

Co-diversification of *Camponotus* and Its Primary Endosymbiont *Blochmannia* in the USA

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Abstract

Blochmannia is a primary intracellular endosymbiotic bacteria found within the ant tribe Camponotini (Family Formicidae: subfamily Formicinae). *Blochmannia* is transmitted vertically through maternal host lineages and synthesizes essential amino acids for its host species. The high fidelity of vertical transmission observed means that close co-diversification is expected between *Blochmannia* and their Camponotini hosts. This study expands on past data on USA Camponotini-*Blochmannia* associations and adds 23 new species to the host COI mitochondrial phylogeny and the 16S ribosomal DNA bacterial phylogeny. Using statistical analyses we found evidence of *Blochmannia* co-diversification with the host with enhanced species coverage, and one clear host shift is documented.

Keywords: horizontal transmission, phylogeny, symbiosis

Introduction

Many insects have ‘primary’ endosymbionts that are closely linked to host survival and fecundity. Primary endosymbionts often show very close diversification with their hosts, in which case their respective phylogenies will be congruent (Baumann & Baumann 2005, Moran et al. 1993, Munson et al. 1991, Spaulding & von Dohlen 2001, Thao et al. 2000). Vertical transmission can lead to co-diversification as host and endosymbiont. Taxonomic groups that have extensive co-diversification with primary symbionts are often much more diverse than their sister taxa, as in the case of the Order Hemiptera within the Superorder Acercaria (Johnson et al. 2018). Here we focus on the co-diversification of the endosymbiotic bacteria *Blochmannia* found in the ant tribe Camponotini (Family Formicidae: subfamily Formicinae).

There have been extensive studies on the co-diversification of bacteria and their ant hosts in another bacterial genus *Wolbachia*. Unlike *Blochmannia*, *Wolbachia* is found widely across Insecta and does not always have perfect vertical transmission, and does not show close co-diversification. *Wolbachia* is a commonly found

endosymbiont in ants, including Camponotini (Russel 2012). There are many documented cases of horizontal *Wolbachia* transmission within Hymenoptera (Gerth et al. 2013, Raychoudhury et al. 2009, Tseng et al. 2019), including ants (Boivin et al. 2014, Frost et al. 2010, Tolley et al. 2019). This contrasts with *Blochmannia*, in which the existence of horizontal transmission has been suggested (Degnan et al. 2004) but has not been observed experimentally (Ramalho et al. 2017, Saur et al. 2000).

Blochmannia

Blochmannia is an endosymbiotic bacteria that resides in bacteriocytes (Saur et al. 2002) of its host ants. It was discovered to reside in the midgut lining of all castes and the ovarioles of *Camponotus* (Blochmann 1887, Saur et al. 2000). Their proximity to the ovarioles plays a direct role in their vertical transmission (Ramalho 2018). The common ancestor of *Blochmannia* was likely horizontally transferred from hemipterans (Gil et al. 2003, Rafiqi 2020, Ward et al. 2016, Wernegreen et al. 2009). They are endemic endosymbionts to the Camponotini clade of ants and likely play a nutritional synthesis role, providing hosts with vitamins (Degnan et al. 2005, Gil et al. 2003).

Nutritional endosymbionts are often found in herbivorous taxa and can help the host synthesize nutrients missing in their diet (Baumann et al. 2006, Feldhaar & Gross 2009). This can help the host species expand to new niches previously inaccessible due to a lack of nutrients. *Blochmannia* crosses over to the next generation in the egg (Rafiqi 2020, Ramalho et al. 2018), where they contribute to larval nutrition and survival, especially in the absence of essential amino acids (Feldhaar 2007). *Camponotus* are known to be omnivorous but have a lower $^{15}\text{N}/^{14}\text{N}$ ratio than other ants, which indicates a diet skewed towards herbivory (Moreau 2020). There is evidence that many *Blochmannia* supply their Camponotini hosts with riboflavin and vitamin B6 (Williams & Wernegreen 2015), which the ants may lack in their plant-heavy diet.

Perfect co-diversification between *Camponotus* and their endosymbiont *Blochmannia* has been observed in analyses with fewer species (Degnan et al. 2004). The purpose of this study is to generate new host and endosymbiont sequence data for USA Camponotini-*Blochmannia* relationships and use the larger dataset to test if co-diversification is still supported. To accomplish this, Camponotini were collected from the continental USA. We then sequenced and reconstructed phylogenies of host COI mitochondrial DNA and endosymbiont *Blochmannia* 16S rDNA using maximum-likelihood and Bayesian methods. COI mitochondrial and 16S rDNA genes were chosen for their known phylogenetic signal and cost-effectiveness (Mardulyn & Whitfield 1999, Nunez & Oleksiak 2016).

Materials and Methods

Collection of Specimens

Ants were collected during June 2021 from various sites in the USA. Ten species were collected by S. Wang from sites in the states of New Mexico, Texas, Louisiana, Mississippi, and Pennsylvania. An additional 11 species were collected by collaborators in Texas, Florida, and Arizona (see Table 1). Ants were collected directly into

95% EtOH. These samples were combined with the 16 species sequenced in Degnan et al. 2004, and unpublished data from Schluns et al., which brings this study to a total of 39 sequenced Camponotini. This makes up 62% of the 63 species of valid extant Camponotini species present in the continental USA. Vouchers were point mounted and identified to species with keys (Macgown n.d., Hansen & Klotz 2005, Snelling 2006, Snelling 1988) and deposited in the Cornell University Insect Collection (CUIC) under voucher numbers CUIC000007039-CUIC000007062.

DNA Isolation

Ant petiole + gaster segments were dissected for genetic analysis. These dissected samples were rinsed for 10 seconds each in 95% ethanol, 10% bleach, and pure water. Each cleaned sample was placed in a 1.5mL tube with a ceramic bead. The OMNI Bead Mill Homogenizer was used to homogenize the samples. Samples were checked visually, and sterile pipette tips were used to crush any remaining large tissue pieces. Vials were incubated with the lysis buffer at 56°C overnight in a VWR hybridization oven. Genomic DNA was then extracted from the samples using a Qiagen DNEasy™ DNA extraction kit (Qiagen Inc., Valencia, CA), according to the manufacturer protocols.

Polymerase Chain Reaction (PCR) Amplification

PCR reactions were performed in 0.2mL strip tubes, each with 5.5μL water, 12.5μL Taq PLUS Master Mix (Invitrogen, Waltham, MA), 2.5μL 10mM forward primer, 2.5μL 10mM reverse primer, 1.0μL bovine serum albumin (BSA) from Thermo Scientific (Thermo Scientific, Vilnius, LT), and 1.0μL DNA product from the Qiagen DNEasy™ (Qiagen Inc., Valencia, CA) extraction. CAS 7732-18-5 Molecular Biology Water was obtained from VWR Life Sciences (VWR Chemicals, LLC, Solon, OH) and UV sterilized for 10 min. before use.

The primers C1-J-1754F, C1F356F, and C2-N-3661R (Integrated DNA Technologies, Inc., Coralville, IA) were used to amplify the COI/II

mitochondrial genes (Simon et al. 1994, Degnan et al. 2004, Gómez-Valero 2008, Wernegreen et al. 2009). However, when the primers were used with the same methods in the referenced papers, they failed to produce adequate concentrations of PCR product. Instead, LCO-1490 and HCO-2198 primers were used to sequence 626 to 743bp long fragments of the COI mitochondrial gene (Supplementary material, Fig. 1). This resulted in a 267bp overlapping region with the COI/II fragments in Degnan et al. 2004. COI fragments were initially denatured with a Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad, Hercules, CA) at 94°C for 2 min., then 35 cycles (94°C denaturation for 1 min, 45°C annealing for 1 min, 72°C extension for 2 min), then a final 72°C extension for 4 min. before going to a holding temperature of 4°C. 16S rDNA was amplified with

Bloch16S-462F and Bloch16S-1299R primers (Degnan et al. 2004, Ramalho 2013, Wernegreen et al. 2009), which produced fragments from 763 to 824bp in length. 16S rDNA samples were denatured on the Bio-Rad C1000 Touch™ at 94°C for 2 min., then 30 cycles (95°C denaturation for 20 sec.0, 60°C annealing for 50 sec, 72°C extension for 90 sec), then a final 72°C extension for 5 min. before going to a holding temperature of 4°C.

DNA Yield Assays and Cleaning

Gel electrophoresis of all PCR products was run with VWR Agarose gel loading dye + GelRed (VWR Chemicals, LLC, Solon, OH) to confirm sufficient yield. Loading dye mix was prepared with 100µL 6X Loading dye, 500µL UV sterilized VWR pure.

Table 1: Camponotini collection localities and data, organized by subgenus

	Species	CUIC Database #	Collection Location	Latitude/Longitude	Accession/Collector #	Collector	
Camponotus	subgenus <i>Camponotus</i>						
	<i>Camponotus americanus</i>	—	Falmouth, MA, USA	—	AY334395, AY334379	Degnan et al. 2004	
	<i>Camponotus chromaiodes</i>	—	Falmouth, MA, USA	—	AY334392, AY334376	Degnan et al. 2004	
	<i>Camponotus laevigatus</i>	—	—	—	JN134908, GU226268	Schluns et al. 2011 (submitted)	
	<i>Camponotus laevisinus</i>	—	Portal, AZ, USA	—	AY334384, AY334370	Degnan et al. 2004	
	<i>Camponotus modoc</i>	CUIC000007039	Clouderoft, NM, USA	32°56'28"N 105°44'13"W	SYW0419	Steven Wang	
	<i>Camponotus novaeboracensis</i>	—	Falmouth, MA, USA	—	AY334394, AY334378	Degnan et al. 2004	
	<i>Camponotus pennsylvanicus</i>	—	Concord, MA, USA	—	AY334391, AY196850	Degnan et al. 2004	
	<i>Camponotus schaefferi</i>	—	Portal, AZ, USA	—	AY334388, AY334373	Degnan et al. 2004	
	<i>Camponotus texanus</i>	—	Rumley, TX, USA	31°10'19"N 98°04'06"W	SYW0428, #ALW6281, UTIC ethanol 254719	Alex Wild	
		CUIC000007040					
	subgenus <i>Hypercolobopsis</i>	<i>Camponotus ulcerosus</i>	—	Portal, AZ, USA	—	AY334390, AY334375	Degnan et al. 2004
	subgenus <i>Myrmaphaenus</i>	<i>Camponotus novogranadensis</i>	CUIC000007041	Sun Valley, FL, USA	26°29'47"N 80°12'44"W	SYW0420	Cole Icenhower
	subgenus <i>Myrmecotoma</i>	<i>Camponotus clarithorax</i>	—	—	JN134859, GU226295	Schluns et al. 2011 (submitted)	
		<i>Camponotus cuahuhtemoc</i>	CUIC000007042	Davis Mtns., TX, USA	30°39'56"N 104°01'45"W	SYW0413	Steven Wang
		<i>Camponotus decipiens</i>	CUIC000007043	Cypress, TX, USA	29°56'19"N 95°43'17"W	SYW0414	Steven Wang
		<i>Camponotus essigi</i>	CUIC000007044	Davis Mtns., TX, USA	30°41'47"N 104°04'57"W	SYW0415	Steven Wang
		<i>Camponotus nearcticus</i>	—	Falmouth, MA, USA	—	AY334396, AY334380	Degnan et al. 2004
		<i>Camponotus sayi</i>	—	Portal, AZ, USA	—	AY334385, AY334371	Degnan et al. 2004
		<i>Camponotus snellingi</i>	CUIC000007045	Monroe, LA, USA	32°32'55"N 92°09'56"W	SYW0425	Steven Wang
		<i>Camponotus subbarbatus</i>	CUIC000007046	Amity, PA, USA	40°28'39"N 75°16'00"W	SYW0427	Steven Wang
	subgenus <i>Myrmobrachys</i>	<i>Camponotus mina</i>	CUIC000007047	Camp Creek, AZ, USA	33°52'09"N 111°44'32"W	SYW0418	Jake Nitta
		<i>Camponotus planatus</i>	CUIC000007048	Sarita, TX, USA	27°08'04"N 97°47'34"W	SYW0421	Steven Wang
	subgenus <i>Myrmosphincta</i>	<i>Camponotus sexguttatus</i>	CUIC000007049	Sewall's Point, FL, USA	27°12'25"N 80°12'12"W	SYW0424	Cole Icenhower
	subgenus <i>Myrmothrix</i>	<i>Camponotus atriceps</i>	CUIC000007050	Arroyo City, TX, USA	26°20'25"N 97°25'14"W	SYW0412	Steven Wang
		<i>Camponotus floridanus</i>	—	Gainesville, FL, USA	—	AY334397, AY334381	Degnan et al. 2004
	subgenus <i>Tanaemyrmex</i>	<i>Camponotus castaneus</i>	—	Falmouth, MA, USA	—	AY334393, AY334377	Degnan et al. 2004
		<i>Camponotus festinatus</i>	—	Portal, AZ, USA	—	AY334386, AY196851	Degnan et al. 2004
		<i>Camponotus fragilis</i>	CUIC000007051, CUIC000007052	Scottsdale, AZ, USA	33°46'41"N 111°56'27"W	SYW0416	Jake Nitta
		<i>Camponotus inaequalis</i>	CUIC000007053	Sewall's Point, FL, USA	27°12'25"N 80°12'12"W	SYW0417	Cole Icenhower
	<i>Camponotus ocreatus</i>	—	Portal, AZ, USA	—	AY334387, AY334372	Degnan et al. 2004	
	<i>Camponotus pudorosus</i>	CUIC000007054, CUIC000007055	Portal, AZ, USA	31°53'26"N 109°10'05"W	SYW0422	Tyler Boehmer	
	<i>Camponotus sansabeanus</i>	—	Portal, AZ, USA	—	AY334382, AY334368	Degnan et al. 2004	
	<i>Camponotus semitestaceus</i>	CUIC000007056	Hunter Creek, AZ, USA	34°18'36"N 111°02'18"W	SYW0423	Tyler Boehmer	
	<i>Camponotus socius</i>	CUIC000007057	Paisley, FL, USA	28°59'02"N 81°33'29"W	SYW0426	Jonathan Ross	
	<i>Camponotus vafer</i>	—	Portal, AZ, USA	—	AY334383, AY334369	Degnan et al. 2004	
	<i>Camponotus vicinus</i>	—	Portal, AZ, USA	—	AY334389, AY334374	Degnan et al. 2004	
	<i>Camponotus zonatus</i>	—	Little Havana, FL, USA	25°47'17"N 80°14'23"W	SYW0429	Queen coll. Armando Placeres, workers reared Aaron Stoll	
		CUIC000007058					
Colobopsis	<i>Colobopsis etiolata</i>	CUIC000007059	Corpus Christi, TX, USA	27°40'03"N 97°19'40"W	SYW0433	Steven Wang	
	<i>Colobopsis impressa</i>	CUIC000007060	Pensacola, FL, USA	30°26'35"N 87°19'37"W	SYW0430	Aaron Stoll	
	<i>Colobopsis mississippiensis</i>	CUIC000007061	Scsums, MS, USA	33°23'33"N 88°42'42"W	SYW0431	Steven Wang	
	<i>Colobopsis obliqua</i>	CUIC000007062	Pensacola, FL, USA	30°26'35"N 87°19'37"W	SYW0432	Aaron Stoll	
	<i>Colobopsis papago</i>	—	—	—	JN134903, GU226272	Schluns et al. 2011 (submitted)	

H₂O, and 150µL GelRed. Each agarose well received 3.0µL PCR product and 3.0µL loading dye mix. Electrophoreses were run in 1x TBE for 40 minutes at 80V and 200mA. An AMRESCO N550-300UL 100bp DNA ladder (VWR Chemicals, LLC, Solon, OH) was prepared alongside each gel to check approximate fragment lengths.

PCR products then underwent DNA purification through the ExoSAP-IT™ Protocol. 1.5µL ExoSAP was added per reaction and hydrolysis cleaning was performed in a Bio-Rad C1000 Touch™ Thermal Cycler at 35°C for 15 minutes, and then 80°C for 15 minutes.

DNA samples were then quantified through a Qubit 4 Fluorometer. 16S rDNA fragment yield was measured against BR STD1 and BR STD2 standards from Invitrogen (Thermo Fisher Scientific, Eugene, OR), and COI fragment yield was measured against HS STD1 and HS STD2 standards from Invitrogen.

DNA Sequencing

A BigDye DNA sequencing reaction was performed on the cleaned PCR products for each forward and reverse primer individually, totaling four separate primer batches of LCO-1490, HCO-2198, Bloch16S-462F, and Bloch16S-1299R. Each vial received 0.3µL Big Dye, 2.0µL 5x Buffer, 0.5µL 10 mM respective primer, 5.2µL pure H₂O, and 2.0µL cleaned PCR DNA. Sequencing reactions were performed in a Bio-Rad C1000 Touch™ Thermal Cycler, at 96°C for 1 minute, 25 cycles (96°C for 10 sec, 50°C for 5 sec, 60°C for 4 min). DNA samples were then submitted to the Genomics Facility at the Cornell Institute of Biotechnology for Ready-to-Load Sanger sequencing.

DNA Analysis

Forward and reverse sequences were assembled in Geneious (Kearse et al. 2012). The COI sequences after aligning and trimming were 267bp in length, the 16S rDNA sequences were 749bp in length. These were then aligned with MUSCLE 3.8.425 (Edgar 2004) with 8 maximum iterations. Alignments were visually checked in

Mesquite 3.61 (Maddison & Maddison 2021), and extra spaces at each end were trimmed off. The fully aligned sequences were then run through a RAxML Maximum Likelihood analysis (Stamatakis 2014) as well as a MrBayes Bayesian analysis (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) on the CIPRES Portal (Miller et al. 2010). jModeltest 2.1.10 suggested TP3uf+I+G for COI and TVM+I+G for 16S rDNA. Since RAxML and MrBayes do not have these models implemented, the GTR+I+G model was used for among-site rate variation instead as an approximate equivalent. For Bayesian analysis, the number of substitution types was set at 6, using a 4X4 substitution model. Markov chain Monte Carlo (MCMC) was run for 10,000,000 generations in MrBayes, sampling every 1000 generations and discarding the first 25% as burn-in.

Co-diversification analysis was tested in Jane 4 (Conow et al. 2010) with the completed RAxML trees. For the genetic algorithm parameters, 100 generations with a population size of 1,000 were run in solve mode on the 39 host + 39 parasite tips for a total of 78 tips. In stats mode, 50 generations on a population size of 100 were run to generate a p-value distribution with the data, including original problem instances, with a sample size of 50, and random tip mapping. Two COI sequences from *Ca. decipiens* and *Co. impressa* failed to generate contigs, so they and their bacterial symbionts were excluded from the co-speciation analysis.

Results

The Sanger sequence contigs generated 626 to 743bp reads for *Camponotus* COI and 763 to 824bp reads for *Blochmannia* 16S rDNA. The total sequences represent 39 host species/endosymbiont associations total distributed across two host genera. Twenty novel COI and 16S rDNA associations were sequenced. All 8 *Camponotus* subgenera present in the continental USA are represented, including 4 species of *Colobopsis* (Table 1, Fig. 1). There were 22 total sequences for *Blochmannia*

16S rDNA, but 2 corresponding host COI contigs failed to align, so those 2 *Blochmannia* 16S rDNA were excluded from further analysis.

Outgroup Selection

Colobopsis was recovered to be sister to all Camponotini in Ward et al. 2016. Wernegreen et al. 2009 found that *Blochmannia obliqua* is also the earliest diverging lineage on their analysis across Camponotini, so the phylogenies were rooted on the node between *Camponotus* and *Colobopsis* to keep monophyly in both genera, with *Co. obliqua* and *B. obliqua* as sister to other USA *Colobopsis* and USA *Colobopsis* symbiont *Blochmannia* respectively. This is supported by *B. obliqua* being measured as the earliest diverging *Blochmannia* lineage in a previous Camponotini dataset (Williams & Wernegreen 2015).

Phylogenetic Modelling

In the *Blochmannia* 16S rDNA, the nucleotide frequencies were calculated with RAxML to be A: 0.283335, C: 0.196855, G: 0.290438, T: 0.229371. In the Camponotini COI mitochondrial DNA, the nucleotide frequencies were calculated to be A: 0.281277, C: 0.239281, G: 0.093596, T: 0.385846.

The reconstructed Bayesian and maximum likelihood trees are shown (Supplementary material, Fig 2), recovered with the GTR+I+G (INVGAMMA) model for sequence evolution (Tavaré 1986). The maximum likelihood trees were chosen for subsequent co-diversification analysis because they had no polytomies. Some of these generated trees have low support or polytomies on some nodes, which is likely due to the limitations of 16S rDNA of being a conserved gene (Poretsky et al. 2014) and may not show strong phylogenetic signal in some cases. Additionally, the limitations of the COI mitochondrial gene for phylogenetic signals must be recognized, including its ability to reach saturation quickly due to A+T richness (Morlais & Severson 2002). The incompleteness of the overlapping 267bp section of the COI mitochondrial gene sequenced also reduces available phylogenetic signal in the data.

Co-diversification Analysis

The Jane 4 (Conow et al. 2010) analysis of co-diversification found a significant p-value distribution from the original cost. Out of 69,064 solutions found, the most common isomorph which consisted of 61.6% of the total was chosen. Confidence values are shown on the nodes (Fig. 2). In statistics mode the original randomized cost was 37. With a sample size of 50, the observed cost had a median of 67 with a standard deviation of 2.18. The p-value cost histogram shows that the original randomized cost of no co-divergence does not overlap with the observed cost, which means that the p-value is significant and indicates possible congruence and co-diversification.

Discussion

The goal of this study was to test for co-diversification between US Camponotini and their *Blochmannia* endosymbionts, expanding on previous data. The purpose of this study is to generate new host and endosymbiont sequence data for USA Camponotini-*Blochmannia* relationships and use the larger dataset to test if co-diversification (Degen et al. 2004) is still supported. This study generated 20 new Camponotini COI sequences and 22 new *Blochmannia* 16S rDNA sequences.

Most of the divergences that Jane 4 shows may result from the analysis picking up minor differences in the topologies of species divergences within a clade, since the *Blochmannia* does not tend to move out of an immediate clade. However, there is one such case where the *Blochmannia* not only jumps out of a clade but between subgenera. It was found that *Blochmannia novogranadensis* jumped between two unrelated subgenera, from *Myrmentoma* to *Myrmaphaenus*. Both the ML topology and the Bayesian bootstrap values showed high confidence in *B. novogranadensis* 16S rDNA within a group of *Myrmentoma*, in a clade with *B. snellingi*, *B. essigi*, and *B. cuauhtemoc*. In contrast, the COI mitochondrial sequences place *Ca. novogranadensis* outside of any *Myrmentoma* species. *Ca. novogranadensis* is introduced to Florida (Deyrup & Belmont 2013, Wetterer 2019)

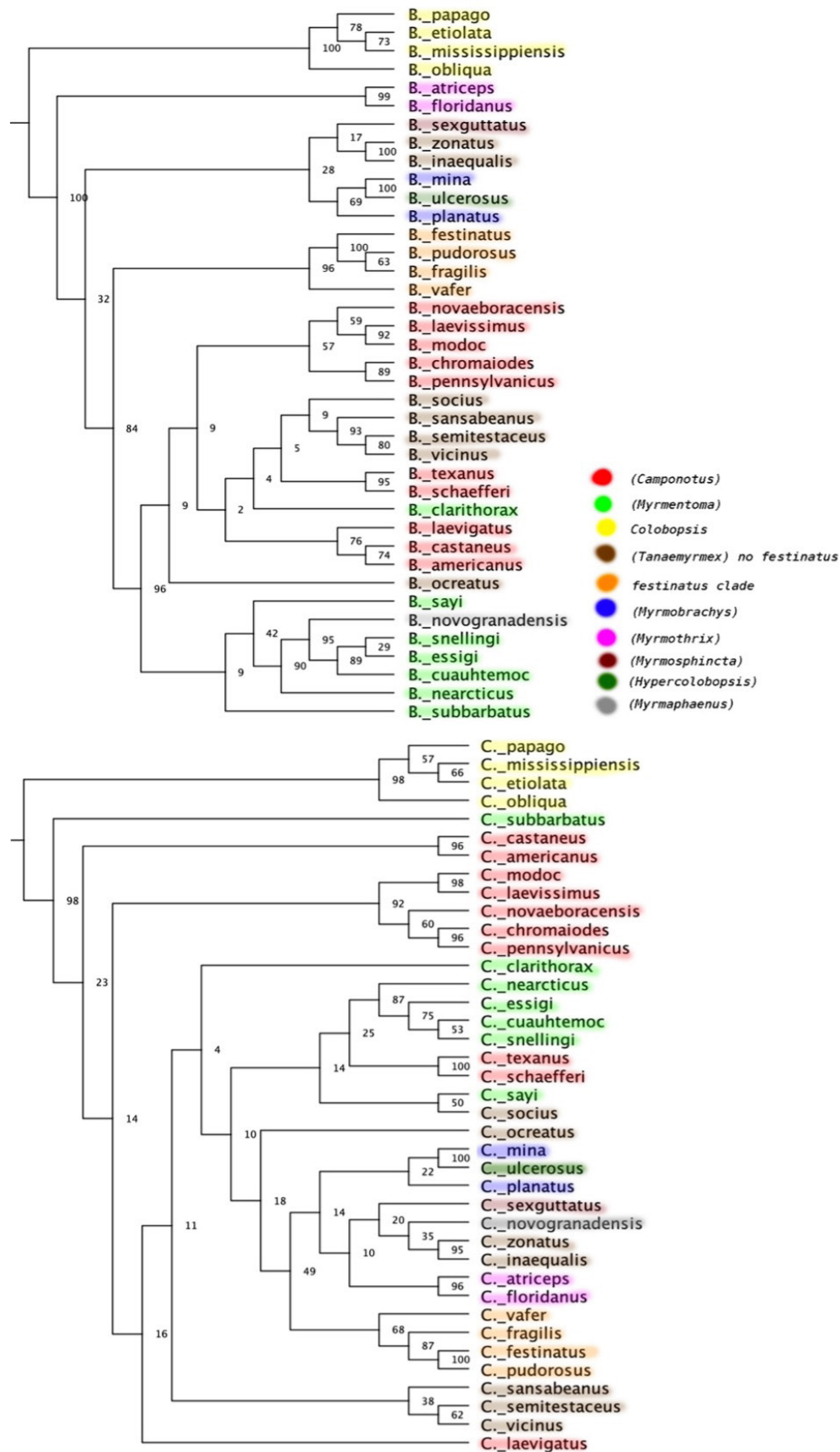


Fig. 1: Host genus and subgenus (in parenthesis) groupings mapped onto maximum likelihood cladograms of COI mitochondrial Camponotini and 16S rDNA *Blochmannia* trees. Calculated percent probabilities shown on nodes. Colors indicate subgenus groupings

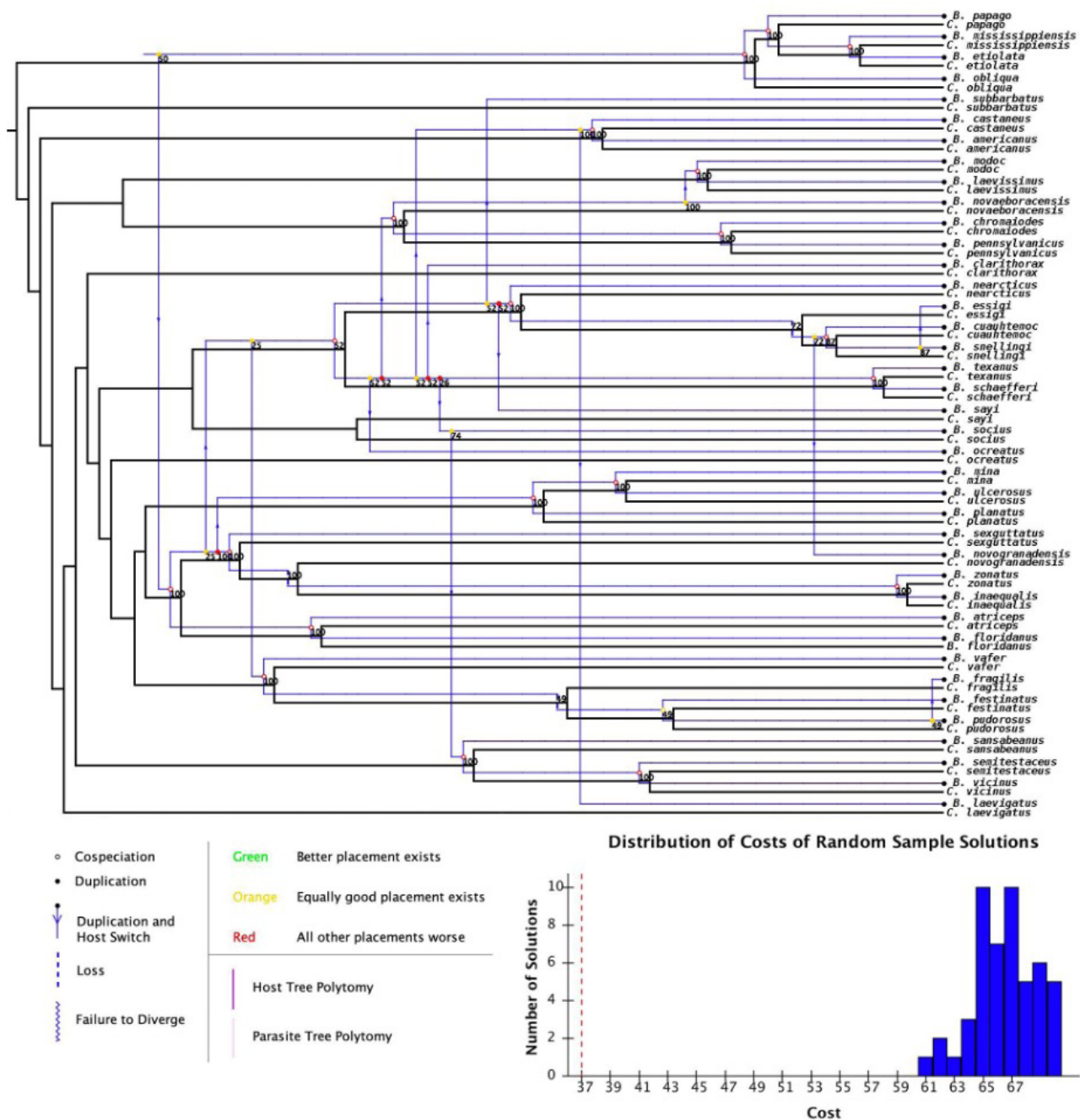


Fig. 2: Jane 4 co-diversification analysis. Original randomized cost is shown as a red dotted line on p-value cost histogram.

and lives arboreally, potentially in close contact with native *Myrmentoma* species. It is possible *Ca. novogranadensis* may have picked up this strain of *Blochmannia* from shared arboreal habitat, but more data is needed to conclude how and when this strain could have been horizontally transmitted.

Coverage of subgenera is increased in this study (Fig. 1), and this data supports many previous taxonomic groupings, like the *Ca. (Tanaemyrmex) festinatus* complex (Snelling 2006), the close

relationship of *Ca. atriceps* and *Ca. floridanus* in the subgenus *Myrmotherix*, and the genus *Colobopsis*. It continues to give evidence that some broad subgeneric categories are inaccurate when grouping species of *Camponotus* by molecular phylogenetic relationships. The subgenera *Camponotus*, *Myrmentoma*, and *Tanaemyrmex* were recovered as polyphyletic, and *Ca. ulcerosus* could prove to be closely related to *Ca. mina* and not a true *Hypercolobopsis*. Like previous studies (Degnan et al. 2004), *Ca. schaefferi* arises from a group of *Myrmentoma* instead of

from the nominate *Camponotus* subgenus, but in this study *Ca. texanus* comes sister to *Ca. schaefferi*, something hypothesized from COI mitochondrial data and morphology (Gadau et al. 1999, Mackay 2019). The recovered trees do not support *Camponotus laevigatus* (=past *quercicola*) as it is in the same clade as *Ca. texanus* and *schaefferi* (Gadau et al. 1999). In this study, *Ca. laevigatus* mitochondrial COI and its 16S rDNA *Blochmannia* symbiont were recovered within or close to a group of temperate nominate *Camponotus*, and never in a monophyletic clade with *Ca. texanus* and *schaefferi*.

Similar to previous studies on only *Camponotus* (Degnan et al. 2004), we have found evidence of co-diversification between *Camponotus/Colobopsis* and *Blochmannia*, which also used a GTR+I+G model for among-site rate variation and an ML phylogenetic tree. The co-diversification found in this study indicates a lack of horizontal gene transfer in *Blochmannia* (Degnan et al. 2004), like genome stability in the related bacterial endosymbionts *Buchnera* and *Wigglesworthia* (Akman et al. 2002 Funk et al. 2000, Tamas et al. 2002, Wernegreen & Moran 2001). Jane 4 codiversification analysis shows that *Blochmannia* have high fidelity of vertical transmission within a species of Camponotini and are tightly bound to their host species, but may be able to horizontally transmit, as observed for the first time in this study.

Acknowledgements

Thanks to Corrie Moreau for funding this project and guidance, and for Manu Ramalho's comments, intensive teaching of lab techniques, and help during analysis for this project. Thanks to the Moreau Lab undergraduate members Corey Reese and Jenna Webb, who helped me in the molecular lab and during data analysis. Thanks to my collaborators Aaron Stoll, Cole Icenhower, Jake Nitta, Jonathan Ross, and Tyler Boehmer, who collected and submitted numerous Camponotini specimens for sequencing. Thanks to Alex Wild at Brackenridge Field Laboratory for providing

the *Camponotus texanus* specimen, Rob Plowes for allowing me to collect on the BFL campus, as well as Joe MacGown for letting me collect on his property in Mississippi.

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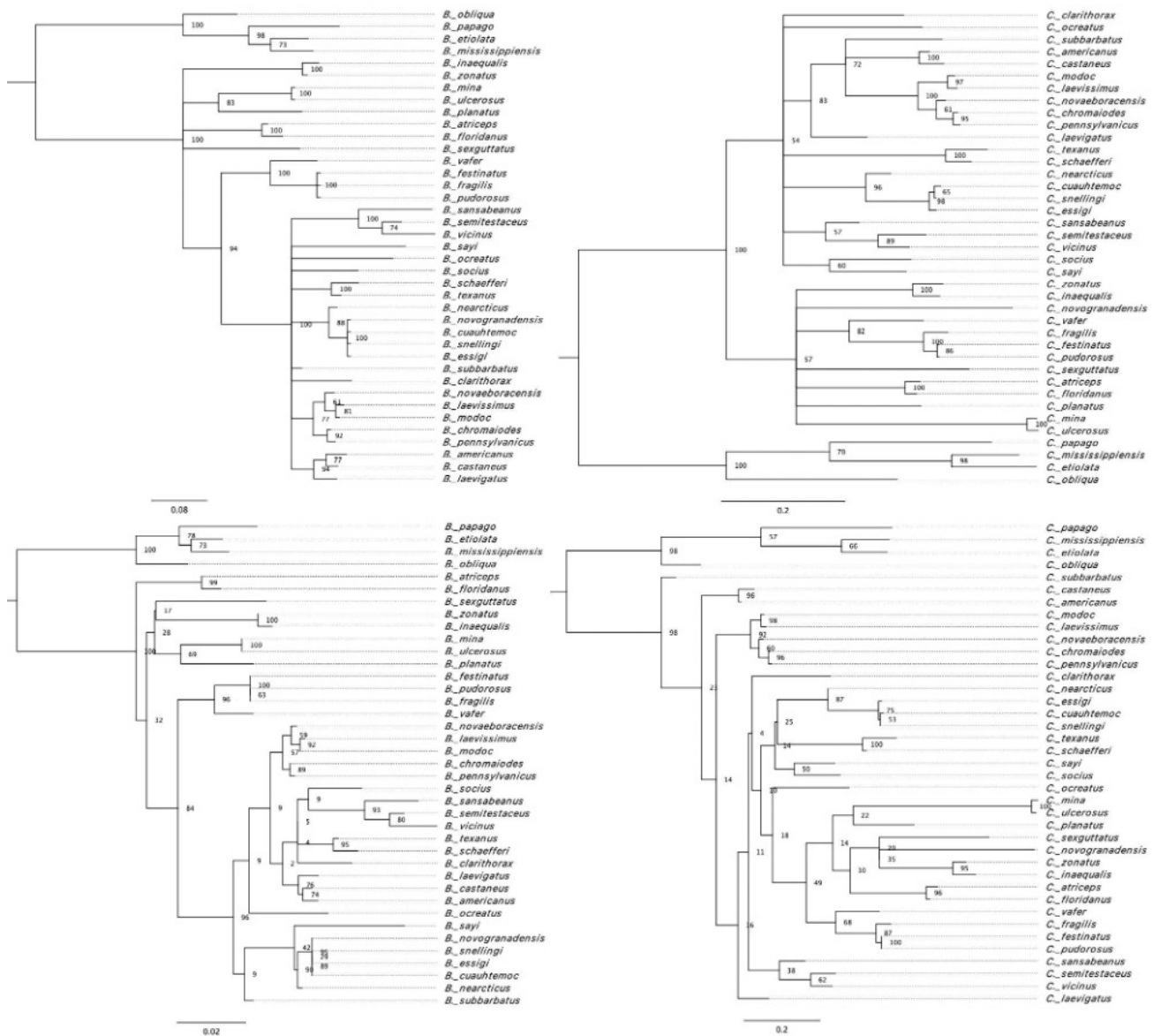
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Supplementary Figures

COI/II	C1-J-1754Fb	CCACGTTTAAATAATATAAGATTTTGAC
	C2-N-3661Rb	CCACAAATTTCTGAACATTGACCA
	C1F356F	GGATCAGGAACAGGTTGAAC
	LCO1490	GGTCAACAAATCATAAAGATATTGG
	HCO2198	TAACTTCAGGGTGACCAAAAAATCA
16S rDNA	Bloch16S_462F	AAACCCTGATGCAGCTATACCGTGTGTG
	Bloch16S-1299R	CCATTGTAGCACGTTTGTAGCCCTACTCA

Supplementary Fig. 1: Primer Sequences



Supplementary Fig. 2: Bayesian analysis (top) and maximum likelihood (bottom) endosymbiont 16S rDNA and host COI consensus trees, with genetic distances to scale and calculated percent probabilities.