0.0	0.0	f__Enterobacteriaceae; g__; s__
AJ245591.1.12	313.888				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
15	878494	0.0	0.0	0.0	f__Enterobacteriaceae; g__Candidatus Blochmannia; s__
KF249887.1.1	161.420				k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales;
350	533873	0.0	0.0	0.0	f__Rickettsiaceae; g__Wolbachia; s__
EU348326.1.1	153.918				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria;
455	834949	0.0	0.0	0.0	o__Pasteurellales; f__; g__; s__
AF495758.1.1	209.205				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
401	244929	0.0	0.0	0.0	f__Enterobacteriaceae; g__Candidatus Blochmannia; s__

G-test	Colony Test-			Bonferroni	
OTU	Statistic	P	FDR_P	_P	taxonomy
CP010049.668	775.208				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
121.669704	425876	0.0	0.0	0.0	f__Enterobacteriaceae; g__; s__
AJ245591.1.12	972.952				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
15	399294	0.0	0.0	0.0	f__Enterobacteriaceae; g__Candidatus Blochmannia; s__
KF249887.1.1	185.346				k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales;
350	602067	0.0	0.0	0.0	f__Rickettsiaceae; g__Wolbachia; s__
AF495758.1.1	937.084				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
401	624465	0.0	0.0	0.0	f__Enterobacteriaceae; g__Candidatus Blochmannia; s__
New.Referenc	115.929				k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;
eOTU1	152324	0.0	0.0	0.0	f__Enterobacteriaceae; g__Candidatus Blochmannia; s__

EU348326.1.1455	30.0301280157	1.3600645 9484e-06	9.5431199 0709e-05	0.0005725 87194426	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pasteurellales; f__; g__; s__

G-test ***Camponotus*** - Sample Type					
OTU	Test-Statistic	P	FDR_P	Bonferroni_P	taxonomy
GAUE0201437 2.1.1238	146.361 125266	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Rickettsiaceae; g__Wolbachia; s__
CP010049.668 121.669704	158.339 976979	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__; s__
AJ245591.1.12 15	402.599 85135	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus Blochmannia; s__
AF495758.1.1 401	47.7322 497722	2.8657959 8718e-07	2.5577229 1856e-05	0.0001023 08916742	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus Blochmannia; s__

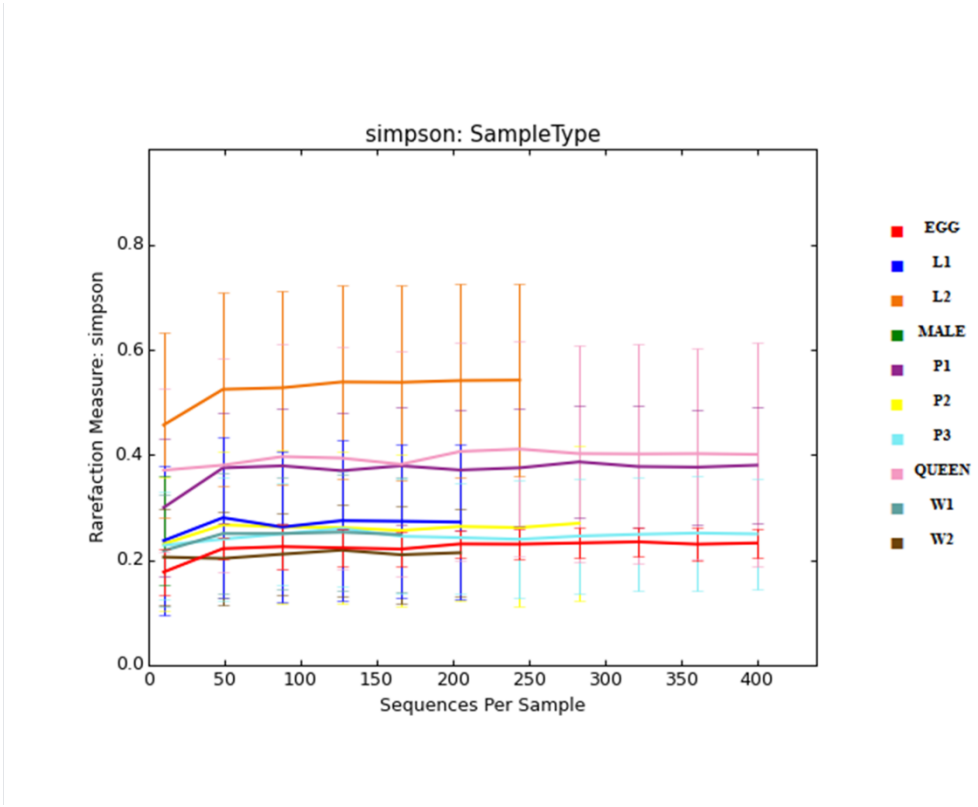
G-test ***Colobopsis*** - Sample Type					
OTU	Test-Statistic	P	FDR_P	Bonferroni_P	taxonomy
KF249887.1.1 350	107.335 400841	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Rickettsiaceae; g__Wolbachia; s__
EU348326.1.1 455	305.800 226718	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Pasteurellales; f__; g__; s__
CP010049.668 121.669704	31.1714 088255	5.7807014 8608e-05	0.0014837 1338143	0.0044511 4014428	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__; s__

S6 Table. Bacterial Quantification through 16S rRNA gene (qPCR) of all samples. Each sample was analyzed in triplicate therefore follows the values of average and standard deviation for each sample.

Samples	Average	Standard deviation	Samples	Average	Standard deviation
M59L1A	84960,1671	73905,29381	M69MALEC	194983,5504	46236,24462
M59L1B	14020,40779	12485,39882	M69P1A	92211,80741	18291,24585
M59L1C	208047,4363	184857,9127	M69P1B	87700,0441	3456,19783
M59L2A	0	0	M69P2A	1587078,908	517487,9081
M59L2B	0	0	M69P2B	841967,1999	408850,2758
M59L2C	0	0	M69P2C	532437,1322	67020,5031
M59P1A	0	0	M69P3A	345587,9462	33748,05656
M59P1B	0	0	M69QUEENA	3410470,875	967014,3097
M59P1C	0	0	M69QUEENB	2556235,658	2223688,328
M59P2A	0	0	M69QUEENC	1854596,072	1595752,798
M59P2B	0	0	M69W1A	288472,5921	63969,20214
M59P2C	0	0	M69W1B	1542197,209	354161,6722
M59W1A	29052,72225	25257,52396	M69W1C	619959,7671	83359,74321
M59W1B	5493,844557	2720,619273	M69W2A	1164147,413	193366,0074
M59W1C	242771,4779	203984,7457	M69W2B	284577,3033	53088,51398
M59W2A	262940,3512	235740,1746	M69W2C	1011528,826	312862,9247
M59W2B	11006,42582	9475,883715	M73EGGA	36511,9523	12220,92324
M59W2C	77,805295822	0	M73EGGB	120946,6279	22555,99727
M62L1A	23108,8176	18011,91932	M73L1A	85865,86329	22344,69687
M62L1B	39702,00133	7825,588274	M73L1B	14082,92447	3889,136516
M62L1C	36499,69133	9193,022231	M73L1C	10805,00187	3403,751297
M62L2A	228541,1743	43064,44659	M73L2A	542,7653656	233,6497994
M62L2B	339043,5672	107237,1432	M73MALEA	1137341,973	320060,0544
M62L2C	108270,0302	21292,74633	M73MALEB	1180361,408	33983,76507
M62P1A	101750,0552	16377,74691	M73MALEC	147195,5051	57568,47862
M62P1B	115306,6351	65320,8167	M73P2A	515367,0479	93304,19102
M62P1C	275033,717	71072,40606	M73P2B	832573,204	45127,41781

	5			2	
M62P2A	293219,2578	199087,3706	M73P2C	371595,9773	86960,08781
M62P2B	31389,69755	13624,0977	M73P3A	74559,63048	28432,81776
M62P3A	70466,16795	25200,63548	M73P3B	514418,2626	29117,21772
M62QUEENA	605937,1489	61260,27439	M73P3C	264094,8086	80133,00871
M62W1A	160151,3408	109115,3676	M73W1A	284804,0451	211381,5586
M62W1B	287938,9511	134254,4236	M73W1B	768578,1412	28727,82801
M62W1C	183831,3032	45684,17255	M73W1C	851757,7967	217102,1631
M62W2A	1166475,53	42797,97375	M73W2A	237374,0462	82119,74772
M62W2B	1097470,381	102373,3927	M73W2B	181597,8701	119521,0938
M62W2C	1783547,596	260353,7077	M73W2C	608839,5842	162445,8138
M69EGGA	913730,6198	390542,006			
M69EGGB	430555,0053	170114,7769			
M69EGGC	259559,0949	117418,1248			
M69L1A	7957,940434	1656,703284			
M69L1B	17947,49623	3926,895806			
M69L1C	4153,912358	1721,693295			
M69L2A	85990,54024	42243,24688			
M69L2B	23248,45649	7658,234897			
M69L2C	6195,768574	2290,664446			
M69MALEA	702919,0519	271116,9398			
M69MALEB	201872,6109	88124,70484			

S7 Fig. Simpsons Index by sample type. Through this image it is possible to visualize that the L2 larvae have a greater diversity in comparison with the other stages of development.



S8 Table. Bacteria found in Camponotini samples. Bacteria and the quantities identified in Camponotini samples in the present study.

	Total
Unassigned;Other;Other;Other;Other;Other	0.2%
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__	0.1%
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__	0.1%
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Flavobacterium	0.2%
k__Bacteria;p__Cyanobacteria;c__Chloroplast;o__Streptophyta;f__;g__	0.1%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus	0.1%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Aerococcaceae;g__Facklamia	0.2%
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus	0.1%
k__Bacteria;p__OD1;c__ZB2;o__;f__;g__	0.1%
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__Rickettsiaceae;g__Wolbachia	6%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;Other	0.1%
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__	0.1%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;Other	0.1%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__	19.8%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Candidatus Blochmannia	69.5%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Enterobacteriales;f__Enterobacteriaceae;g__Sodalis	0.5%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__;g__	0.9%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Moraxellaceae;g__Acinetobacter	0.2%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas	0.1%
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Xanthomonadaceae;g__Stenotrophomonas	0.1%

S9 Table. Samples excluded after the depth of 400 reads. These 10 samples from different colonies did not reach our cutoff of 400 reads and they were excluded from the subsequent analyses.

Counts/sample detail:
M69MALEb: 10.0
M59W1c: 172.0
M62L1c: 206.0
M59W2b: 231.0
M73L2a: 252.0
M59L1c: 272.0
M59W2a: 292.0
M73P2c: 317.0
M73P2b: 365.0
M59W1a: 391.0

Capítulo 3

The potential role of environment in structuring the microbiota of *Camponotus* across parts of the body

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Resumo

Vários estudos têm tentado entender o que pode influenciar a comunidade bacteriana do hospedeiro, mas estudos que examinam se diferentes espécies de bactérias são encontradas em diferentes partes do corpo de insetos são limitados e várias questões permanecem sem resposta. Nas formigas, o gênero *Camponotus*, considerado hiper-diverso, é comumente estudado por suas interações simbióticas. No presente estudo abordamos as seguintes questões: 1) Como as comunidades bacterianas estão distribuídas em diferentes partes do corpo (cabeça, mesossoma e gáster) de *Camponotus* e 2) A diversidade encontrada é explicada pelo ambiente em que essas formigas foram coletadas? Nossos resultados foram capazes de diferenciar as comunidades bacterianas presentes nas diferentes partes do corpo e isso pode ser explicado da seguinte maneira: cada parte do corpo tem órgãos únicos com funções diferentes e provavelmente um pH diferente; e o proventriculum complexo de *Camponotus* pode estar agindo como um filtro e estruturação da comunidade bacteriana encontrada no gáster. Além disso, um resultado inesperado do presente estudo foi a alta diversidade encontrada associada à cabeça e mesosoma, e nossos resultados foram capazes de confirmar que essa diversidade está associada com o ambiente onde as formigas foram coletadas. Saber mais sobre os fatores que podem influenciar comunidades bacterianas pode revelar mais sobre a importância dessas associações na natureza.

Palavras-chave: Camponotini, *Blochmannia*, *Wolbachia*, *Sodalis*,

Abstract

Several studies have attempted to understand what may influence the bacterial community of the host, but studies examining whether different bacterial species are found in different parts of the body of insects are limited and several questions remain unaddressed. In ants, the genus *Camponotus*, considered hyper-diverse, is commonly studied for its symbiotic interactions. In the present study we address the following questions: 1) How are bacterial communities distributed across different parts of the body (head, mesosoma and gaster) of *Camponotus* and 2) Is the diversity found explained by the environment in which these ants were collected? Our results were able to differentiate the bacterial communities present in the different parts of the body and can be explained in the following way: each part of the body has unique organs with different functions and likely a different pH; and the complex proventriculum of *Camponotus* may be acting as a filtering and structuring the bacterial community found in the gaster. In addition, an unexpected finding of the present study was the high diversity found associated with the head and mesosoma, and our findings were able to confirm that this diversity is associated with the environment where the ants were collected. Knowing more about the factors that can influence bacterial communities may reveal more about the importance of these associations in nature.

Key-words: Camponotini, *Blochmannia, Wolbachia, Sodalis,*

Background

Symbiotic microbes can influence the host and provide direct benefits through nutrition, defense, or even environmental tolerance (CHARLAT; HURST; MERÇOT, 2003; ENGELSTÄDTER; HURST, 2009; FELDHAAR, 2011; OLIVER et al., 2010; STOUTHAMER; BREEUWER; HURST, 1999; WERREN, 1997). Little is known about the factors that may affect or drive bacterial community membership (LEY et al., 2008; RAMALHO; BUENO; MOREAU, 2017; SANDERS et al., 2014), although several studies have attempted to tease this apart including its relation to the geography and phylogeny of the host (LEY et al., 2008; LINNENBRINK et al., 2013; RAMALHO; BUENO; MOREAU, 2017; SANDERS et al., 2014). In addition, few studies have investigated the bacterial community within a colony comparing different stages of development (ontogeny) (RAMALHO; BUENO; MOREAU, [s.d.]; RUBIN et al., 2014; SARAITHONG et al., 2017) and examined whether different bacterial species are found in different parts of the body of insects (KAUTZ et al., 2013; LANAN et al., 2015).

There are several ways of acquiring microbes, and clearly the path of acquisition is a determining factor in the structure and composition of the bacterial community, and consequently, can influence host biology. These include: 1) environmental acquisition, 2) social transmission, or 3) specialized maternal transmission (BROWN; WERNEGREEN, 2016). Acquiring microbes from the environment, also called horizontal transfer or secondary interaction, is usually facultative. These bacteria have part or all of their life cycle outside the host and can be transient in the host compared to those vertically transmitted by the mother (SALEM et al., 2015). Socially transmitted microbiota may represent the transition between free living and inherited bacteria, a factor that may be common among social insects such as ants. Specialized associations often characterized this third primary interaction where the phylogenetic trees of the symbionts are often congruent with their hosts across long periods in evolutionary time. This suggests high levels of host fidelity. Lastly for microbes that have specialized maternal transmission, the symbiont may become localized in a specialized organ inside the host (BAUMANN, 2005; MUNSON et al., 1991).

With a worldwide distribution, and commonly known as carpenter ants, *Camponotus* Mayr, 1861 is well known genera for having symbiotic bacteria localized

in specialized organs (FELDHAAR et al., 2007; RAMALHO et al., 2017; SAMESHIMA et al., 1999; WERNEGREEN et al., 2009). It is considered a hyper-diverse genus and has generalized feeding and nesting habits. Their diet is derived from the exudate of plants and phytophagous insects and can include scavenged prey (BLÜTHGEN; GEBAUER; FIEDLER, 2003; COOK; DAVIDSON, 2006; DAVIDSON et al., 2003). Another striking feature is the absence of the metapleural gland in some species of *Camponotus.* Production of antimicrobials, chemical defense, odor recognition and territorial marking are some of the possible functions of this gland (YEK; MUELLER, 2011).

Recently Brown and Wernergreen (BROWN; WERNEGREEN, 2016) evaluated the gut microbiota of *Camponotus chromaiodes* Bolton, 1995, and they found that 95 - 98% of the reads were dominated by the bacteria *Blochmannia* and *Wolbachia*. Even within *Camponotus textor* Forel 1899, a Neotropical species, these infections seem dominate in the genus (RAMALHO et al., 2017). Another study involving colonies of *Camponotus planatus* Roger, 1863 and *Camponotus floridanus* (Buckley, 1866) also reported high infection by these bacteria in addition to other bacteria in smaller quantities, but observed variation across the different stages of colony development (RAMALHO; BUENO; MOREAU, [s.d.]). These studies corroborate that these bacteria are highly associated with this genus, but these studies have included entire workers or only the digestive tract.

The present study intends to characterize the microbiota of different *Camponotus* species and to answer the following questions: 1) How are bacterial communities distributed across different parts of the body (head, mesosoma and gaster)? 2) Is the diversity found explained by the environment in which these ants were collected? Addressing these questions will advance our knowledge of the natural variation of insect-associated microbiota and may reveal important aspects of host biology that contribute to these associations.

Methods

Sample collection and determination of the different stages of development

The 58 specimens used in this study were collected in several locations from South and North America (Additional File 1). The samples were preserved in 95%

ethanol and stored at -20 °C before DNA extraction. The head, mesosoma and gaster were dissected and included separately totaling 174 samples (58 heads, 58 mesossoma and 58 gaster) (Additional File 2). The taxonomic identifications for the USA ants follow keys to species in the southeastern US (available from: http://mississippientomologicalmuseum.org.msstate.edu//Researchtaxapages/Formicidaepages/Identification.Keys.htm#.WE7qIH31-3H - from Creighton 1950, Snelling 1988; Mark Deyrup, pers. comm.; William MacKay's *Camponotus* website). Ants from South America were identified to the genus following Baccaro et al (2015) and by using the collections at the University of São Paulo (USP) Zoology Museum. All vouchers were deposited in the collection of the USP Zoology Museum in São Paulo, Brazil.

DNA Extraction and Bacterial DNA Sequencing

Total DNA was extracted from the head, mesosoma and gaster separately with Qiagen DNeasy Tissue kit following the manufacturer's recommendations with slight modifications following MOREAU (2014) and we did not use the modification of the Quigen DNeasy kit for gram-positive bacteria. We amplified the bacterial V4 region of 16S rRNA through primers described in Caporaso et al. (CAPORASO et al., 2012), following the Earth Microbiome Project protocol (515f primer and 806r; http://www.earthmicrobiome.org/emp-standard-protocols/16s/). PCR was performed in triplicate, each 25 µl PCR reaction contained 12 µl of MO BIO PCR Water (Certified DNA-free), 10 µl of 5 Prime HotMasterMix (1x), 1 µl of forward primer (5 mM concentration, 200 final pM), 1 µl Golay barcode tagged reverse primer (5 mM concentration, 200 pM final) and 1 µL of template DNA, under the following conditions 94˚C for 3 with 35 cycles at 94˚C for 45 s, 50˚C is 60 s, and 72˚C for 90 s, with a final of 10 min at 72˚C. After, the triplicate reactions were combined.

The samples were quantified via qPCR and Qubit (Thermo Fisher Scientific), and only then pooled with different samples after controlling for volume. For purification, only 100 µL of each pool was cleaned using the UltraClean PCR Clean-Up Kit (MO BIO), following the manufacturer's recommendations. After quantification, the molarity of the pool was determined and diluted down to 2 nM, denatured, and then diluted to a final concentration of 6.1 pM with a 10% PhiX for sequencing on the Illumina MiSeq. A 151 bp x 12 bp x 151 bp MiSeq run was performed using the custom sequencing primers and procedures described in the supplementary methods in

Caporaso et al. (CAPORASO et al., 2012) on the Illuminia MiSeq at the Field Museum of Natural History, Chicago, IL, USA. All raw sequence data is available publicly in Figshare [https://figshare.com/s/290531bea3dee984444e] and NCBI SRA accession number SRR5136256 and study SRP095836.

Bacterial Quantification

We measured the quantity of bacterial DNA present with quantitative PCR of the bacterial 16S rRNA gene using 515f (5' - GTGCCAGCMG CCGCGGTAA) and 806r (5' - GGACTACHVGGGTWT CTAAT) universal bacterial primers of the EMP (http://www.earthmicrobiome.org/emp-standard-protocols/16s/). All qPCRs were performed on a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using SsoAdvanced 2X SYBR green supermix (Bio-Rad) and 2 µL of DNA. Standard curves were created from serial dilutions of linearized plasmid containing inserts of the *E. coli* 16S rRNA gene and melt curves were used to confirm the absence of qPCR primer dimers. The resulting triplicate quantities were averaged before calculating the number of bacterial 16S rRNA gene copies per microliter of DNA solution (Additional File 2).

Bioinformatic Analysis

The sequences were analyzed in QIIME 1.9.1 (CAPORASO et al., 2010). The forward and reverse sequences were merged through SeqPrep. Demultiplexing was completed with the split_libraries_fastq.py command. QIIME defaults were used for quality filtering of raw Illumina data. For calling the OTUs, we chose the pick_open_reference_otus.py command against the SILVA 128 reference database at 97% identity (QUAST et al., 2013; YILMAZ et al., 2014) and UCLUST to create the OTU table. Sequences with less similarity were discarded. Chimera checking was performed in QIIME and PyNAST (v1.2.2) was used for sequence alignment (CAPORASO; BITTINGER; BUSHMAN, 2010). The summarize_taxa_through_plots.py command was used to create a folder containing taxonomy summary files. We joined head, mesosoma and gaster results to visualize the profile of the entire microbiome to be able to compare it with other studies that have examined whole workers.

We used Analysis of Similarity (ANOSIM) to test whether two or more predefined groups of samples are significantly different, and Adonis (MCARDLE;

ANDERSON, 2001) to determine sample grouping all calculated by compare_categories.py command in QIIME. The G test of independence (P, FDR_P and Bonferroni_P) was carried out to determine whether OTU presence/absence is associated with different colonies or different sample type, through the group_significance.py command also in QIIME.

At a sequencing depth of 400, 99 samples passed this cutoff and were included in downstream analyses, including 30 from the head, 18 from the mesosoma and 51 from the gaster. Alpha diversity was quantified using observed species richness, Shannon diversity, the Chao1 nonparametric richness estimator, whole-tree phylogenetic diversity and Simpson as implemented in equitability metric. We also compared alpha diversities based on a two-sample t-test using non-parametric (Monte Carlo) methods to test differences in OTU richness. Unweighted and weighted UniFrac distance matrices (LOZUPONE; KNIGHT, 2005), which uses phylogenetic information to calculate community similarity, were produced through the QIIME pipeline. These beta diversity metrics were used to compare community level differences between two categories: sample type (head, mesosoma and gaster) and host localities. A matrix of community pairwise distances was generated by UniFrac and used to cluster samples by principal coordinates analysis (PCoA).

To illustrate the relationship between ecological communities (CLARKE, 1993; MCCUNE, B. & GRACE, 2002), we implemented the analysis of multidimensional nonmetric scaling (NMDS) and related statistics in the PAST3 software package (HAMMER, Ø., HARPER, D. A. T. & RYAN, 2001). Sorensen (Dice coefficient) and Bray-Curtis similarity indices (MCCUNE, B. & GRACE, 2002) were used to test the variation and the structure of the bacterial community, respectively. The samples were grouped according to the sample type and host localities, and after viewing the plots, analyzes of similarity (ANOSIM) with Bonferroni correction was used to determine statistical significance (CLARKE, 1993; MCCUNE, B. & GRACE, 2002). The SIMPER analysis was conducted to verify the contribution of each OTU for grouping between colonies and different sample type (CLARKE, 1993).

Networks were visualized using Cytoscape3.2.1 (SHANNON et al., 2003) with the edge-weighted spring embedded algorithm to display the OTUs and sample nodes (LEY et al., 2008). Each host-bacterial network was constructed as a graph, in which

each node represented a host sample. Connections were drawn between samples representing the shared significant OTUs. A heatmap was constructed with all OTUs that had at least 100 reads represented in the main dataset using heatmap.2 and the vegan package (OKSANEN et al., 2007) in R ("R Development Core Team (2015) R: A Language and Environment for Statistical Computing. Available from http://www. R-project.org/.," 2016). The dendrogram of the samples shown in the heatmap was created with Bray-Curtis dissimilarity hierarchical clustering of bacterial communities in hclust. We also added a column dendrogram to cluster the bacterial genera that occur more often together.

Results

A total of 131 samples were successfully sequenced (head, mesosoma and gaster) resulting in 85530 reads and 2686 OTUs. From the heads we obtained 28871 reads, with variation from 104 reads for the lowest sample to 2524 reads for the most abundant. In the mesosoma we obtained 26283 reads, ranging from 50 to 2764 reads per sample. The gaster as expected was the most abundant in quantity with 51,958 reads, ranging from 131 to 3586 reads per sample. A summary of the total diversity of OTUs recovered across samples can be found in Figure 1.

Across the different sampled body parts, there was a clear distinction of the bacterial communities, where the gaster despite having the largest amount of bacteria (reads) has less diversity in comparison to the head and mesosoma samples. The high diversity found in these two tissues was unexpected. But as expected for the whole worker (combined body parts) and the gaster most of the bacteria were from *Blochmannia,* followed by *Wolbachia* (Wernegreen and Brown et al 2016), being 44.7% and 84.10% from *Blochmannia*, and 20.3% and 7.10% from *Wolbachia* in whole workers and gasters, respectively. In whole worker samples it was also possible to identify *Sodalis* (3.3%), followed by Enterobacteriaceae (2.4%), *Lactobacillus* (2.3%), and others bacteria in smaller quantities. In the gaster we also found Enterobacteriaceae (2%), *Sodalis* (1.7%), *Lactobacillus* (1.0%). In the samples of the head and mesosoma, the bacterial communities were more diverse. For the head we obtained *Wolbachia* (25% - 7217 reads), *Candidatus Blochmannia* (5.4%), *Sodalis* (5.1%), *Lactobacillus* (4.5%), Enterobacteriaceae (4%), *Acinetobacter* (2.5%), *Nocardia* (1.9%), Acetobacteraceae (1.8%), followed by others in smaller quantities. For the mesosoma

we obtained *Wolbachia* (32%), *Candidatus Blochmannia* (6.7%), *Sodalis* (4.3%), Enterobacteriaceae (3.9%), *Streptococcus* (3.4%), *Corynebacterium* (1.9%), Acetobacteraceae (1.8%), *Nocardia* (1.5%), *Acinetobacter* (1.5%), followed by others in smaller amounts.

Alpha and beta diversity

The diversity found in *Camponotus* is not very high and rarefaction curves confirm that our sequencing was sufficient to recover most of the diversity of the bacterial community associated with this genus. However, despite sequencing thousands of read, the rarefaction curves of several samples did not reach a plateau (Additional file 3). Likely due to the small number of OTUs per sample, we did not obtain significant results when comparing alpha diversities based on a two-sample t-test using non-parametric (Monte Carlo) methods to test differences in OTU richness among different sample type. The PCoA was calculated with the weighted distance values of the beta diversity and suggest head and mesosoma samples almost completely overlap and there is separation from the gaster samples (Figure 2A). This can also be observed by the NMs analysis (Figure 2B).

Network analysis was performed using the spring-embedded edge-weighted algorithm to facilitate the visualization of the associations of the shared bacterial community between the samples - OTUs with less than 100 reads were hidden (Figure 3). In this analysis each vertice is represented by a host and the edges are the shared bacterial communities, colored with different categories: Different body parts (Figure 3A), different locations where the host was collected (Figure 3B), and the main bacteria found in this study (Figure 3C). This analysis reinforces our findings that the bacterial community of the gaster is different from the head and thorax, which overlap in bacterial diversity (Figure 3A). Our results also confirm the influence of the locality (Figure 3B). In Figure 3C, it is possible to observe another aspect of the bacterial community where *Blochmannia* and the gaster samples practically overlap (see Figure 3A and 3C), this is due to the fact that *Blochmannia* is very common in the gaster samples. But when analyzing *Wolbachia*, we find this bacterium is associated with the head, mesosoma, and gaster.

Our statistical data support our findings that the microbiome differs across

different parts of the ant's body (Adonis, unweight R2= 0.16769 and P=0.001, weight R2=0.3619 and P= 0.001; Anosim, unweight R2=0.49622 and P=0.001, weight R2= 0.58026 and P= 0.001; RDA, unweight Pseudo F=9.5275 and Significance=0.001, weight Pseudo F=25.287 and Significance=0.001). This corroborates our data as visualized by sample type (Figure 1), PCoA and NM analyses (Figure 2) and the network analysis (Figure 3). What we did not expect, and indeed is the most striking is the highest diversity is found in the head and mesosoma.

To test what might be contributing to the high diversity found in the mesosoma and head, we tested whether these bacteria were being acquired from the local environment in which the ant was collected potentially through horizontal transfer by feeding. Our data partially confirmed this hypothesis, but only when we did not consider abundance (unweight) (Adonis, unweight R2= 0.24019 and P=0.006; RDA, unweight Pseudo F=1.314 and Significance=0.003). The main bacteria across all of these body parts are *Wolbachia* and others in smaller quantities, but the OTU (weighted) may not be appropriate to test for significant differences across the different localities. Outside of *Wolbachia* it is likely that the diversity of the head and the mesosoma comes partly from the acquisition of these bacteria from the host's diet and environment.

No significant changes in the composition of the bacterial community (Soresen index) were observed across all samples (R= -0.0367 and P= 1). This may be explained because the main bacteria are the same across the different parts of the body, but in varying concentrations. However, when we analyzed the total bacterial community structure (Bray-Curtis index) we obtained significant results (R= 0.6015 and P= 0.0001), and when analyzing each part of the body of the ant we found that head (R= 0.6609 and P = 0.0001) and mesosoma (R= 0.7363 and P= 0.0001) are different from the gaster.

In each part of body the most common bacteria, which are shared across all samples, are responsible for structuring bacterial communities; this was reinforced by the SIMPER (Additional file 4) analysis. This analysis recovered that the head and the mesosoma are more similar to each other (89.39%), compared to the gaster. Also for the head and mesosoma *Wolbachia* (multiple strains) and *Sodalis* represent 42% of the contribution of the difference found, with *Blochmannia* accounting for less

representation in these sampled body regions (different strains inclusive). When the comparison involves the gaster, several *Blochmannia* OTUs are present representing more than 40% of the reads if we include multiple OTUs.

Differences in bacterial presence/absence was tested using the G test and we found significant differences for the categories "different locations" and "head, mesosoma and gaster" (P, FDR_P and Bonferroni_P) (Additional file 5). The category "different locations" showed significantly more bacteria compared to different parts of the body. However, in both of these two categories the main bacteria were: *Candidatus Blochmannia* (multiple strains), *Wolbachia* (multiple strains) and *Sodalis*.

In the heatmap analysis the amount of bacteria present in each part of the body was investigated (Figure 4). In each part of the body, we grouped the samples according to the quantity and type of associated bacteria. Through this analysis it is possible to visualize the presence of individuals with multiple strains of *Wolbachia* (n = 38, 70.37%) and *Blochmannia*: there are six main *Blochmannia* OTUs - individuals who presented OTU AJ245591.1.1215 presented simple infection, and OTU AF495758 .1.1401 and New.ReferenceOTU1 were shown to be related to several individuals presenting with double infections (n = 23, 60.86%). There are also several individuals with triple infections with OTUs AY334369.1.1410, AY196851.1.1402 and New.ReferenceOTU55 (n = 24 individuals = 66.66%).

Discussion

Besides the bacteria already well-known as associates of *Camponotus*, as is the case of *Blochmannia* and *Wolbachia* (BROWN; WERNEGREEN, 2016; RAMALHO et al., 2017), our study also recovered Enterobacteriaceae, *Sodalis* and *Lactobacillus* in large quantities. In addition, we also analyzed separately the body parts of the ant: head, mesosoma and gaster, and verified the presence of different bacterial communities associated with each body part.

Enterobacteriaceae is the bacterial family that *Blochmannia* belongs to and has been found frequently in recent studies of the bacteria associated with *Camponotus, Colobopsis* and *Polyrhachis* (RAMALHO; BUENO; MOREAU, 2017). As this bacterium can have a high mutational rate (Degnan et al 2004) this could explain our inability to assign most "Enterobacteriaceae" to lower taxonomic categories. Therefore,

it possible that these Enterobacteriaceae may actually be OTUs from *Blochmannia*.

Although not documented in high quantities before in *Camponotus*, we commonly recovered *Sodalis*, which may act as facultative or obligate endosymbiont in other organisms (HEDDI et al., 1998; SNYDER et al., 2011). It has been found in several insect hosts including tsetse flies (DALE; MAUDLIN, 1999), aphids (BURKE et al., 2009) and beetles (GRÜNWALD; PILHOFER; HÖLL, 2010), but the role of this bacterium in these associations is not yet clear.

Another bacterium that has recently become commonly identified as one of the major bacteria found in ant microbiomes, and also was evident in our samples is *Lactobacillus.* This bacteria has been identified in *Cephalotes* (HU et al., 2014; LANAN et al., 2015; SANDERS et al., 2014), leaf-cutting ants (KELLNER et al., 2015; VIEIRA et al., [s.d.]), and also in other Camponotini ants such as *Polyrhachis* (RAMALHO; BUENO; MOREAU, 2017). Its function in these groups is still being discussed, but it is believed that this bacterium could bring benefits to nutrition, or confer defenses against other microorganisms, altering pH with the production of latic acid (KELLNER et al., 2015; LANAN et al., 2015).

Many studies to date have analyzed the bacterial community of the insect gut and found it is less diverse than that found in vertebrates (ENGEL; MARTINSON; MORAN, 2012; FELDHAAR, 2011; HOSOKAWA et al., 2006; JING et al., 2014; MCFALL-NGAI, 2007; MORAN; MCCUTCHEON; NAKABACHI, 2008). However, it is already known that several factors can contribute to the gut bacterial community such as diet, physiology, immunity and physical barriers (HEGEDUS et al., 2009; KWONG; MORAN, 2015; MUEGGE et al., 2011; NYHOLM; GRAF, 2012).

Kautz et al. (KAUTZ et al., 2013) and Lanan et al. (LANAN et al., 2015) taking into account different parts of the digestive tract of two different species of an herbivorous ant, *Cephalotes*, found different bacterial communities across digestive compartments. In addition, Lannan et al. (2015) identified a possible anatomical filter - called the proventriculum that hinders the passage of bacteria transferred horizontally, and guarantees the specificity of the vertically transferred bacterial community - often called microbial signatures.

In general, the present study was able to differentiate the bacterial communities

present in the different parts of the body of the ant. This is likely explained because each part of the body has different organs with unique functions and likely a different pH. Additionally, anatomic filters have been observed for *Cephalotes* (LANAN et al., 2015) and could also be a factor structuring bacterial communities in other ant species and the proventriculum found in *Camponotus* (see additional file 6) has four hair-lined, sclerotized channels (EISNER; WILSON, 1952), which may also play a role in filtering. As expected the gaster is the part of the body that contains the largest number of bacteria, although with low diversity. An unexpected finding of the present study was the high diversity found associated with the head and mesosoma.

The microbial diversity found in the head and mesosoma of ant may be explained by the horizontal acquisition of microbes with ingested food (PERNICE; SIMPSON; PONTON, 2014) or the local environment. Our results showed the relationship of the bacterial community to the collection location where the ants were collected. These microbes are being picked up in the environment, and therefore are less stable, relative to the host.

Although uncovering the functional role, if any, in host-associated microbial communties is critical to understanding how they may influence aspects of host biology, documenting the diversity of microbial communities associated with hosts and across body parts is an important first step. We found that the gaster of ants in the genus *Camponotus* have very dense bacterial communities, but these were simple communities dominated by *Blochmannia* and *Wolbachia*. We did find much higher diversity in the head and mesosoma, but in lower abundance. When we examined the similarity of communities based on host collection location we found that locality did explain similarity of samples suggesting that many of the bacteria, especially for the head and thorax, are likely acquired in the environment or through the food they ingest, but we cannot rule out that some of these bacteria still play important functional roles for the host.

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Authors' contributions: MOR, CSM and OCB designed the experiments, analyzed the data and wrote the manuscript. MOR performed the experiments. CSM and OCB assists in data analysis and discussions. All authors read and approved the final manuscript.

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List of figure

Figure 1. Summary graph of bacterial OTUs found in *Camponotus* samples with 16S rRNA amplicon sequencing. A. Bacterial communities from whole worker, head, mesosoma and gaster samples. Bar graphs for each library show the percentage of sequence reads classified to selected 97% OTUs. Each color represents a distinct bacterium. B. Summary of all OTUs found in this study in each part of the body analyzed with legend ordered in proportion of reads found across all 131 samples. Orders that accounted for less than 0.8% in a sample are summarized in a category termed "Other."

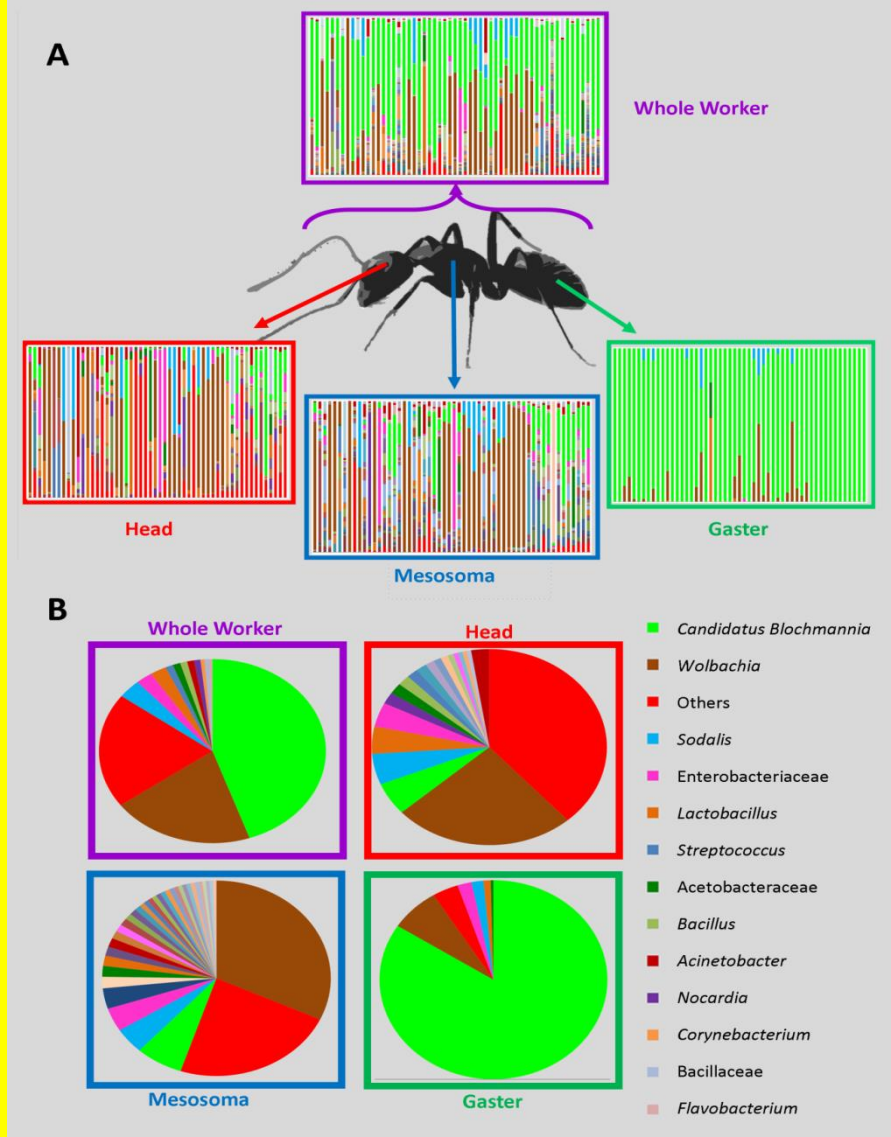


Figure 2. Beta diversity of head, mesosoma and gaster samples of *Camponotus* (depth of 400 reads). **A.** PCoA plots (weighted UniFrac method) of bacterial communities grouped according to different sample type with 95% ellipses. Note that there are a clustered of gaster samples, and a mix of head and mesosoma samples. This suggests that different parts of the body plays an important role in structuring the bacterial community. B. Nonmetric multidimensional scaling (NMS) plot illustrating bacterial community structure among different body parts, Bray-Curtis, Axis 1: 0.9524, Axis 2: 0.017 and stress 0.051. The dots were colored according to the sample type (red = head, blue = mesosoma and green = gaster).

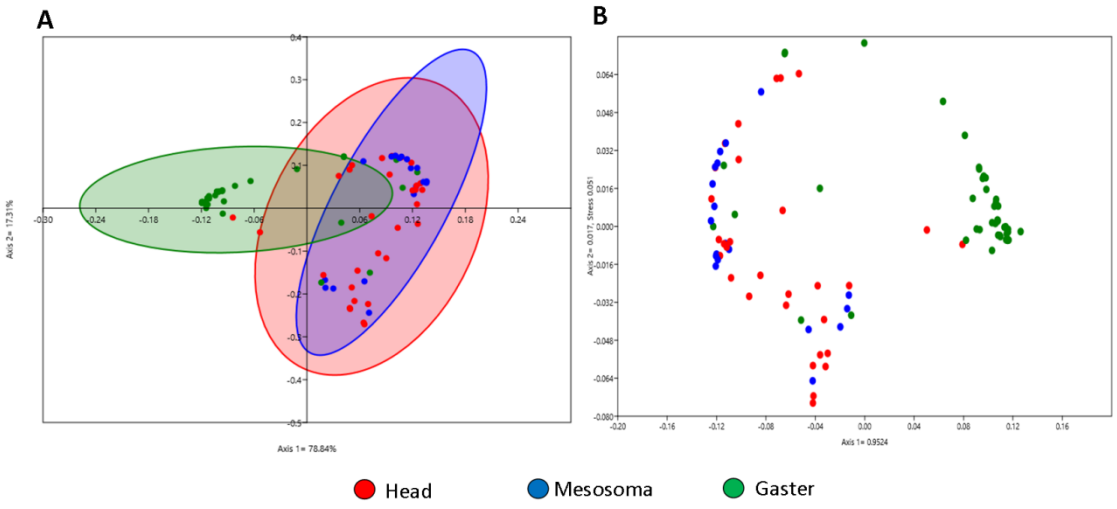


Figure 3. Network analysis of *Camponotus* samples with edges representing the main community bacterial members. **A.** The edges were colored according to the different sample type: head, mesosoma and gaster of *Camponotus*. **B.** The edges were colored according to the different localities. **C.** The edges were colored according to the different bacteria. Note that it is the same image, but colored according to the different sample type, localities and bacteria. For the former, it is easy to see structuring. For the locality, it is not possible to find a pattern easily, outside of the samples from the Florida Keys, USA. And for the identity of the bacteria, it is perceived that there is a certain overlap of *Blochmannia* associated with the gaster.

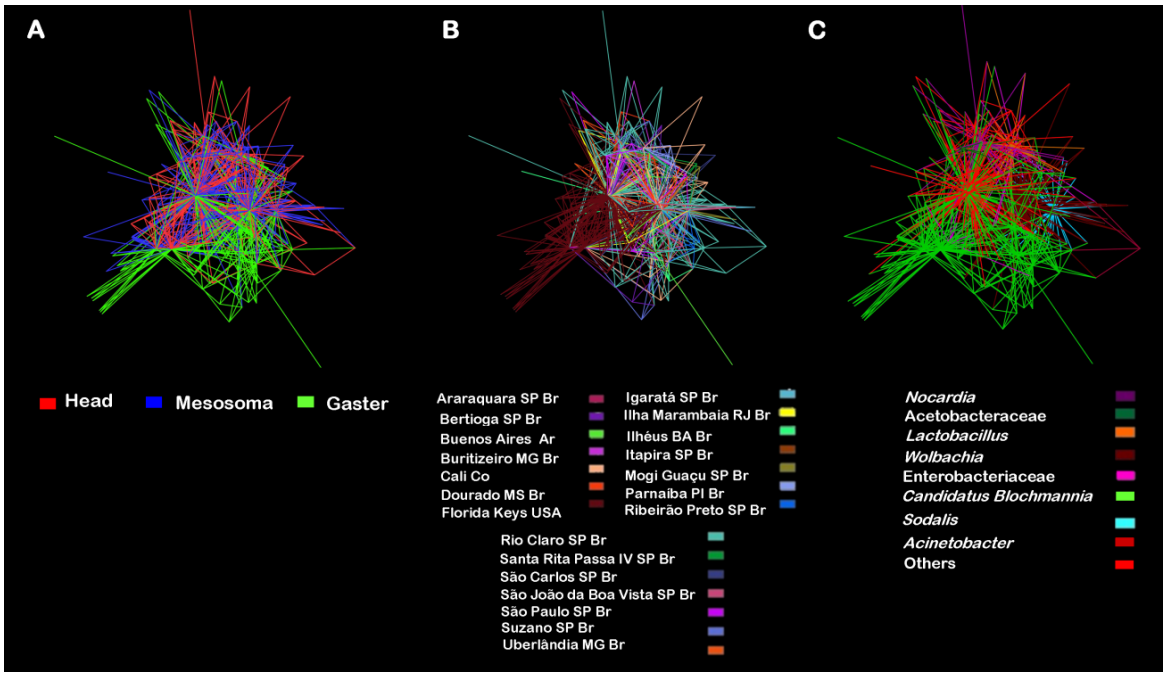
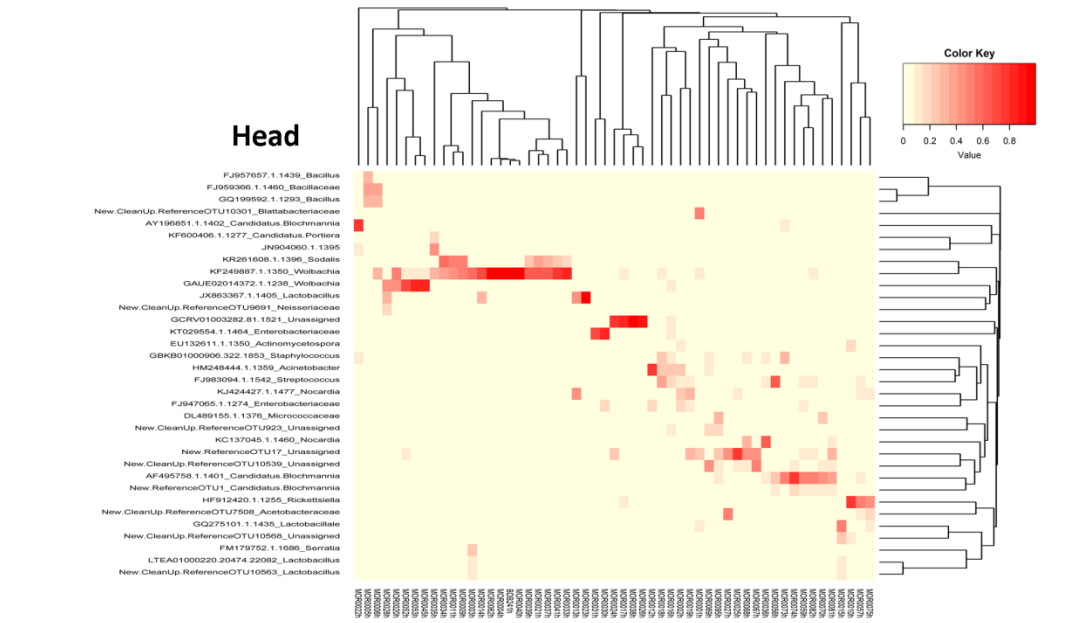
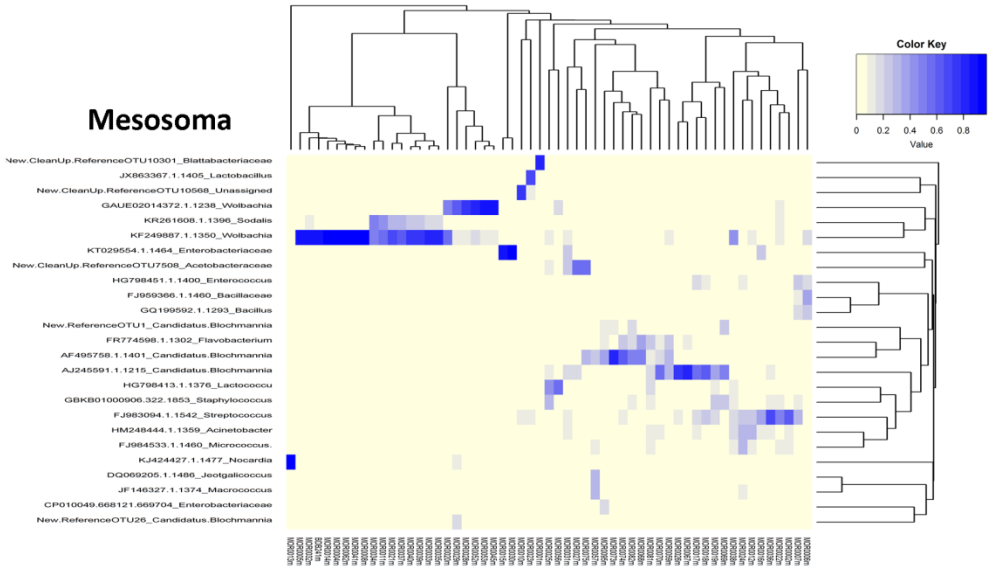
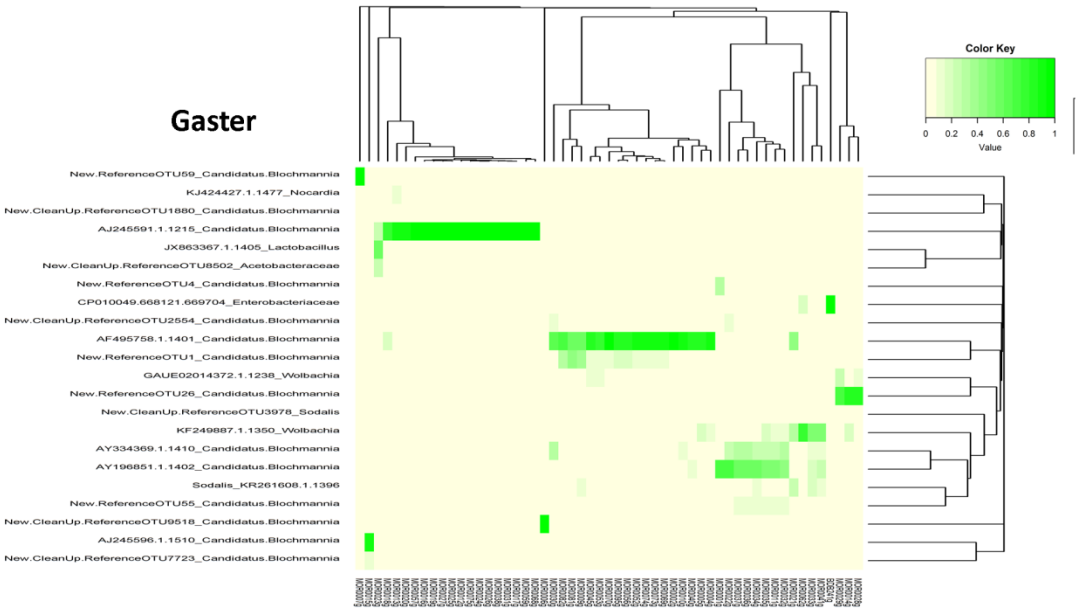


Figure 4. Heatmap of the different sample types – **A.** head, **B**. mesosoma and **C**. gaster of *Camponotus*. The colors in the heatmap indicate variation in the relative abundance of different bacteria in different sample types. We choose to show only OTUs with more than 100 reads, for easy viewing. Dendrograms were generated from Bray–Curtis distance matrices.


Head
Color Key
0 0.2 0.4 0.6 0.8
Value
FJ957657.1.1439_Bacillus
FJ959366.1.1460_Bacillaceae
GQ199592.1.1293_Bacillus
New.CleanUp.ReferenceOTU10301_Blattabacteriaceae
AY196851.1.1402_Candidatus.Blochmannia
KF600406.1.1277_Candidatus.Portiera
JN934060.1.1395
KR261608.1.1396_Sodalis
KF249887.1.1350_Wolbachia
GAUE02014372.1.1238_Wolbachia
JX863367.1.1405_Lactobacillus
New.CleanUp.ReferenceOTU9691_Neisseriaceae
GCRV01003282.81.1521_Unassigned
KT029554.1.1464_Enterobacteriaceae
EU132611.1.1350_Actinomycetospora
GBKB01000906.322.1853_Staphylococcus
HM248444.1.1359_Acinetobacter
FJ983094.1.1542_Streptococcus
KJ424427.1.1477_Nocardia
FJ947065.1.1274_Enterobacteriaceae
DL469155.1.1376_Micrococcaceae
New.CleanUp.ReferenceOTU923_Unassigned
KC137045.1.1460_Nocardia
New.ReferenceOTU17_Unassigned
New.CleanUp.ReferenceOTU10539_Unassigned
AF495758.1.1401_Candidatus.Blochmannia
New.ReferenceOTU1_Candidatus.Blochmannia
HF912420.1.1255_Rickettsiella
New.CleanUp.ReferenceOTU7508_Acetobacteraceae
GQ275101.1.1435_Lactobacillale
New.CleanUp.ReferenceOTU10568_Unassigned
FM179752.1.1686_Serratia
LTEA01000220.20474.22082_Lactobacillus
New.CleanUp.ReferenceOTU10563_Lactobacillus


Mesosoma
Color Key
0 0.2 0.4 0.6 0.8
Value
New.CleanUp.ReferenceOTU10301_Blattabacteriaceae
JX863367.1.1405_Lactobacillus
New.CleanUp.ReferenceOTU10568_Unassigned
GAUE02014372.1.1238_Wolbachia
KR261608.1.1396_Sodalis
KF249887.1.1350_Wolbachia
KT029554.1.1464_Enterobacteriaceae
New.CleanUp.ReferenceOTU7508_Acetobacteraceae
HG798451.1.1400_Enterococcus
FJ959366.1.1460_Bacillaceae
GQ199592.1.1293_Bacillus
New.ReferenceOTU1_Candidatus.Blochmannia
FR774598.1.1302_Flavobacterium
AF495758.1.1401_Candidatus.Blochmannia
AJ245591.1.1215_Candidatus.Blochmannia
HG798413.1.1376_Lactococcu
GBKB01000906.322.1853_Staphylococcus
FJ983094.1.1542_Streptococcus
HM248444.1.1359_Acinetobacter
FJ984533.1.1460_Micrococcus.
KJ424427.1.1477_Nocardia
DQ069205.1.1486_Jeotgalicoccus
JF146327.1.1374_Macrococcus
CP010049.668121.669704_Enterobacteriaceae
New.ReferenceOTU26_Candidatus.Blochmannia


Gaster
Color Key
0 0.2 0.4 0.6 0.8 1
Value
New.ReferenceOTU59_Candidatus.Blochmannia
KJ424427.1.1477_Nocardia
New.CleanUp.ReferenceOTU1880_Candidatus.Blochmannia
AJ245591.1.1215_Candidatus.Blochmannia
JX863367.1.1405_Lactobacillus
New.CleanUp.ReferenceOTU8502_Acetobacteraceae
New.ReferenceOTU4_Candidatus.Blochmannia
CP010049.668121.669704_Enterobacteriaceae
New.CleanUp.ReferenceOTU2554_Candidatus.Blochmannia
AF495758.1.1401_Candidatus.Blochmannia
New.ReferenceOTU1_Candidatus.Blochmannia
GAUE02014372.1.1238_Wolbachia
New.ReferenceOTU26_Candidatus.Blochmannia
New.CleanUp.ReferenceOTU3978_Sodalis
KF249887.1.1350_Wolbachia
AY334369.1.1410_Candidatus.Blochmannia
AY196851.1.1402_Candidatus.Blochmannia
Sodalis_KR261608.1.1396
New.ReferenceOTU55_Candidatus.Blochmannia
New.CleanUp.ReferenceOTU9518_Candidatus.Blochmannia
AJ245596.1.1510_Candidatus.Blochmannia
New.CleanUp.ReferenceOTU7723_Candidatus.Blochmannia

Supplementary Material

Additional file 1. *Camponotus* specimens used in this study.

Additional file 1. *Camponotus* specimens used for the development of this study.

Collection Code	SUBFAMILY	SPECIES	Country	City	Lat	Long	Collector
MOR0001	Formicidae	*C. mus*	Argentina	Buenos Aires	34.59972S	58.37306W	Roxana Josens
MOR0002	Formicidae	*C. spp. 1*	Colombia	Cali	4.98333S	76.65W	James Montya
MOR0003	Formicidae	*C. spp. 1*	Colombia	Cali	2.90028S	76.98361W	James Montya
MOR0004	Formicidae	*C. spp. 1*	Colombia	Cali	2.90028S	76.98361W	James Montya
MOR0005	Formicidae	*C. spp.1*	Colombia	Cali	2.90028S	76.98361W	James Montya
MOR0006	Formicidae	*C. vittatu*	Brazil	Parnaíba PI	2.90561S	41.7545W	Cintia Martins
MOR0007	Formicidae	*C. spp. 2*	Brazil	Bertioga SP	23.7475S	46.14417W	Maria Santina C Morini
MOR0008	Formicidae	*C. spp. 9*	Brazil	Igaratá SP	23.20472S	46.15167W	Maria Santina C Morini
MOR0009	Formicidae	*C. senex*	Brazil	São Paulo SP	23.58778S	46.64833W	Maria Santina C Morini
MOR0010	Formicidae	*C. spp. 3*	Brazil	Suzano SP	23.53361S	46.3275W	Maria Santina C Morini
MOR0011	Formicidae	*C. textor*	Brazil	Mogi Guaçu SP	22.36747S	46.94325W	Maria Santina C Morini
MOR0012	Formicidae	*C. atriceps*	Brazil	Rio Claro SP	22.37553S	47.55222W	Manuela O Ramalho
MOR0013	Formicidae	*C. atriceps*	Brazil	Rio Claro SP	22.37553S	47.55222W	Manuela O Ramalho
MOR0014	Formicidae	*C. spp. 4*	Brazil	São Carlos SP	21.88881S	47.87372W	Larissa M R Silva
MOR0015	Formicidae	*C. spp. 5*	Brazil	São Carlos SP	21.88881S	47.87372W	Larissa M R Silva
MOR0016	Formicidae	*C. rufipes*	Brazil	Itapira SP	22.43362S	46.82992W	Marcela Ceccato
MOR0017	Formicidae	*C. textor*	Brazil	Uberlandia MG	18.91863S	48.25908W	Kleber Del-Claro
MOR0018	Formicidae	*C. atriceps*	Brazil	Ilha marambaia RJ	23.06808S	43.93956W	Larissa M R Silva
MOR0019	Formicidae	*C. rufipes*	Brazil	São Paulo SP	23.58861S	46.64844W	Amanda Ap. Oliveira
MOR0020	Formicidae	*C. balzani*	Brazil	Rio Claro SP	22.39586S	47.54417W	Manuela O Ramalho
MOR0021	Formicidae	*C. sericeiventris*	Brazil	Rio Claro SP	22.39583S	47.545W	Manuela O Ramalho
MOR0022	Formicidae	*C. substitutus*	Brazil	Rio Claro SP	22.39583S	47.545W	Manuela O Ramalho
MOR0023	Formicidae	*C. rengggeri*	Brazil	Rio Claro SP	22.39383S	47.54472W	Manuela O Ramalho
MOR0024	Formicidae	*C. rufipes*	Brazil	Rio Claro SP	22.39592S	47.54267W	Manuela O Ramalho
MOR0025	Formicidae	*C. rufipes*	Brazil	Rio Claro SP	22.3685S	47.53928W	Manuela O Ramalho

MOR0026	Formicidae	*C. rengggeri*	Brazil	Rio Claro SP	22.39578S	47.54328W	Manuela O Ramalho
MOR0027	Formicidae	*C. rengggeri*	Brazil	Rio Claro SP	22.36692S	47.53847W	Manuela O Ramalho
MOR0028	Formicidae	*C. blandus*	Brazil	Dourado MS	22.21606S	54.81556W	William F A Junior
MOR0029	Formicidae	*C. blandus*	Brazil	Dourado MS	22.21675S	54.81575W	William F A Junior
MOR0030	Formicidae	*C. substitutus*	Brazil	Dourado MS	22.21714S	54.81497W	William F A Junior
MOR0031	Formicidae	*C. rengggeri*	Brazil	Buritizeiro MG	16.89094S	44.92258W	Odair Correa Bueno
MOR0032	Formicidae	*C. textor*	Brazil	Ribeirão Preto SP	21.21167S	47.80667W	Manuela O Ramalho
MOR0033	Formicidae	*C. textor*	Brazil	Araraquara SP	21.72473S	48.01875W	Manuela O Ramalho
MOR0034	Formicidae	*C. textor*	Brazil	Santa Rita Passa IV SP	21.70098S	47.48954W	João Nascimento
MOR0035	Formicidae	*C. textor*	Brazil	Rio Claro SP	22.39508S	47.54261W	Manuela O Ramalho
MOR0036	Formicidae	*C. textor*	Brazil	Ilheus BA	14.3125S	39.88694W	Jacques Delabie
MOR0037	Formicidae	*C. textor*	Brazil	São João da Boa Vista SP	21.96944S	46.79889W	Manuela O Ramalho
MOR0038	Formicidae	*C. spp. 7*	Brazil	Uberlândia MG	18.88603S	48.26639W	Kleber Del-Claro
MOR0039	Formicidae	*C. textor (male)*	Brazil	Rio Claro SP	22.39919S	47.57192W	Manuela O Ramalho
MOR0040	Formicidae	*C. senex*	Brazil	Suzano SP	23.53361S	46.3275W	Maria Santina C Morini
MOR0041	Formicidae	*C. textor*	Brazil	Rio Claro SP	22.39611S	47.54356W	Manuela O Ramalho
MOR0045	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	25.12404N	080.40276W	Manuela O Ramalho
MOR0052	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	25.12404N	080.40276W	Manuela O Ramalho
MOR0053	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	25.09034 N	080.44412W	Manuela O Ramalho
MOR0056	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0057	Formicidae	*C. tortuganus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0059	Formicidae	*C. floridanus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0067	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0068	Formicidae	*C. floridanus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0069	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0070	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0073	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho

MOR0074	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.69786N	081.34054W	Manuela O Ramalho
MOR0075	Formicidae	*C. spp. 8*	USA	Florida Keys, Florida, USA	25.09034N	080.44412W	Manuela O Ramalho
MOR0081	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.55793N	081.7627W	Manuela O Ramalho
MOR0082	Formicidae	*C. floridanus*	USA	Florida Keys, Florida, USA	24.55793N	081.7627W	Manuela O Ramalho
MOR0095	Formicidae	*C. planaltus*	USA	Florida Keys, Florida, USA	24.55793N	081.7627W	Manuela O Ramalho
BDW0010	Formicidae	*C. floridanus*	USA	Florida Keys, Florida, USA	25.12404'N	080.40276'W	Brian Wray

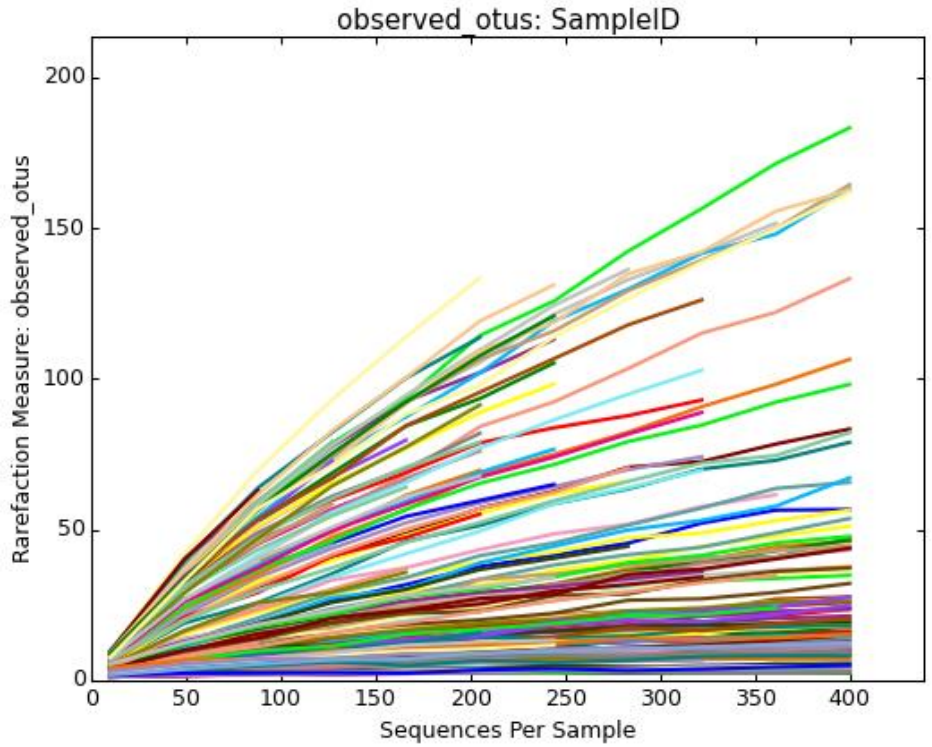
Additional file 2. Bacterial quantification through 16S rRNA gene (qPCR) of all *Camponotus* samples separated into whole worker, head, mesosoma and gaster. Each sample was analyzed in triplicate therefore follows the values of average and standard deviation for each sample.

Sample	**average**	**mistake**	Sample	**average**	**mistake**	Sample	**average**	**mistake**
M1G	874873,3	88435,67912	**M17G**	3071806	207194,2	**M34G**	910220	42539,9053
M1H	921,2907	118,97401	**M17H**	624,494	162,6925	**M34H**	30711,63	1053,682295
M1M	818,715	103,3419952	**M17M**	226,9669	25,16057	**M34M**	20435,45	1711,623184
M2G	254599,1	19891,01686	**M18G**	81,22816	34,23891	**M35G**	450056,4	29519,19398
M2H	321,9773	74,15484	**M18H**	40,53105	8,57569	**M35M**	86622,24	1146,802452
M2M	321,4497	158,9633666	**M18M**	132,934	15,54298	**M35M**	33753,29	2129,28995
M3G	743,1619	364,43608	**M19G**	324252,6	19632,78	**M36G**	187043,8	20228,33369
M3H	14594,66	1594,53042	**M19H**	358,7999	315,8526	**M36H**	388,4966	19,33514728
M3M	850,8956	52,71518668	**M19M**	418,5788	12,68454	**M36M**	386,7954	55,14590535
M4G	417751,9	70859,26213	**M20G**	9381907	1297652	**M37G**	1981548	58566,03661
M4H	3945,597	516,04767	**M20H**	149655,5	45033,17	**M37H**	15514,87	492,897422
M4M	6911,37	526,5694198	**M20M**	441727,9	27511,19	**M37M**	12628,19	1494,95141
M5G	3797,298	411,88245	**M21G**	1496764	124008,6	**M38G**	1936967	69741,45099
M5H	366976,3	34344,36239	**M21H**	120829,6	5036,075	**M38H**	670834,9	326,8675199
M5M	49739,27	5327,552944	**M21M**	97597,66	6585,284	**M38M**	454,4652	8,143870074
M6G	659415	77687,22772	**M22G**	2488197	430967,8	**M39G**	58470,67	10210,94574
M6H	14677,79	2153,3494	**M22H**	6398,506	1850,233	**M39H**	11758,48	1115,086388
M6M	22776,89	89,24543589	**M22M**	180,3291	53,97559	**M39M**	62768,28	2649,4818
M7G	2734180	639354,3913	**M23G**	13821,43	2355,793	**M40G**	901428,8	422286,5376
M7H	2360,496	640,84043	**M23H**	9110,933	1703,585	**M40H**	4311,83	124,0348266
M7M	271,1061	20,87055714	**M23M**	410,3729	42,39151	**M40M**	4182,871	364,6657634
M8G	64863,22	10490,11925	**M24G**	6832815	1240598	**M41G**	415227,1	27277,57642
M8H	668733,3	23845,26235	**M24H**	14009,41	1254,913	**M41H**	58073,12	163,3546631
M8M	91673,34	6837,054368	**M24M**	590,9476	22,53848	**M41M**	116926,6	3588,467135
M9G	701923	105099,9921	**M25G**	2684774	480262,2	**M45G**	1307756	294032,01
M9H	7417,591	1706,68126	**M25H**	7548,85	1394,723	**M45H**	11097,88	3346,642281
M9M	10883,4	853,4498387	**M25M**	308,7638	46,23676	**M45M**	7965,841	1313,45
M10G	474373,1	64331,58682	**M26G**	636450,5	41209,03	**M52G**	391474,8	48571,01
M10H	6908,085	866,78738	**M26H**	4422,307	1241,614	**M52H**	5196,458	1144,272045
M10M	334,0736	26,69977377	**M26M**	757,5005	428,0112	**M52M**	5401,645	181,01
M11G	1275854	69681,74745	**M27G**	2718611	823501	**M53G**	502644,2	176417,24
M11H	16636,11	7327,79976	**M27H**	798,238	129,0605	**M53H**	5862,984	3113,109259
M11M	16817,97	961,5503073	**M27M**	290,7331	24,88798	**M53M**	11473,82	1443,06
M12G	228720,6	22971,27295	**M28G**	57760,41	8888,956	**M56G**	608402,3	34416,61
M12H	2878,807	442,15706	**M28H**	4433,724	451,7523	**M56H**	1026,593	235,691768
M12M	332,5045	16,63376267	**M28M**	1282,063	46,42601	**M56M**	289,3929	63,31
M13G	302621,9	14367,21593	**M29G**	31104,96	4736,712	**M57G**	14278,53	981,44
M13H	25084,66	4185,33477	**M29H**	4102,854	867,2286	**M57H**	1557,427	295,0567341

M13M	1333,075	73,87087807	**M29M**	2142,719	50,96176	**M57M**	365,7961	70,26144254
M14G	1593991	78939,86555	**M30G**	95118,79	8993,562	**M59G**	28988,99	3049,43
M14H	101482,5	14128,28402	**M30H**	25670,46	4150,715	**M59H**	945,3085	193,4699934
M14M	64317,16	6258,878876	**M30M**	573,2927	33,43998	**M59M**	743,0425	229,0667047
M15G	1039749	29948,18106	**M31G**	17635,63	2234,081	**M67G**	144181,4	1188,17
M15H	6338,492	1080,72626	**M31H**	1476,841	502,0849	**M67H**	726,6945	285,4189073
M15M	1017,802	312,0647868	**M31M**	548,1112	33,39451	**M67M**	301,1311	77,82734302
M16G	315118,7	35955,08519	**M32G**	84882,85	8615,544	**M68G**	269288,9	12557,96
M16H	76,08216	13,36004	**M32H**	3344,775	120,1965	**M68H**	485,7193	83,71203883
M16M	308,3352	5,5190624	**M33M**	23743,65	468,498	**M68M**	257,3993	81,72135885

Sample	**average**	**mistake**
M69G	553688,7	10103,06
M69H	1254,992	109,9883988
M69M	4965,075	2088,83293
M70G	339630,3	58635,21
M70H	3525,943	785,6590949
M70M	364,3957	96,19625125
M73G	477291,2	106680,88
M73H	649,0001	218,7182693
M73M	458,2997	242,1742801
M74G	683055	143203,46
M74H	1561,928	390,9451292
M74M	306,8567	63,11408043
M75G	1220529	62056,51
M75H	2526,584	184,8661455
M75M	408,125	112,4050687
M81G	307325,7	94574,65
M81H	812,2269	159,9683126
M81M	325,8265	142,9741976
M82G	389072,5	52663,74
M82H	85,96409	148,8941642
M82M	572,0676	284,2536264
M95G	321718,1	3846,61
M95H	1666,431	217,753248
M95M	263,4466	94,93963177
BDW0010G	316349,4	22075,74
BDW0010H	5739,712	283,7949104
BDW0010M	2275,99	958,5544147

Additional file 3. Rarefaction curves were used to estimate richness in the observed OTUs. The vertical axis shows the observed bacterial OTUs and the number of sequences per sample is shown on the horizontal axis. Note that although sequencing covers thousands of Illumina reads, some samples have not reached the plateau.



Additional file 4. SIMPER analyses indicating the contribution of specific operational taxonomic units (OTUs) to the observed differences in community structure among different sample type of *Camponotus.*

	Overall Average Dissimilarity	**Most Influential Taxonomy / OTUs**	**Percent Contribuition to Difference**
Head vs. Mesosoma	**89,39**	*Wolbachia* / KF249887.1.1350	27.09
		Wolbachia / GAUE02014372.1.1238	9.033
		Sodalis / KR261608.1.1396	6.371
		Candidatus *Blochmannia* / AF495758.1.1401	4.404
		Unassigned / GCRV01003282.81.1521	4.114
		Enterobacteriaceae / KT029554.1.1464	3.12
		Lactobacillus / JX863367.1.1405	2.989
		Unassigned / New.ReferenceOTU17	2.916
		Candidatus *Blochmannia* / AJ245591.1.1215	2.261
		Nocardia / KJ424427.1.1477	1.967
Head vs. Gaster	**96,34**	Candidatus *Blochmannia* / AJ245591.1.1215	20.42

		Candidatus *Blochmannia* / AF495758.1.1401	20.1
		Wolbachia / KF249887.1.1350	11.56
		Candidatus *Blochmannia* / AY196851.1.1402	7.06
		Candidatus *Blochmannia* / AY334369.1.1410	3.315
		Wolbachia / GAUE02014372.1.1238	3.301
		Sodalis / KR261608.1.1396	3.201
		Acetobacteraceae / New.ReferenceOTU1	3.004
		Candidatus *Blochmannia* / New.ReferenceOTU26	2.932
		Unassigned / GCRV01003282.81.1521	2.032
Mesosoma vs. Gaster	**95,46**	Candidatus *Blochmannia* / AJ245591.1.1215	22.15
		Candidatus *Blochmannia* / AF495758.1.1401	21.39
		Wolbachia / KF249887.1.1350	13.27
		Candidatus *Blochmannia* / AY196851.1.1402	7.244
		Wolbachia / GAUE02014372.1.1238	3.605
		Candidatus *Blochmannia* / AY334369.1.1410	3.419
		Candidatus *Blochmannia* / New.ReferenceOTU26	3.255
		Candidatus *Blochmannia* / New.ReferenceOTU1	3.165
		Sodalis / KR261608.1.1396	2.725
		Enterobacteriaceae / CP010049.668121.669704	1.961

Additional file 5. Results of G test of independence (P, FDR_P and Bonferroni_P) across *Camponotus* samples to determine whether OTU presence/absence is associated with different sample type (head, mesosoma and gaster), and host localities.

G-test					
Differents locations					
OTU	Test-Statistic	P	FDR_P	Bonferroni_P	taxonomy
KR261608.1.1396	1053.06322485	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__*Sodalis*; s__
FJ959366.1.1460	485.413990216	0.0	0.0	0.0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__; s__
GQ275101.1.1435	194.122905671	0.0	0.0	0.0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__; g__; s__
GAUE02014372.1.1238	525.942507737	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Rickettsiaceae; g__*Wolbachia*; s__
GCRV01003282.81.1521	527.306265723	0.0	0.0	0.0	Unassigned
AJ245596.1.1510	403.945776339	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
GQ132270.1.1377	139.911265473	0.0	0.0	0.0	k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__Coprococcus; s__
AJ245591.1.1215	3515.07330375	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
KF249887.1.1350	2195.96145608	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Rickettsiaceae; g__*Wolbachia*; s__
AY334369.1.1410	924.031144076	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
HF912420.1.1255	228.89063382	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Legionellales; f__Coxiellaceae; g__Rickettsiella; s__
AF495758.1.1401	1646.02741909	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
GQ199592.1.1293	379.809	0.0	0.0	0.0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales;

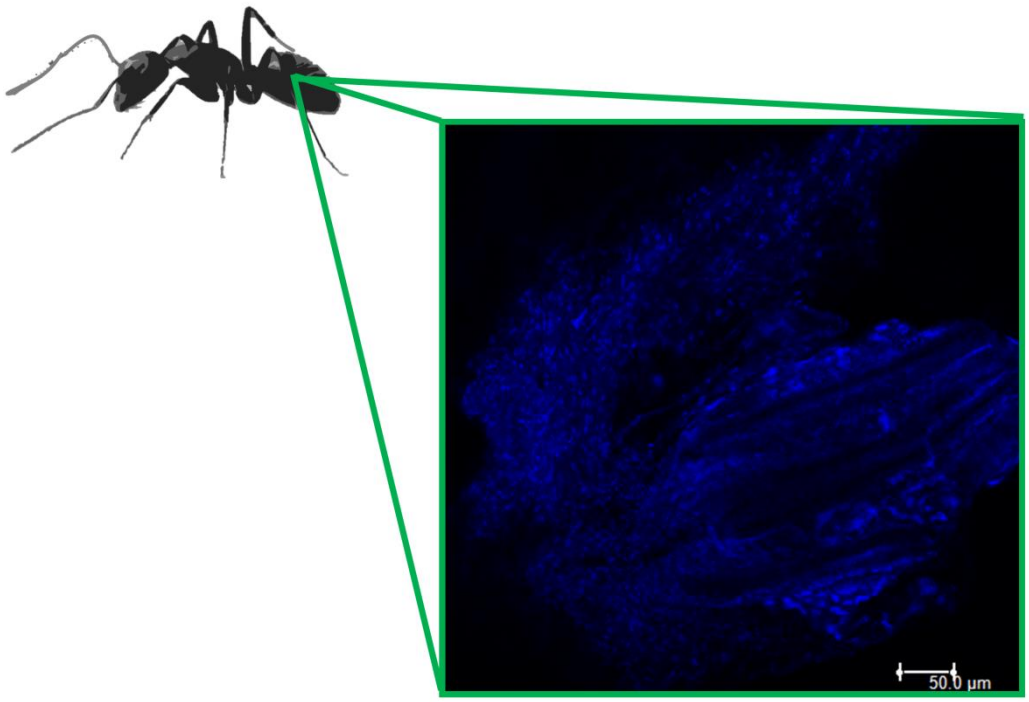
	606018				f__Bacillaceae; g__Bacillus
AY196851.1.1402	2448.52923542	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
JX863367.1.1405	237.560799415	0.0	0.0	0.0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
FJ957657.1.1439	144.684126594	0.0	0.0	0.0	k__Bacteria; p__Firmicutes; c__Bacilli; o__Bacillales; f__Bacillaceae; g__Bacillus; s__
New.ReferenceOTU55	306.341175697	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.ReferenceOTU59	2337.03442928	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.ReferenceOTU26	663.954948889	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.ReferenceOTU1	293.000876565	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.ReferenceOTU4	710.573373061	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.CleanUp.ReferenceOTU9518	706.58218659	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.CleanUp.ReferenceOTU10568	275.631253219	0.0	0.0	0.0	Unassigned
FM179752.1.1686	87.3841493551	9.61075663497e-11	5.73842260746e-09	1.37722142579e-07	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Serratia; s__marcescens
HG798451.1.1400	84.5983417615	2.96025870483e-10	1.69682028961e-08	4.24205072402e-07	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Enterococcaceae; g__Enterococcus; s__
CP010049.668121.669704	79.7798225531	2.02937844307e-09	1.1184997342e-07	2.90809930892e-06	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__; s__
KJ424427.1.1477	71.3888960817	5.39708874259e-08	2.86445487708e-06	7.73402816813e-05	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Nocardiaceae; g__Nocardia; s__
New.CleanUp.Refe	62.6583	1.4588146	7.4660048	0.0020904	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales;

renceOTU1880	360383	1566e-06	0087e-05	8134424	f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
New.CleanUp.Refe renceOTU9691	54.2423 982974	3.0339037 3126e-05	0.0014991 6691272	0.0434758 40469	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Neisseriales; f__Neisseriaceae; g__; s__
LTEA01000220.204 74.22082	53.9501 059023	3.3609927 1366e-05	0.0016054 3418623	0.0481630 255868	k__Bacteria; p__Firmicutes; c__Bacilli; o__Lactobacillales; f__Lactobacillaceae; g__Lactobacillus; s__
Sample Type -Head, Mesosoma and Gaster					
OTU	Test-Statistic	P	FDR_P	Bonferroni _P	taxonomy
AJ245591.1.1215	235.004 17354	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
KF249887.1.1350	156.706 847304	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Rickettsiaceae; g__*Wolbachia*; s__
AF495758.1.1401	126.723 996514	0.0	0.0	0.0	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
AY196851.1.1402	59.1965 13646	1.3988810 1103e-13	5.0114912 2201e-11	2.0045964 888e-10	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
GAUE02014372.1. 1238	41.7377 755344	8.6448448 3998e-10	2.4776125 3114e-07	1.2388062 6557e-06	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rickettsiales; f__Rickettsiaceae; g__*Wolbachia*; s__
AY334369.1.1410	27.9828 910973	8.3867250 3112e-07	0.0002003 02949493	0.0012018 1769696	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__Candidatus *Blochmannia*; s__
KR261608.1.1396	23.8972 122985	6.4682423 4096e-06	0.0013241 4161066	0.0092689 912746	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Enterobacteriales; f__Enterobacteriaceae; g__*Sodalis*; s__

Additional file 6. The complex proventriculum of *Camponotus* with the nucleus stained in blue (DAPI).

Confocal Microscopy

Workers gasters were dissected in 1X PBS (Fig 1A). The midgut was separated and fixed in 4% (w/v) paraformaldehyde in PBS at room temperature for two hours. Subsequently, they were washed in 50%, 70% and 100% ethanol baths for 3 min each. The material was placed on StarFrost slides (Knittel Glass, Germany), and dried at room temperature. The DAPI (Molecular Probes, USA) (1:500) which stains host nuclei blue, was placed directly into midgut for 5 min, and then washed 3x in miliQ water. Prolong Gold (Thermo Fisher Scientific, USA) was used to mount the slide, which was overlaid with cover slip and sealed with clear nail polish. For the whole-mount laser the Leica TCS SP5II confocal microscope was used to obtain the photomicrographs (lasers 405 nm) and Leica TCS SP5II software was used for the confocal analysis using maximum projection.



Capítulo 4

Transovarian transmission of *Blochmannia* and *Wolbachia* endosymbionts in the Neotropical weaver ant *Camponotus textor* (Hymenoptera, Formicidae)

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Resumo

Camponotus é um gênero de formiga hiper-diverso que é sempre associado com a presença do endossimbionte *Blochmannia* e também frequentemente com *Wolbachia*, mas os estudos morfológicos sobre posição destas bactérias na ovogenesis da rainha permanecem limitados. No presente estudo, utilizou-se a formiga-tecelã Neotropical *Camponotus textor* para caracterizar o ovário utilizando técnicas de histologia (HE) e documentar a localização de *Blochmannia* e *Wolbachia* na ovogênese por hibridização in situ de fluorescência (FISH) uma vez que esta formiga apresenta alta taxa de infecção por essas bactérias. Este é o primeiro relato morfológico dessas duas bactérias no mesmo hospedeiro com ovários meroísticos politróficos e revelou que *Blochmannia* foi encontrada dentro do ovócito tardio e *Wolbachia* sempre foi encontrada ao redor dos núcleos das células nutridoras. Nossos resultados fornecem uma sugestão da seqüência de desenvolvimento de quando essas bactérias atingem o ovo, onde *Blochmannia* se estabelece no ovócito primeiramente, e *Wolbachia* só atinge o ovócito pouco antes de completar seu desenvolvimento. Estudos como este podem revelar mais sobre os mecanismos e o momento do estabelecimento desses endossimbiontes no hospedeiro.

Palavras-chave: células nutridoras, ovogênese, transferência vertical, ovócito, células foliculares.

Abstract

Camponotus is a hyper-diverse ant genus that is always associated with the presence of the endosymbiont *Blochmannia* and often also with *Wolbachia*, but morphological studies on the location of these bacteria in the queen's oogenesis remain limited. In the present study we used the Neotropical weaver ant *Camponotus textor* to characterize the ovary using histology (HE) techniques, and to document the location *Blochmannia* and *Wolbachia* in oogenesis through fluorencence in situ hybridization (FISH) since it presents a high rate of infection by of these bacteria. This is the first morphological report of these two bacteria in the same host with polytrophic meroistic ovaries and revealed that *Blochmannia* was found inside of late oocyte and *Wolbachia* was always found around the nuclei of the nurse cells. Our results provide a suggestion of the developmental sequence of when these bacteria reach the egg, where *Blochmannia* first establishes itself in the egg, and *Wolbachia* only reachs the egg shortly before completing its development. Studies like this may reveal more about the mechanisms and timing of the establishment of these endosymbionts in the host.

Key words: nurse cells, oogenesis, vertical transfer, oocyte, follicular cells.

Background

The ant genus *Camponotus* Mayr, 1861 is one of the most diverse and has a worldwide distribution (ANTWEB, [s.d.]; BOLTON, 2016). They have generalist diets and can nest in cavities in trees, in hollow or rotten twigs or in the ground (FERNANDES et al., 2012; MATTA et al., 2013) and some species construct nests in trees with silk produced by larvae, as is the case of *Camponotus textor* Forel, 1899 (RAMALHO et al., 2016b; SANTOS; DEL-CLARO, 2009). This group of ants is also known to have obligatory symbiotic relationships with bacteria (DEGNAN; LAZARUS; WERNEGREEN, 2005; FELDHAAR et al., 2007; GIL et al., 2003; WERNEGREEN et al., 2009) and studies have shown that the main associated groups are *Blochmannia* and *Wolbachia*, corresponding to about 95-98% of all sequencing reads of *Camponotus chromaiodes* Bolton, 1995 (BROWN; WERNEGREEN, 2016).

These symbiotic bacteria may have positive or negative effects on the host. *Blochmannia*, for example, is known for its beneficial effect because it provides a number of aminoacids to the host, thus it has a nutritional role, especially in the early developmental stages of host life (DEGNAN; LAZARUS; WERNEGREEN, 2005; FELDHAAR et al., 2007; GIL et al., 2003; WOLSCHIN et al., 2004). For many arthropod hosts *Wolbachia* is known for its negative effect in manipulating host reproduction, such as parthenogenesis, death of males, feminization, and cytoplasmic incompatibility (CI) (BARR, 1980; FENN; BLAXTER, 2004; ROUSSET; RAYMOND, 1991; STOUTHAMER; BREEUWER; HURST, 1999). For bedbugs, it may aid in nutrition with vitamin B supplementation (HOSOKAWA et al., 2010). However, its function in ants is not known, especially in the workers, who are not able to reproduce themselves (ANDERSEN et al., 2012; RUSSELL, 2012; RUSSELL; SANDERS; MOREAU, 2017). Both bacteria can be transmitted vertically (maternal inheritance), with *Blochmannia* acting as a primary and obligatory endosymbiont and *Wolbachia* as secondary and facultative (ANDERSEN et al., 2012; FELDHAAR et al., 2007; WERNEGREEN et al., 2009).

A previous study by Ramalho et al. [s.d.] (in prep, Chapter II) using next-generation sequencing techniques surveyed the bacterial community present across all stages of development of *Camponotus* colonies. The main bacteria found in the egg and queen were *Blochmannia* and *Wolbachia*, reinforcing the idea that the route of

acquisition of these endosymbionts occurs through maternal inheritance. Thus, morphological studies of the reproductive organs of *Camponotus* ant queens may inform the strategies of the establishment of these endosymbiotic bacteria in the host.

The acquisition of endosymbionts in oviparous insects can occur either at the beginning or in the late stages of oogenesis, although there are few studies on Hymenoptera that present polytrophic meroistic ovary (KUPPER et al., 2016). Insects with polytrophic meroistic ovaries have nurse cells (grouped inside the nurse chamber) and oocytes that alternate along the length of ovariole. This set of nurse chamber (containing the nurse cells) plus the egg chamber (containing the oocyte) is called the ovarian follicle. To investigate tramsmission of *Wolbachia*, several studies have been carried out in *Drosophila* and tsetse flies, which have meroistic telotrophic ovaries, in which the nurse chamber is located in the basal, and in the apical region is where the germarium (containing the stem cells) is found (BALMAND et al., 2013; CASPER-LINDLEY et al., 2011; FRYDMAN et al., 2006). Frydman et al. (2006) were able to experimentally add *Wolbachia* to the *Drosophila melanogaster* abdomen and to monitor their tissue distribution. They found that only 15 days after the infection, *Wolbachia* was detected in the germ line and the transmission route was through the somatic stem cell in the germarium.

Blochmannia has been identified through the fluorescence in situ hybridization (FISH) technique during the oogenesis of *Camponotus floridanus*, a species commonly found in the Nearctic region (KUPPER et al., 2016). These authors found that the bacterium was not present in the germarium, nor in the nurse cells, but they were identified within the oocyte and believed to have been transferred via follicular cells (KUPPER et al., 2016). As both bacteria, *Blochmannia* and *Wolbachia*, were found in large numbers infecting workers of *Camponotus textor*, an exclusively Neotropical species (RAMALHO et al., 2017), this presents the opportunity to investigate the localization of these bacteria in the reproductive tracts of this species. As the genus *Camponotus* is very species rich, it is unclear if this pattern is common across the group.

There are few studies addressing the location of these bacteria in host tissues (ANDERSEN et al., 2012; FELDHAAR et al., 2007; FRYDMAN et al., 2006; KUPPER et al., 2016; STOLL et al., 2010). Other studies that have included insects with telotrophic meroistic ovaries have found *Wolbachia* associated with the nucleus of

the nurse cells, and this bacterium could reach the egg through the cytoplasmic bridges that exist between the nurse chamber and the egg chamber (BALMAND et al., 2013; ZCHORI-FEIN; ROUSH; ROSEN, 1998). But this bacterium has not been localizated in insects that exhibit polytrophic meroistic ovaries. For *Blochmannia*, in *Camponotus floridanus* it is already known that it is present in the young and mature oocyte, via follicular cells in polytrophic meroistic ovaries (KUPPER et al., 2016). This leads to the question of whether other species of *Camponotus* have the same pattern of distribution for *Blochmannia* and what is the location of *Wolbachia* within polytrophic merotistic ovarian tissues. In this study, we used fluorescence in situ hybridization (FISH) to document the distributions of *Blochmannia* and *Wolbachia* symbionts in detail during the oogenesis of the queen of *Camponotus textor* and provide a possible developmental mechanism of how these bacteria reach the egg.

Materials and methods

The silk nest of *Camponotus textor* containing adult, immature individuals, breeding and several queens (polygeny) was collected in August 2016 in Araraquara São Paulo, Brazil (Lat. -21.8262, Long. -48.2001). Eight workers were collected and stored in 95% ethanol for screening of *Wolbachia* and *Blochmannia* infections. Total DNA extraction was performed following the same parameters described by Ramalho et al (RAMALHO et al., 2016a). For the confirmation of these bacteria the primers Bloch 16S-462F and Bloch 16S-1299R (WERNEGREEN et al., 2009); and Wsp81f and Wsp691r (ZHOU; ROUSSET; O'NEILL, 1998), for *Blochmannia* and *Wolbachia* respectively, were used to amplify this target region through PCR, following the same parameters of Ramalho et al. (RAMALHO et al., 2017). Three *C. textor* queens from the same colony were dissected in 1X PBS (Fig 1A). Some ovarioles were submitted to the histological technique Hematoxylin and Eosin (HE) and to the total assembly technique for FISH.

Morphology - Hematoxylin and Eosin

For this technique, the protocol described by Junqueira and Junqueira (1983) was followed. The extracted ovarioles were fixed in 4% paraformaldehyde (w/v) for 24 hours (h), and then transferred to buffer solution (Sodium Phosphate pH 7.4) for 24h. Subsequently, they were dehydrated in an increasing series of alcohols (50% to 95%)

over fifteen minute intervals.

At the end of the dehydration process, the material was transferred to the embedding historesin and held for 5 days. Subsequently, the organs were included in plastic molds containing historesin (Leica Historesin) and polymerizer 3-6 μm thick. The blocks were sectioned on a LEICA RM 2255 microtome. The histological sections were placed on glass slides and hydrated for 1 min in dH_2O and then stained by Harris hematoxylin for 10 minutes. After washing for five minutes with miliQ water, they were stained by aqueous eosin for another five minutes, and again washed in miliQ water. After drying the slides they were dipped in xylol and then covered with Canada balsam and a cover slip. The permanent slides were examined and photographed under a LEICA DM750 light microscope.

FISH technique (Fluorescence in situ Hybridization) and confocal microscopy

The ovarioles were separated and fixed in 4% (w/v) paraformaldehyde in PBS at room temperature for two hours. Subsequently, they were washed in 50%, 70% and 100% ethanol baths for 3 min each. The material was placed on StarFrost slides (Knittel Glass, Germany), and dried at room temperature. After drying 40 μL of hybridization buffer (35% formamide, 900 mM NaCl, 20 mM Tris / HCl pH 7.5, 5 mM EDTA, 0.2% SDS) preheated together with 2 ng/μL probe (*Wolbachia* 5' CTAACCCGCCTACGCGCC 3' (ANDERSEN et al., 2012) with Alexa 488-Invitrogen, and *Blochmannia* 5' CCTATCTGGGTTCATCCAATGGCATAAGGC 3' (FELDHAAR et al., 2007) with Alexa 647 - Invitrogen) were added and kept in a humid chamber in the dark at 46 °C for two hours. The slides were then washed with wash buffer (70 mM NaCl, 20 mM Tris/HCl pH 7.5, 5 mM EDTA, 0.01% SDS) and heated again for 30 min at 48 °C and also kept in a humid chamber. Subsequently, the excess washing buffer was removed with miliQ water, and it was allowed to dry at room temperature.

The DAPI (Molecular Probes, USA) (1:500) which stains host nuclei blue, and is thus possible to infer whether bacteria are intra- or extracellularly are present, was placed directly onto the organ for 5 min, and then washed 3x in miliQ water. Prolong Gold (Thermo Fisher Scientific, USA) was used to mount the slide, which was overlaid with a cover slip and sealed with clear nail polish. For the whole-mount laser the Leica

TCS SP5II confocal microscope was used to obtain the photomicrographs, and Leica TCS SP5II software was used for the confocal analysis using maximum projection. To guarantee the specificity of the analyzed probes, a negative control of the material was performed without any probe used, only with the wash buffer, subjected to wavelength lasers 405 nm, 488 nm, 545 nm and 647 nm.

Results

Our PCR-based screening results confirmed 100% *Blochmannia* and *Wolbachia* infection in *C. textor* workers as found in a previous study (RAMALHO et al., 2017). The queens of *C. textor* used in the present study showed that the two ovaries developed with more than hundred ovarioles each (see Fig. 1A). Histological techniques using HE staining showed the *C. textor* ovary exhibits polytrophic meroistic ovaries, composed of several ovarioles (Fig. 1), with each ovariole composed of the germarium in the distal region (Supplementary File Fig. 1), late or mature oocytes flow into a common oviduct in which the spermatheca is present (see scheme Fig. 1B), oocyte, which can be visualized in the early and late stages of development (Fig. 1C), a chamber of nurse cells with nurse cells (Fig. 1D) and a chamber of eggs surrounded by follicular cells with simple cubic epithelium (Fig. 1E).

Additionally, the probe based technique, Fluorescent in situ hybridization (FISH), permitted rapid visualization of ovarioles of *Camponotus textor* queens infected with *Wolbachia* and *Blochmannia*. Through the analysis of the apical region, which is where the germarium (stem cells) of *Camponotus* queens (Supplementary File Fig. 1) reside we did not detect the presence of *Blochmannia* or even of *Wolbachia* (Supplementary File Fig. 1). Also even when the stem cells begin to differentiate in the egg chamber with the oocyte and follicular cells, and the nurse chamber with nurse cells (Fig. 1F and Fig. 2A) the presence of *Blochmannia* and *Wolbachia* could not be found. Later, with the advancement of oocyte development, which is correlated with a decrease of the nurse cells (since there are nutrient passages from this cell to the oocyte), the *Blochmania* and *Wolbachia* markings began to appear, but each in a different location: *Blochmannia* is always found inside the oocyte in a central region (Fig.1G, H, I, and J), and *Wolbachia* is always around the nuclei of the nurse cells (Fig. 2B, C, D, F, G, H). In our results we did not find any polarization of *Blochmannia*, that is, it did not appear displaced in any specific region of the oocyte in this stage.

Discussion

Several endosymbionts that are transmitted maternally use different strategies to establish themselves in the oocyte. For example, in *Marchalina hellenica* (Insecta, Hemiptera), which has a meroistic telotrophic ovary, the infection already appears in the germarium cell, and consequently both the oocytes and the nurse cells have endosymbionts (SZKLARZEWICZ et al., 2013). In the ant, *Cardiocondyla obscurior* Wheeler, 1929, the *Westeberhardia* endosymbiont is found in the nurse cells and is only transmitted to the oocytes in the final stages of their development (KLEIN et al., 2016).

To better understand how *Wolbachia* and *Blochmannia*, two bacteria commonly associated with *Camponotus*, are maternally transferred (see RAMALHO; BUENO; MOREAU, [s.d.] in prep - Chapter II) it is important to include studies of ovarioles of queens using FISH technique. Here we identified when and where these bacteria are located in the oogenesis process and provide a possible developmental mechanism of how these bacteria reach the egg. In addition, this is the first study to show the transovarian transmission of *Blochmannia* and *Wolbachia* during oocyte ovulation in a single host species with polytrophic meroistic ovary (Fig. 2E).

Blochmannia

The results revealed *Blochmannia* present in the already developing oocytes. This could be explained because at this stage of development *Blochmannia* is present in large quantities, permitting detection with these methods. Kupper et al (KUPPER et al., 2016) detected in *Camponotus floridanus* that *Blochmannia* infection is more prevalent at the beginning of oocyte development, still in the follicular cells, but not in the germarium. The *C. textor* germarium region of ovary showed no *Blochmannia* infection, corroborating data from *Camponotus floridanus* (Buckley, 1866) (KUPPER et al., 2016). This may suggest that the infection by this bacterium occurs later in development. Some studies of *Camponotus* have been able to detect infection in young oocytes (BLOCHMANN, F., 1882; BUCHNER, 1918; KUPPER et al., 2016) while others are only able to detect in mature oocytes (SAUER et al., 2002; SCHRÖDER et al., 1996, present study). But *Blochmannia*, whether in the young or mature oocyte, may access the oocyte via follicular cells (BLOCHMANN, F., 1882; BUCHNER, 1918) as the nurse cells do not have this endosymbiont (KUPPER et al., 2016), corroborating

our results.

Additionally, Kupper and collaborators (2016) in a more advanced stage of ovarian development were able to visualize the displacement of *Blochmannia* to the posterior pole of the oocyte, which according to the authors may be explained by the high quantity of yolk present in the late oocyte, moving away *Blochmannia* to this region. In our observations, *Blochmannia* always appears in the central region, and never concentrated towards any of the poles. But this still may occur because our oocytes had not yet arrived at this later developmental stage, instead being distributed in the center of the oocyte, where oocytes are surrounded by follicle cells.

Wolbachia

Our current understanding of the mechanisms involved in *Wolbachia* localization are limited (FRYDMAN et al., 2006; HE; WANG; MONTELL, 2011; TOOMEY et al., 2013). Our results showed that *Wolbachia* were always present inside the nurse chamber, around the nuclei of the nurse cells, intracellularly. This provides additional evidence for the vertical transference of these bacteria. It must then be passed to the oocyte at a later developmental stage. We know that this bacterium has been found in eggs of *Camponotus* even if in low quantities (RAMALHO; BUENO; MOREAU, [s.d.] in prep, Chapter II).

At the beginning of oogenesis, *Wolbachia* was not found in the germarium. This suggests that this bacterium must have an alternative mechanism to ensure that it arrives in the nurser chamber later to insure its vertical transference. Other studies performed with Chalcidoidea wasps and *Glossina* tsetse flies have also found this same distribution pattern of *Wolbachia* associated with nurse cells, and being transferred only late in the stages of oogenesis or even in young embryos (BALMAND et al., 2013; ZCHORI-FEIN; ROUSH; ROSEN, 1998). Zchori-Fein and collaborators (ZCHORI-FEIN; ROUSH; ROSEN, 1998) have been able to demonstrate that the bacterium is passed to the oocyte at later stages, via nurse cells, through cytoplasmic bridges. Our data from *Wolbachia* is the first study to account for meroistic politrophic ovaries in the ant, *Camponotus textor*, and suggests that the same mechanism happens, since we only observe *Wolbachia* around the nucleus of the nurse cells.

For *Wolbachia* to be passed vertically to the progeny, it seems reasonable that it

is present in the stem cells, in the germarium. The low amount of *Wolbachia* in these stem cells could result in the loss of infection that has been observed by Werren (2005). However some studies have demonstrated that there are different strategies and pathways to ensure that this bacterium is passed to the next generation (FRYDMAN et al., 2006; GENTY et al., 2014). Therefore, although *Wolbachia* appears to pass into the oocyte via nurse cells shortly before completing development, another strategy could be for different strains of *Wolbachia* to concentrate at different locations in the oocyte, in order to ensure greater chances of success in the passage to the progeny, as has been observed for *Drosophila* (KOSE; KARR, 1995; VENETI et al., 2004). The results presented here support the hypothesis of *Wolbachia* being passed in the late stages of oocyte development for *C. textor*.

Conclusion

Our study is the first use histology techniques and fluorencence in situ hybridization to visualize the two main bacteria of *Camponotus* during oogenesis of a polytrophic meroistic ovary that presented different strategies. From our findings, we demonstrated that *Blochmannia* appears first in the oocyte, with *Wolbachia* only present in the final stages, before the oocyte completes development. Our results corroborate the idea that *Blochmannia* is transferred into the oocyte via follicular cells, and *Wolbachia* passes laterally straight from the nurse cells to the oocyte through the cytoplasmic connections between the egg chamber and the nurse chamber. Understanding more about the mechanisms of ovarian transmission of these endosymbionts may reveal more about their adaptations that permit the failure or success of *Blochmannia* and *Wolbachia* to colonize new hosts.

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List of Figures

Figure 1

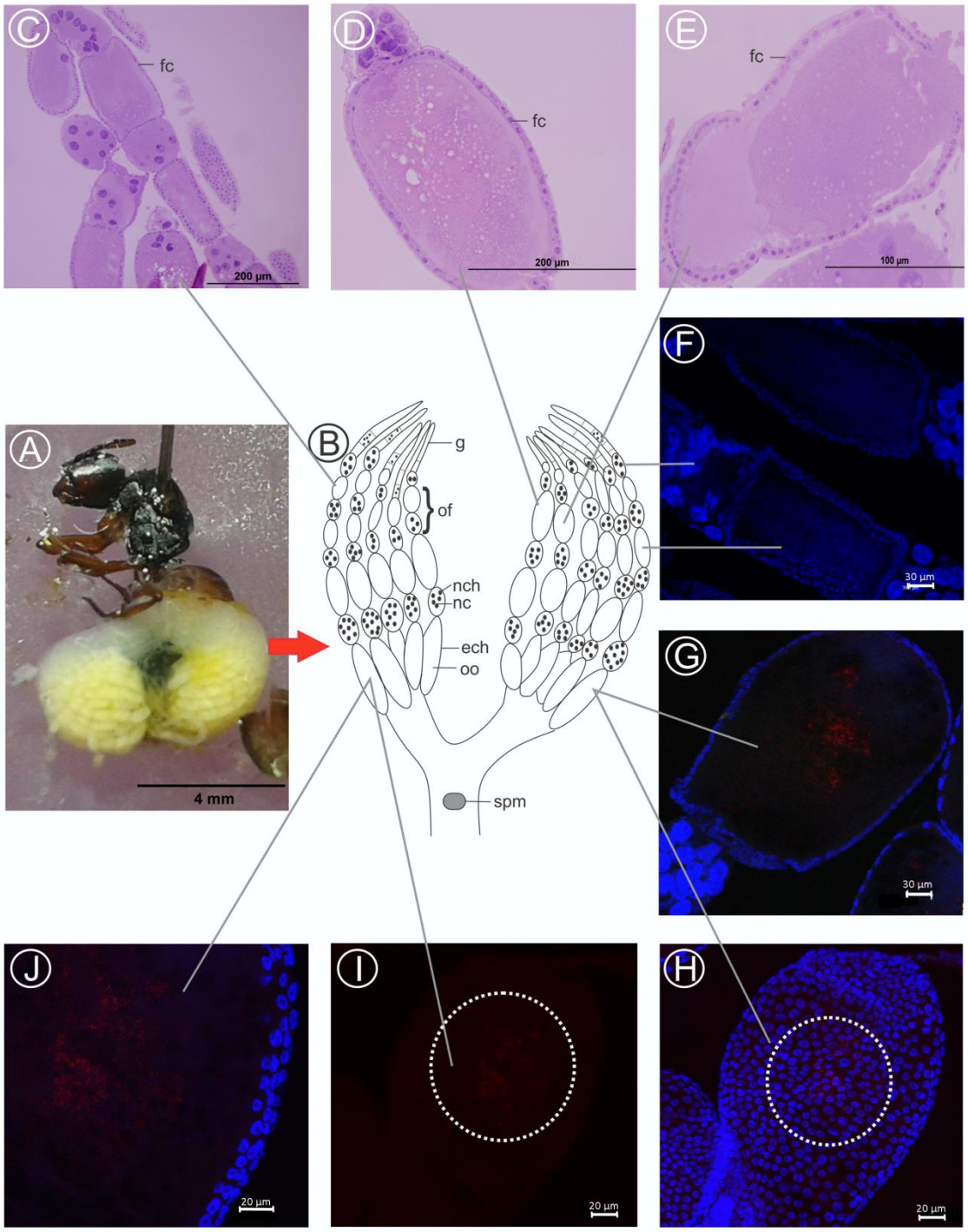
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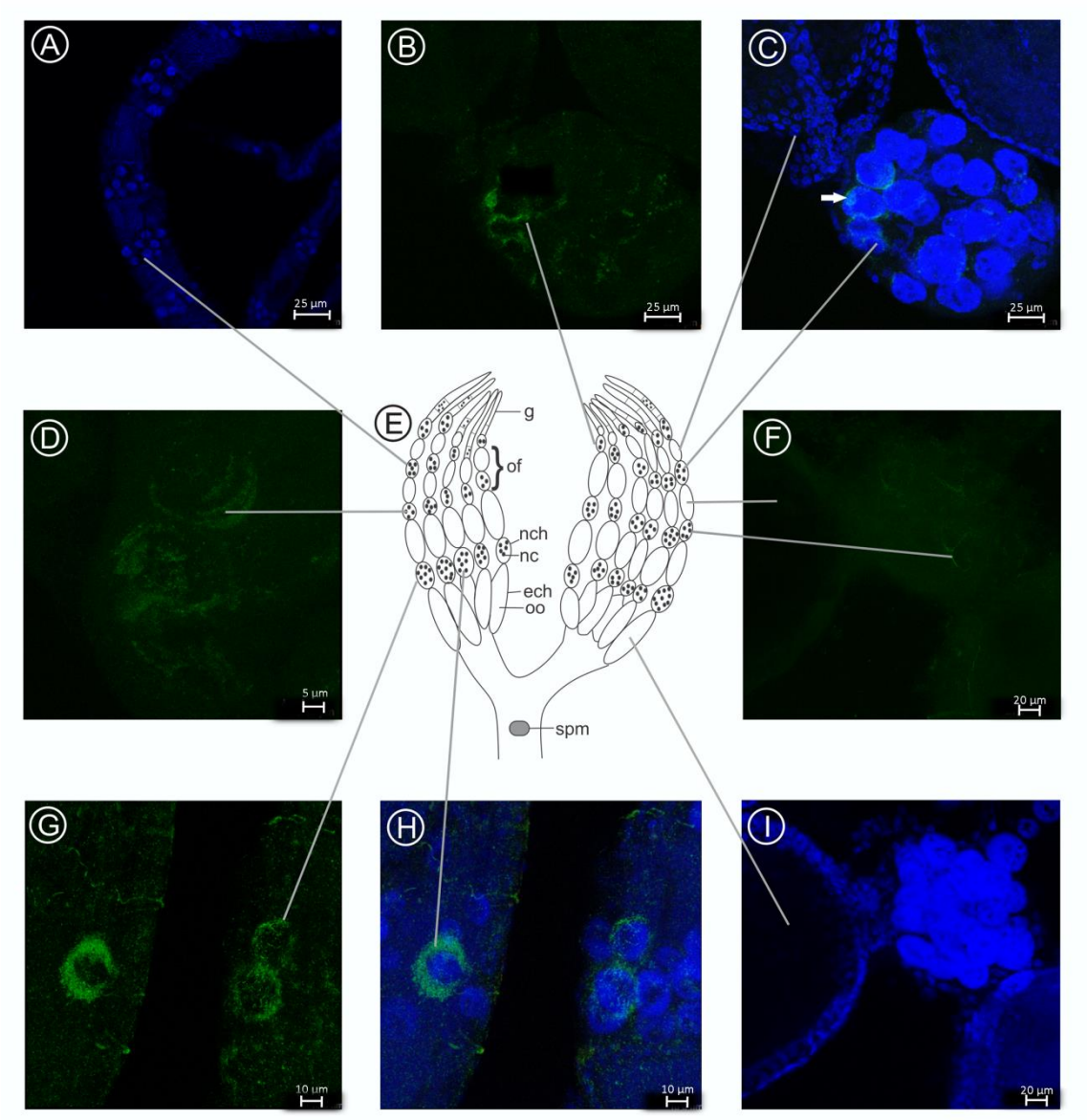
Fig. 2. Schematic representation and photomicrography of the ovaries of *Camponotus textor* submitted to Flourescence in situ hybridization (FISH) with presence of *Wolbachia* in the nurse cells. **A.** Young oocytes and nurse cells without the presence of *Wolbachia*. **B, C, D, F, G** and **H**. Presence of *Wolbachia* around the nucleus of the nurse cells. Arrow is highlighting *Wolbachia* around the nucleus **E.** Scheme of the polytrophic meroistic ovary of *C. textor*, with representation of some ovarioles. **I.** Note that there is no presence of *Wolbachia* within the oocytes. of - ovarian follicle, nch – nurse chamber, ech – egg chamber, oo - oocyte, nc - nurse cells, g - germarium, spm - spermatheca. The process of maturation of the oocyte occurs in the germarium-to-spermatheca direction. Cell nuclei were stained with DAPI and are in blue, and *Wolbachia* is shown in green (Alexa 488 Invitrogen).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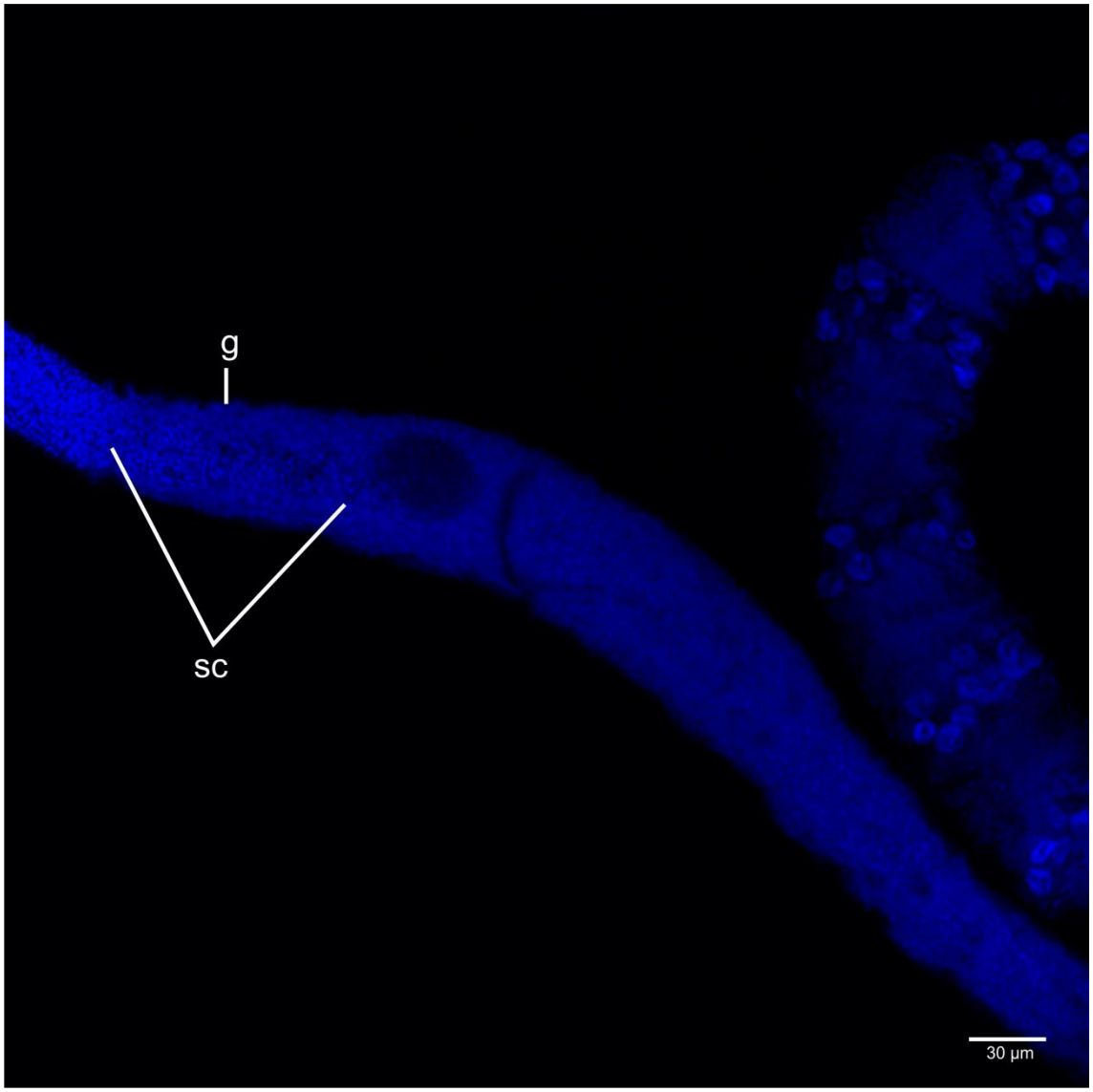
Supplementary Material

Supplementary Fig. 1 - Photomicrography of the region of the *Camponotus textor* germarium submitted to Flourescence in situ hybridization (FISH) without the presence of *Blochmannia* and *Wolbachia*. g- germarium region, sc - stem cells. Cell nuclei were stained with DAPI and are blue.



Considerações Finais

Os resultados presentes nesse estudo permitiram avançar no conhecimento a respeito da variação natural da microbiota dos insetos, o que permite entender importantes aspectos da biologia do hospedeiro. Estes estudos demonstraram que existem diversos fatores que podem influenciar a comunidade bacteriana associada a formiga, como a filogenia do hospedeiro, o gênero, a colônia, a ontogenia, diferentes partes do corpo e o ambiente em que a formiga foi coletada. Adicionalmente, na analise da ovogênese de *C. textor* é possível sugerir mecanismos adaptativos que garantem que as principais bactérias cheguem ao ovo. Da mesma forma, verificou se que o técnicas de sequenciamento de nova geração, hibridização "in situ" fluorescente (FISH) e microscopia confocal podem ser utilizadas como excelentes ferramentas para estudos de relações simbióticas.

Para *Polyrhachis*, o estudo revelou as principais bactérias associadas aos subgêneros e mostrou a presença diversas bactérias nunca antes associadas a formigas. Também mostrou que existem algumas bactérias especificas para um determinado subgênero, o que corrobora os resultados obtidos em que a filogenia do hospedeiro é um dos fatores relevantes que interferem na comunidade microbiana deste grupo.

O presente estudo também mostrou que *Colobopsis* possui uma microbiota distinta de *Camponotus*, apesar serem gêneros relacionados filogeneticamente. Nas análises da microbiota das colônias, verificou se a presença de um padrão específico da espécie, que exerce uma forte influencia na composição da comunidade bacteriana. Ao analisar os diferentes estágios de desenvolvimento dentro de uma colônia, percebeu se como a comunidade bacteriana é conservada, reforçando que a trofalaxia e o intenso contato dentro de uma colônia podem atenuar as diferenças nas microbiotas.

Ao analisar separadamente as três partes do corpo de *Camponotus*, verificou-se que o gáster possui comunidades bacterianas mais abundantes, porém principalmente composta por *Blochmannia* e *Wolbachia*. O presente estudo também revelou uma diversidade bacteriana maior na cabeça e mesossoma, mas em menor abundância. Ao relacionar a similaridade de comunidades com base no local de coleta do hospedeiro, observou-se que a localidade é um fator determinante na similaridade de amostras, sugerindo que muitas das bactérias, especialmente aquelas da cabeça e o tórax, são provavelmente adquiridas no ambiente ou pelos alimentos que ingerem.

Após a confirmação de que as duas principais bactérias de *Camponotus* – *Blochmannia* e *Wolbachia* já estavam presentes no ovo, a marcação dessas bactérias

pela técnica de FISH nos ovários de *C. textor* permitiu ampliar os conhecimentos dos mecanismos de transmissão ovariana desses endossimbiontes e sobre suas adaptações. Pela primeira vez foi possível marcar concomitantemente as duas principais bactérias de *Camponotus* durante a ovogênese no ovário meroístico politrófico. Assim, foi observado que *Blochmannia* aparece primeiro no ovócito e *Wolbachia* só deve ser transferido nos estádios finais, antes do ovócito completar o desenvolvimento. Esses resultados corroboram a ideia de que *Blochmannia* é transferida para o ovócito via células foliculares e *Wolbachia* passa lateralmente direto das células nutridoras para o ovócito através das conexões citoplasmáticas entre a câmara de nutridora ee câmara de ovo.

As principais bactérias associadas a essas formigas pertencentes a tribo Camponotini devem ter um papel importante na sobrevivência, apesar de ainda serem desconheciadas. Podem também influenciar a ecologia e a evolução do hospedeiro. Nos últimos anos, abordagens genômicas tem se tornado cada vez mais acessíveis, podendo ser utilizadas como excelentes ferramentas para explicar diversos fenômenos biológicos, como as relações intra e interespecíficas, a ecologia de espécies, além dos mecanismos moleculares, fisiológicos, comportamentais e evolutivos do hospedeiro. Essas abordagens têm o potencial de gerar metadados que podem contribuir para o entendimento das interações simbióticas que envolvem o campo da mirmecologia. Portanto, unir informações do hospedeiro com o microbioma permitirá entender a complexidade evolutiva dessas associações na natureza.