Impact of host demography and evolutionary history on endosymbiont molecular evolution: A test in carpenter ants (genus *Camponotus*) and their *Blochmannia* endosymbionts

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**Abstract**
Obligate endosymbioses are tight associations between symbionts and the hosts they live inside. Hosts and their associated obligate endosymbionts generally exhibit codiversification, which has been documented in taxonomically diverse insect lineages. Host demography (e.g., effective population sizes) may impact the demography of endosymbionts, which may lead to an association between host demography and the patterns and processes of endosymbiont molecular evolution. Here, we used whole-genome sequencing data for carpenter ants (Genus *Camponotus*; subgenera *Camponotus* and *Tanaemyrmex*) and their *Blochmannia* endosymbionts as our study system to address whether *Camponotus* demography shapes *Blochmannia* molecular evolution. Using whole-genome phylogenomics, we confirmed previous work identifying codiversification between carpenter ants and their *Blochmannia* endosymbionts. We found that *Blochmannia* genes have evolved at a pace ~30x faster than that of their hosts' molecular evolution and that these rates are positively associated with host rates of molecular evolution. Using multiple tests for selection in *Blochmannia* genes, we found signatures of positive selection and shifts in selection strength across the phylogeny. Host demography was associated with *Blochmannia* shifts toward increased selection strengths, but not associated with *Blochmannia* selection relaxation, positive selection, genetic drift rates, or genome size evolution. Mixed support for relationships between host effective population sizes and *Blochmannia* molecular evolution suggests weak or uncoupled relationships between host demography and *Blochmannia* population genomic processes. Finally, we found that *Blochmannia* genome size evolution was associated with genome-wide estimates of genetic drift and number of genes with relaxed selection pressures.

**KEYWORDS**
Codiversification, genome evolution, genomics, mutualism

**TAXONOMY CLASSIFICATION**
Genomics
1 | INTRODUCTION

Symbiotic associations between eukaryotic and prokaryotic organisms are ubiquitous in nature and highly variable (Dimijian, 2000; Moya et al., 2008). Through symbiotic associations with their single-celled partners, eukaryotes may increase their repertoire of metabolic functions, broadening the range of resources or environments they can exploit (Gil et al., 2004; Moya et al., 2008). Endosymbioses, where one of the symbiotic organisms lives inside the other, are common in insects, may be intra- or extracellular, and the mutualisms or commensalisms range in dependence from facultative to obligate (Kikuchi, 2009). Obligate endosymbioses in insects are particularly common and are often implicated in host nutrition and resistance to pathogens (Anbutsu et al., 2017; Brownlie & Johnson, 2009; Moreau, 2020; Perlmutter & Bordenstein, 2020). When obligate endosymbionts are vertically transmitted from host mothers to their offspring (Bright & Bulgheresi, 2010; Clark et al., 2000), this vertical transmission leads to codiversification between the host and its endosymbiont, and has been demonstrated across insects in the orders Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera, among others (Chen et al., 1999; Clark et al., 2001; Clark et al., 2000; Gil et al., 2004; Gueguen et al., 2010; Heddi et al., 1999; Lo et al., 2003; Moran et al., 2008; Moreau, 2020; Russell et al., 2012; Sauer et al., 2000).

Obligate endosymbionts not only share linked evolutionary histories with their hosts, but endosymbiont demography may also be impacted by host demography (Wernegreen, 2002). Because host and endosymbiont effective population sizes are (at least partially) intrinsically linked, host demographic history may influence the potential strength of selection and rate of genetic drift in endosymbiont genomes. Effective population size is linked with the potential strength of selection and in general, we may expect relaxed selection strengths in relatively smaller population sizes. Additionally, selection in endosymbiont genomes will be partially affected by host-level selection because endosymbiont fitness is partially linked with host fitness (Wernegreen, 2002). In addition to selection, rates of genetic drift are strongly linked with effective population sizes. Asexual organisms—including bacterial endosymbionts—are also subject to accumulation and fixation of deleterious mutations due to their lack of recombination during reproduction (Moran, 1996; Muller, 1964; Pettersson & Berg, 2007). In a simulation study, Rispe and Moran (2000) showed that endosymbiont mutation fixation rate was higher in relatively smaller host populations. However, endosymbiont population sizes could also be decoupled from host population sizes. For example, endosymbiont transmission population bottlenecks (Mira & Moran, 2002) that vary in different host populations or species would lead to variance in the relationship between host and endosymbiont population sizes. Additionally, endosymbionts may be subject to within-host selection (Perreau et al., 2021) that could differentially change endosymbiont population sizes in different host populations. Despite the potential for host demography to shape endosymbiont molecular evolution, this topic has been largely unexplored in wild host and obligate endosymbiont systems.

The symbiotic relationship between carpenter ants (genus Camponotus Mayr, 1861) and their Blochmannia bacterial endosymbionts is an ideal system for investigating the influence of host demographic history on its associated endosymbioses. Camponotus is the second largest ant genus with over 2000 species grouped in 45 subgenera (AntWeb, 2022; Ward et al., 2016); these ants are common in woodlands across most of the world (Mackay, 2019; Wilson, 1976). Camponotus belongs to the formicine tribe Camponotini that is composed of eight extant genera (Ward et al., 2016). Camponotines have maintained a relationship with Blochmannia for about 40 million years (Wernegreen et al., 2009); Blochmannia is a vertically transmitted, obligate intracellular bacterial symbiont (Ward et al., 2016) that was first recognized during the late 1800s (Blochmann, 1892). Blochmannia are found in specialized cells (bacteriocytes) associated with host midgut tissue and found in the ovaries and oocytes of reproductive females (Ramalho et al., 2018; Wernegreen et al., 2009; Wolschin et al., 2004). Blochmannia provide amino acids to their hosts (Feldhaar et al., 2007), and, consistent with a long-term endosymbiosis, there is evidence that Camponotus and Blochmannia have histories of co-speciation because the evolutionary history of the symbionts reflects that of the ants (Degnan et al., 2004; Sauer et al., 2000; Wernegreen et al., 2009).

Several aspects of the Camponotus–Blochmannia relationship have been studied in detail, including location of the symbionts in the host body (Kupper et al., 2016), transmission method (Ramalho et al., 2018), relative abundance and transcriptional variation across host developmental stages (Ramalho et al., 2017; Stoll et al., 2009), the effects of host development and reproduction on symbiont replication (Wolschin et al., 2004), and the endosymbiont’s beneficial role in host nutrition (Feldhaar et al., 2007). One aspect of the Camponotus–Blochmannia relationship that remains poorly known is how host evolutionary and demographic histories affect endosymbiont molecular evolution. A few studies to date have examined the evolution of entire Blochmannia genomes; in Blochmannia vafer, there is some evidence of ongoing purifying selection (Williams & Wernegreen, 2012), and in comparative analyses across three Blochmannia genomes, gene loss patterns differ across lineages, suggestive of differential selective pressures in different host lineages (Williams & Wernegreen, 2015).

Here, we aimed to study how Camponotus demography and evolutionary history impacts molecular evolution in Blochmannia endosymbions using data from seven Camponotus species (Figure 1; Table 1). Our study taxa include seven species from two subgenera in the genus Camponotus: Tanaemyrmex Ashmead, 1905 (N = 3 species) and Camponotus (N = 4 species). All the species included here are large ants (major workers >1 cm) associated with woodlands in western North America (Table 1; Table 2). Western North American species of the subgenus Tanaemyrmex tend to nest in the soil including soil under rocks or logs while the subgenus Camponotus tends to nest in decaying logs and stumps (Mackay, 2019). Carpenter ants are omnivorous; their feeding is comprised of opportunistic predation and foraging of animal and plant-derived resources (Mackay, 2019) and they are thought to have nitrogen-poor diets (Moreau, 2020).
FIGURE 1  Phylogenetic congruence of host ants and their *Blochmannia* endosymbionts. Branch labels indicate proportion of trees supporting this phylogenetic hypothesis. The ASTRAL species tree topologies were identical to these phylogenies and exhibited 100% quartet support for every relationship. Host trees were rooted with the *Cataglyphis nigra* sample. The *Blochmannia* tree was midpoint rooted. Orange branches in the *Blochmannia* tree indicate branches that vary between the host and endosymbiont phylogenies. Ant photos by JCG and JDM

TABLE 1  Carpenter ant host and *Blochmannia* endosymbiont characteristics

| Species            | Host characteristics | Blochmannia genome characteristics |
|--------------------|----------------------|-----------------------------------|
|                    |                      | Genome-wide $K_p / K_s$ | Genes increased selection | Genes relaxed selection | Gene count* | Genome size* |
| *C. herculeanus*   | 30.58 | 0.2021 | 0.238 | 28 | 13 | 599 | 790,985 |
| *C. laevissimus*   | 10.09 | 0.0638 | 0.277 | 9 | 29 | 578 | 783,856 |
| *C. modoc*         | 22.42 | 0.1637 | 0.218 | 15 | 18 | 600 | 790,461 |
| *C. sp. (1-JDM)*   | 14.11 | 0.0879 | 0.195 | 18 | 10 | 598 | 789,892 |
| *C. sp. (2-JDM)*   | 13.88 | 0.1655 | 0.219 | 21 | 16 | 597 | 780,578 |
| *C. vicinus*       | 40.37 | 0.2393 | 0.207 | 42 | 29 | 596 | 776,986 |

Note: An asterisk (*) indicates calculations that were measured per individual and reported here as the mean per species. $N_e$ = harmonic mean effective population size; $H_O$ = observed heterozygosity (×100).
Because *Blochmannia* provide essential amino acids and may play a role in nitrogen recycling within their hosts (Feldhaar et al., 2007), we may expect species- or environment-specific selective pressures—both increased or relaxed selection—on endosymbiont function in our focal species, and that these selective pressures may be influenced by host demography. To address how *Camponotus* demography and evolutionary history shape *Blochmannia* evolution, we assembled a de novo *Camponotus* genome and 17 de novo *Blochmannia* genomes, as well as resequenced genomes for 17 *Camponotus* individuals. We aimed to address the following questions: (1) Do hosts and endosymbionts exhibit strict phylogenomic codiversification histories? (2) How fast do *Blochmannia* genes evolve and is there rate variation across the genome? (3) Does the rate of host evolution impact the rate of *Blochmannia* evolution? (4) Does host demography shape the strengths of natural selection and genetic drift in endosymbiont genomes?

### TABLE 2 Sampling information for the *Camponotus* individuals used in this study

| Catalog # | ID    | Subgenus | Species   | Lat.  | Long.  | Elev.   | Region          | Nest found or group foraging? |
|-----------|-------|----------|-----------|-------|--------|---------|-----------------|-------------------------------|
| TTU-Z_268552 | C-002 | Camponotus | modoc     | 32.667 | -109.873 | 2761    | Pinañeno        |                              |
| TTU-Z_268553 | C-003 | Camponotus | laevisimus | 32.634 | -109.816 | 2226    | Pinañeno        |                              |
| TTU-Z_268554 | C-005 | Tanaemyrmex | vicinus   | 31.43  | -110.29  | 2012    | Huachucha       | found foraging at night      |
| TTU-Z_268555 | C-006 | Tanaemyrmex | ocreatus   | 31.726 | -110.875 | 1513    | Santa Rita      | found foraging at night      |
| TTU-Z_268556 | C-010 | *Camponotus* | sp. (1-JDM) | 32.419 | -110.735 | 2486    | Santa Catalina  | dried out old small stump     |
| TTU-Z_268557 | C-016 | *Camponotus* | sp. (1-JDM) | 33.812 | -109.159 | 2589    | White Mountains | large pine stump             |
| TTU-Z_268558 | C-018 | *Camponotus* | sp. (1-JDM) | 34.327 | -110.832 | 2303    | White Mountains | large burned pine log         |
| TTU-Z_268559 | C-019 | *Camponotus* | modoc     | 34.369 | -110.996 | 2390    | Central Mogollon | large log                  |
| TTU-Z_268560 | C-024 | Tanaemyrmex | sp. (2-JDM) | 34.909 | -111.529 | 2252    | Central Mogollon | found foraging at night      |
| TTU-Z_268561 | C-028 | Tanaemyrmex | vicinus   | 35.928 | -111.914 | 2279    | South Rim       | nest in soil                 |
| TTU-Z_268562 | C-029 | Tanaemyrmex | sp. (2-JDM) | 35.928 | -111.913 | 2278    | South Rim       | found foraging at night      |
| TTU-Z_268563 | C-036 | *Camponotus* | modoc     | 36.529 | -112.177 | 2672    | North Rim       | large pine stump             |
| TTU-Z_268564 | C-039 | Tanaemyrmex | vicinus   | 36.381 | -112.35  | 2325    | North Rim       | found foraging at night      |
| TTU-Z_268565 | C-046 | *Camponotus* | laevisimus | 37.923 | -109.488 | 2395    | Manti La Sal    | dried pinon log              |
| TTU-Z_268566 | C-049 | *Camponotus* | herculeanus | 38.508 | -109.29  | 2464    | Manti La Sal    | small aspen log              |
| TTU-Z_268567 | C-050 | *Camponotus* | herculeanus | 38.526 | -109.281 | 2815    | Manti La Sal    | adjacent large logs          |
| TTU-Z_268568 | C-056 | *Camponotus* | herculeanus | 38.401 | -108.325 | 2846    | Uncompahgre     | base of small stump          |

Note: ID and Catalog #s = collection IDs that may be associated with specimens deposited at the Texas Tech Natural Science Research Laboratory.

2 | METHODS

2.1 | Field work

We collected *Camponotus* ant specimens from 17 colonies for this study in summer 2018 from Arizona, Colorado, and Utah, USA (Table 2). We actively searched for *Camponotus* colonies by spotting woody debris during the day and tracking *Camponotus* activity during the night. Specimens were placed in cryotubes and frozen with dry ice in the field. Specimens were identified using keys (Mackay, 2019) or comparison with available sequences on NCBI’s GenBank. All specimen collection numbers (Table 2) and throughout the manuscript are associated with voucher specimens housed in the Invertebrate Zoology Collection of the Natural Science Research Laboratory, Museum of Texas Tech University. One individual of each of the sequenced colonies was mounted and photographed using a Macropod Pro 3D system (see ant pictures in Figure 1).

2.2 | *Camponotus* de novo genome assembly and annotation

As of January 2022, the only available genome of a *Camponotus* species is that of *Camponotus* (*Myrmothrix*) *floridanus* (Buckley, 1866) (Genome Accession: GCA_003227725.1 Cflo_v7.5). We decided to assemble the genome of a member of the subgenus *Camponotus* because our research group is focusing on this subgenus in this and several other projects. In April 2022, a somewhat contiguous genome of *C. pennsylvanicus* (a member of the subgenus *Camponotus*) was reported in a preprint (Faulk, 2022); our genome is assembled into scaffolds –10x more contiguous than that of this reported *C. pennsylvanicus* genome (Table 3).

2.2.1 | Genome sample and sequencing

For the reference genome, we chose a North American species in the subgenus *Camponotus* that is currently undescribed (based on
TABLE 3 Camponotus sp. (1-JDM) genome assembly statistics as output by the bbmap stats.sh script

| Statistic                  | CaSp_TTU_1.0 |
|----------------------------|--------------|
| # scaffolds / contigs      | 695/1738     |
| Largest scaffold / contig  | 28.327 Mbp / 2.935 Mbp |
| Scaffold / contig NSO      | 11/164       |
| Scaffold / contig N90      | 29/785       |
| Scaffold / contig L50      | 10.362 Mbp/501.17 kbp |
| Scaffold / contig L90      | 3.583 Mbp/73.89 kbp |
| GC (%)                     | 34.46        |

phylogenomic analyses; unpublished results). We refrain from naming this reference genome species until we complete a thorough genomic and morphological analysis in the future that includes all Camponotus species in the Camponotus subgenus described from the USA and Canada. Hereafter in the manuscript, we refer to this species with the code name Camponotus sp. (1-JDM). We extracted DNA from four individuals from a single colony of Camponotus sp. (1-JDM) for use in two sequencing methods for genome assembly: (1) long reads with Pacific Biosciences (PacBio) sequencing, and (2) a Hi-C library sequenced with Illumina technology.

For the PacBio sequencing, we used services of RTL Genomics (Lubbock, TX, USA). They performed a high molecular weight DNA extraction using Qiagen’s (Hilden, Germany) MagAttract HMW DNA Kit. A single major worker, with legs and gaster removed, was used for the extraction. The extracted DNA was then used for PacBio SMRTbell library preparation, size selection using a Blue Pippin (Sage Science), and sequencing on four PacBio Sequel SMRTcells 1 M v3 with Sequencing 3.0 reagents. We used the services of the Texas A&M University Core Facility to prepare a Hi-C library. They used a single major worker as input for the Arima Genomics Hi-C kit (San Diego, CA, USA). The Hi-C library was then sequenced on a partial lane of an Illumina NovaSeq S1 flow cell at the Texas Tech University Center for Biotechnology and Genomics.

2.2.2 | Genome assembly and annotation

We assembled the Camponotus sp. (1-JDM) genome in two stages. First, we used Canu v1.9 (Koren et al., 2017) to de novo assemble the PacBio long reads. Second, we used the Hi-C sequence data to scaffold the initial assembly using the 3D-DNA pipeline (Dudchenko et al., 2017; Durand et al., 2016). All commands for the assembly, annotation, and all further analyses are documented on GitHub (github.com/jdmanthey/camponotus_genomes1). We quality checked the genome assembly for potential contamination with BlobTools v.1.0.1 (DOI:10.5281/zenodo.845347; Laetsch & Blaxter, 2017). Briefly, BlobTools attempts to identify contamination through taxonomic annotation and coverage parsing of resequencing data to the reference genome. With the use of BlobTools, we identified ~37 kbp of potential contamination attributed to chordates or mollusks that we subsequently removed from our assembly.

We used a multistep process to annotate transposable elements (TEs) and repetitive elements in the Camponotus sp. (1-JDM) genome: (1) identify de novo repeats and over-represented sequences, (2) manually curate repetitive elements, and (3) mask the genome with these elements to create a TE and repetitive element summary file. First, we used RepeatModeler’s (v1.0.11; A. F. Smit & Hubley, 2008) implementations of RepeatScout, RECON, and Tandem Repeats Finder to identify repeats based on homology, structure, and repetitiveness in the de novo assembly (Bao & Eddy, 2002; Benson, 1999; Price et al., 2005). We refined the RepeatModeler output by filtering matches to closely related sequences in the RepBase invertebrate database v24.03 (Jurka et al., 2005) and then creating consensus sequences of novel repetitive elements.

First, we removed any RepeatModeler output sequences ≥98% identical to RepBase sequences. Second, we used BLAST and bedtools (Camacho et al., 2009; Quinlan & Hall, 2010) to extract genomic regions matching repetitive elements as well as 1000bp flanking sequences. We used these extracted sequences to develop consensus sequences for novel TEs using the following steps: (1) alignment using MAFFT (Katoh & Standley, 2013) implemented in Geneious (BioMatters Ltd), (2) 50% majority consensus sequences in Geneious, and (3) trimming any ambiguous nucleotides on the ends of newly created consensus sequences. For any incomplete consensus sequences where we did not recover TE endpoints, we repeated this prior process up to two times. In addition to identifying de novo repeats and manual curation in the Camponotus sp. (1-JDM) genome, we also repeated this process for the published Formica selysi Bondroit, 1918 genome (NCBI: GCA_009859135.1: Brelsford et al., 2020). We added this species to increase the diversity of ant TEs in our database, which has been shown to improve annotations by including TEs that may have been missed in other curated species (Boman et al., 2019). To help with naming some of our de novo TEs, we assessed homology of newly curated sequences to the invertebrate RepBase database using BLAST. Lastly, we used a combination TE library including the RepBase invertebrate database and all newly curated TEs described here for use in RepeatMasker v4.08 (A. Smit et al., 2015). Repeatmasker output included a masked genome and summarized repetitive and TE content in the Camponotus sp. (1-JDM) genome.

To annotate genes in the Camponotus sp. (1-JDM) genome, we used the MAKER v2.31.10 pipeline (Cantarel et al., 2008). First, we used MAKER to predict genes using proteins from other closely related ant species: Camponotus floridanus (GCF_003227725.1), Formica exsecta Nylander, 1846 (GCF_003651465.1), Lasius niger Linnaeus, 1758 (GCA_001045655.1), and Nylanderia fulva Mayr, 1862 (GCF_005281655.1) (Bonasio et al., 2010; Dhaygude et al., 2019). We used these initial MAKER predictions to train SNAP and Augustus (Korf, 2004; Stanke & Waack, 2003). Lastly, we used the models trained in SNAP and Augustus in a second iteration of MAKER to predict gene models in the Camponotus sp. (1-JDM) genome. We used BUSCO v3 (Simão et al., 2015) with the Hymenoptera single orthologous gene set (set:odb9) to assess genome assembly completeness.
2.2.3 | *Camponotus* mutation rate

We extracted the putative *Camponotus* sp. (1-JDM) coding sequence (CDS) from the assembly using the MAKER output and bedtools. We downloaded the CDS sequences for *Formica exsecta*, *Lasius niger*, and *Nyländeria fulva* (same versions as proteins) for homology-based comparisons. We performed a reciprocal BLAST of all species versus *Camponotus* sp. (1-JDM) using blastn (Camacho et al., 2009) to identify putative homologues across datasets.

To align putative homologues between the four ant species, we used T-Coffee (Notredame et al., 2000). T-Coffee translates nucleotide sequences, aligns them using several alignment algorithms, takes the averaged best alignment of all alignments, and back translates the protein alignments to provide a nucleotide alignment for each gene. Before the final back-translating, we used trimAl (Capella-Gutiérrez et al., 2009) to remove gaps in the protein alignments.

We tested each gene for selection using gene-wide and branch-specific tests for selection in CODEML (Yang, 1997). After correcting significance values for multiple testing using the Benjamini and Hochberg (1995) method, we removed any alignments with evidence for selection. We then extracted and concatenated four-fold degenerate sites from the alignments (*N* = 806,844) using custom R scripts and the R packages “Biostrings” and “seqinr” (Charif & Lobry, 2007; Pagès et al., 2017). With this alignment of four-fold degenerate sites, we identified an appropriate model of sequence evolution using jModelTest (Darriba et al., 2012) and used the GTR + I model of sequence evolution in PhyML (Guindon et al., 2010) to estimate a phylogenetic tree.

To put the evolution of the CDS four-fold degenerate sites in a timed evolutionary context, we downloaded a recent phylogenomic tree of formicine ants (Blaimer et al., 2015) and pruned the tree to the four representative lineages covered by our CDS downloads and novel assembly using the R package “ape” (Paradis et al., 2004) using four species as representatives of those lineages: *Camponotus* (Myrmontoma) hyatti Emery, 1893, *Nylanderia dodo* (Donisthorpe, 1946), *Formica neogagates* Viereck, 1903, and *Lasius niger*. We used the *Camponotus*-specific branch length of the four-fold degenerate sites tree along with divergence time estimates from Blaimer et al. (2015) to obtain an estimate of *Camponotus*-specific mutation rates.

2.3 | Resequeencing *Camponotus* genomes

2.3.1 | Lab work

We resequenced genomes for 17 *Camponotus* individuals from 7 species (Table 2) at moderate sequencing coverage (~10–30×). We used a single individual per colony for sequencing. For each individual, we performed two DNA extractions: (1) gaster and (2) head + mesosoma. We did this because the gaster has a plethora of *Blochmannia* DNA relative to ant DNA (Brown & Wernegreen, 2016; Ramalho et al., 2019). For each extraction, we froze the sample with liquid nitrogen and subsequently pulverized the sample with a sterile mortar and pestle. We then used the pulverized material as input for DNA extraction with QIAGEN (Hilden, Germany) DNeasy blood and tissue kits. We quantified DNA concentrations from the extractions with Invitrogen (Carlsbad, California) Qubit fluorescent quantitation, and pooled the head + mesosoma and gaster extracts at a 0.8:0.2 ratio, respectively, to have good representation of both ant and endosymbiotic DNA for sequencing. Genomic DNA extractions were sent to the Texas Tech University Center for Biotechnology and Genomics for standard Illumina shotgun sequencing library creation and subsequent sequencing on a partial lane of an S4 flow cell on the Illumina NovaSeq6000.

2.3.2 | Filtering and genotyping

First, we downloaded illumina sequencing reads from the NCBI SRA of two published datasets to use as outgroups: *Camponotus floridanus* (SRX022802) and *Cataglyphis nigr* (André, 1881) (SRX5650044). With our newly generated data and the downloaded data, we trimmed adapters and quality filtered the raw sequencing data using the bbduk.sh script of the bbmap package (Bushnell, 2014). We then aligned the filtered data to the de novo *Camponotus* sp. (1-JDM) reference genome with BWA (Li & Durbin, 2009) using the BWA-MEM command. We used samtools v1.4.1 (Li et al., 2009) to convert the BWA output SAM file to BAM format, and lastly cleaned, sorted, added read groups to, and removed duplicates from each BAM file using the Genome Analysis Toolkit (GATK) v4.1.0.0 (McKenna et al., 2010). We used GATK’s functions HaplotypeCaller and GenotypeGVCFs to genotype all individuals for both variant and invariant sites on all scaffolds at least two Mbp in length. We measured the distribution of sequencing coverage using the samtools “depth” command. We used VCFtools v0.1.14 (Danecek et al., 2011) to initially filter all variant and invariant site calls using the following restrictions: (1) genotyped in ≥70% of individuals, (2) minimum site quality of 20, (3) minimum genotype quality of 20, (4) minimum depth of coverage of 5, and (5) maximum mean depth of coverage of 70.

2.4 | *Blochmannia* genome assemblies and annotation

With the raw sequencing data, we used the MinYS pipeline (Guyomar et al., 2020) to assemble *Blochmannia* genomes for each sample. MinYS used samples mixed with host and bacterial DNA in a pipeline that allows targeted assembly of bacterial genomes. First, it maps metagenomic reads to a reference genome using BWA. Here, we used a *Blochmannia pennsylvanicus* genome (NC_007292.1: Degnan et al., 2005) as our target. Next, the pipeline assembles these recruited reads using the program Minia (github.com/GATB/minia), followed by gapfilling the contigs using the program MindTheGap (Rizk et al., 2014). Finally, the pipeline simplifies the graphical
fragment assembly (GFA) output of MindTheGap. The resulting GFA output was then visualized in Bandage (Wick et al., 2015), regions with multiple paths merged by coverage, and output in FASTA format. This process assembled a circular genome for each of the samples in our study. We also downloaded the sequence and annotation of the Blochmannia endosymbiont of Camponotus floridanus for use as an outgroup (NC_005061.1; Gil et al., 2003). We used the NCBI prokaryotic genome annotation pipeline (Tatusova et al., 2016) to annotate genes in each of the Blochmannia genomes.

2.5 Camponotus phylogenomics and population genomics

2.5.1 Phylogenomics

We estimated “gene trees” for nonoverlapping 50 kbp sliding windows using RAxML v8.2.12 (Stamatakis, 2014) with the GTRGAMMA model of sequence evolution. From these gene trees (n = 4770), we estimated a species tree using two methods: (1) maximum clade credibility tree of all input trees using DendroPy (Sukumaran & Holder, 2010), and (2) the coalescent-based species tree approach ASTRAL III (Zhang et al., 2018).

2.5.2 Genetic diversity and demography

We estimated genetic diversity for each individual in two ways. First, we estimated observed heterozygosity simply as the proportion of bi-allelic to total genotyped sites (both invariant and variant) for each individual. Because sequencing depth has the potential to impact estimates of genetic diversity, we also used the program ROHAn (Renaud et al., 2019), which uses a Bayesian framework to estimate rates of heterozygosity while accounting for sequencing depth and per-base quality scores. We found the two estimates to be highly correlated in ingroup samples (r = 0.843, p << .001), so we consider only raw estimates of heterozygosity hereafter.

To estimate demography for each individual, we used the program MSMC2 v1.1.0 (Schiffels & Durbin, 2014). For use in MSMC, we masked genomic regions not genotyped, as these would otherwise be mistaken for runs of homozygosity. It is relevant to note that MSMC estimates are accurate in panmictic populations, but population structure or changes in connectivity between populations through time may mimic changes in population sizes (Chikhi et al., 2018; Mazet et al., 2016). Because of this, some caution should be used when interpreting raw demographic history results. We largely used the demographic histories to estimate harmonic mean population sizes over the past 200,000 years, which is highly correlated with observed heterozygosity (r = 0.850, p << .001). When running MSMC, we allowed up to 20 iterations and up to 23 inferred distinct time segments. We performed bootstrap replicates for each individual to see how signal could vary using different genomic regions. For this, we bootstrapped 1 Mbp segments of the genomes, with a total of 10 bootstrap replicates. We decided to use MSMC for each individual rather than aggregating samples per species because of several reasons: (1) uncertainty of population structure among sampling locations, (2) uneven sampling sizes per species, and (3) low certainty with phasing necessary to run the program with multiple individuals, again because of the small sample sizes per species.

MSMC output is presented relative to a species’ generation time and mutation rate. We used the mutation rate calculated from the de novo genome assembly as described above. Because there are no good estimates of generation times in Camponotus ants, we used a conservative proxy for generation time used in other studies: double the age of sexual maturity (Nadachowska-Brzyska et al., 2015). In Camponotus, previous studies have suggested that the earliest age of queens producing winged reproductives is a minimum of 2 years following colony formation, with the first winged individuals overwintering until the third year (Fowler, 1986; Pricer, 1908). As such, we used 3 years as the age of reproductive maturity and 6 years as the generation time for demographic analyses. This value is generally consistent with generation time estimates of 7–8 years in red harvester ant [Pogonomyrmex barbatus (Smith, 1858)] colonies kept in captivity (Ingram et al., 2013).

2.6 Blochmannia phylogenomics and population genomics

2.6.1 Phylogenomics

We extracted coding (CDS) regions from each Blochmannia genome using bedtools. We then used BLAST to match each gene to genes in the outgroup Blochmannia floridanus to identify putatively homologous genes from each Blochmannia genome. For further analysis, we kept 507 genes present in all samples. We aligned all sequences for each gene using T-Coffee and trimmed any portions of the alignments not present in all samples using trimAl. Next, we used RAxML v8.2.12 (Stamatakis, 2014) with the GTRGAMMA model to estimate a phylogeny for each of the Blochmannia genes. From these gene trees, we estimated a species tree using two methods: (1) maximum clade credibility tree of all input trees using DendroPy (Sukumaran & Holder, 2010), and (2) the coalescent-based species tree approach ASTRAL III (Zhang et al., 2018).

2.6.2 Tests for selection

We used the HyPhy software package (Pond & Muse, 2005) to test for selection in the Blochmannia genes in a phylogenetic framework. We tested for positive selection using aBSREL (M. D. Smith et al., 2015) and we tested for shifts in selection strength across the phylogeny using RELAX (Wertheim et al., 2015). We ran aBSREL in exploratory mode where all branches are tested for positive selection. RELAX requires a set of test branches and reference branches.
to identify shifts in selection strength. As such, we ran RELAX six times, once for each Camponotus species in the study with more than a single individual [i.e., excluding C. (Tanaemyrmex) ocreatus Emery, 1893 and the outgroup].

2.6.3 | Genetic drift

For each species’ Blochmannia genomes, we estimated the rate of non-synonymous substitutions per site (Ks) relative to the rate of synonymous substitutions per site (Ks) using the R package “SeqinR” (Charif & Lobry, 2007). Generally, the Ks/Ka ratio is indicative of the strength of selection in coding genes; in a particular gene, we may expect values much less than one under the effects of purifying selection and values greater than one due to positive selection. However, we expect very low genome-wide Ks/Ka ratios due to selection maintaining function of most genes in the genome (Kuo et al., 2009); in species with smaller effective population sizes, we expect increased genetic drift and reduced efficacy of selection that may result in accumulation of slightly deleterious mutations and higher estimates of genome-wide Ks/Ka ratios (Kuo et al., 2009). As such, we used genome-wide Ks/Ka ratios as a proxy for the strength of genetic drift in each species.

2.6.4 | Gene loss

We tested for gene loss in all Blochmannia genomes assembled for this study. First, we performed an all-to-all protein BLAST (blastp) of all amino acid sequences from coding genes annotated from all samples. From the BLAST analysis results, we tabulated a gene presence/absence matrix for each Blochmannia genome (N = 607 unique coding genes identified from all samples).

2.7 | Co-analyses of Camponotus and Blochmannia data

To estimate correlations between host and endosymbiont traits while accounting for the evolutionary history of the samples, we analyzed all host and Blochmannia traits in the context of phylogenetic independent contrasts (PICs). We estimated PICs for each trait in the R package “ape” (Paradis et al., 2004) which uses the method of Felsenstein (1985). This PIC method assumes Brownian motion of trait evolution and transforms the sampled trait data into statistically independent values (contrasts) that may be used in regressions (Felsenstein, 1985).

2.7.1 | Evolutionary rates

We explored evolutionary rates in Blochmannia genomes in multiple ways. First, we examined variation in Blochmannia gene phylogenies relative to the host species tree. To do this, we calculated the Kuhner and Felsenstein (1994); KF94 distance between the host species tree and Blochmannia gene trees, implemented in the R package “ape” (Paradis et al., 2004). Second, we explored rates of evolutionary change in a phylogenetic context by measuring relative branch lengths in the host species tree versus the Blochmannia gene trees. We calculated these rates of evolutionary change in three groups: (1) subgenus Camponotus, (2) subgenus Tanaemyrmex excluding C. ocreatus because it is on a long branch by itself, and (3) the combined group of the subgenera Camponotus and Tanaemyrmex. Third, we measured nucleotide percent identity for all gene alignments, excluding indels, in the same three groups as aforementioned. Fourth, for each sampled individual, we measured the correlation between host and endosymbiont root-to-tip distance in the species tree phylogenies.

2.7.2 | Demographic influences on drift, selection, and gene loss

For each of the six ingroup species (i.e., excluding C. ocreatus), we measured the association between host population sizes and (1) changes in selection strength in Blochmannia genes, (2) Blochmannia genome-wide Ks/Ka ratios, (3) number of complete genes found in each Blochmannia genome, and (4) Blochmannia genome size.

3 | RESULTS

3.1 | Camponotus reference genome characteristics and molecular clock

Our de novo Camponotus sp. (1-JDM) reference genome was highly contiguous (contig L50 – 500 kbp) with a small number of scaffolds composing the majority of the assembly (scaffold L90 – 3.58 Mbp, N90 = 29; see Table 3). Overall, we had 31 scaffolds greater than 2 Mbp. Although there are no karyotypes for North American species in the subgenus Camponotus, there are estimates for Camponotus ligniperda (Latreille, 1802) (haploid N = 14), C. japonicus Mayr, 1866 (N = 13 or 14), and C. obscuripes Mayr, 1866 (N = 14) from the eastern Palearctic (Hauschteck, 1983; Imai, 1969; Imai & Yosida, 1964). As such, it appears we generated a genome with the contiguity of about two scaffolds per chromosome. While we did have additional signal in the Hi-C contacts to additionally scaffold the genome (Figure 2a), we chose to be conservative and only link genomic regions where we were confident of the signal (Figure 2a).

The difficulty in fully scaffolding the genome may relate to the repetitive nature of the genome; the genome averaged 24.7% repetitive element content with many large portions of the genome exhibiting greater than 70% repetitive content (Figure 2b). Overall, about 13% of genomic windows contained more than 60% repetitive content. A large proportion of the repetitive content was DNA transposons, both previously described and those manually curated for this study. The repetitive landscape is consistent with other ant species exhibiting “islands” of extreme repetitive content in a backdrop of lower genomic repetitive content (Schrader et al., 2014).
Coding gene content is heterogeneous across the genome and is negatively correlated with both repetitive element content and GC% in 100 kbp sliding windows (Figure 2b,c). BUSCO results suggest our genome is nearly complete and representative of other hymenopterans, containing 98% complete genes and 1.2% fragmented genes of the 4415 hymenopteran near-universal single-copy orthologs (Table 4). Using the four-fold degenerate sites from the genome’s CDS regions, we estimated a substitution rate of $1.98377 \times 10^{-9}$ substitutions/site/year.

### 3.2 | Phylogenomics

We estimated an ant host species tree using 4770 “gene trees” estimated in 50 kbp sliding windows across the genome. Both methods we used to create the species tree—maximum clade credibility and ASTRAL—identified an identical topology (Figure 1). Here, each species was monophyletic. The four species in the subgenus *Camponotus*, formed a monophyletic group; in contrast, most species in the subgenus *Tanaemyrmex* formed a clade, but *C. ocreatus* was recovered as more closely related to *C. floridanus* (subgenus *Myrmothrix*) than to other species in the subgenus *Tanaemyrmex*. Between 40% and 99% of gene trees supported the relationships identified in the species tree, while each node had 100% support in ASTRAL analyses (Figure 1).
The *Blochmannia* species tree estimated from 507 gene trees identified a strongly supported phylogeny with a nearly identical topology to the host species tree (Figure 1). The only differences were some relationships between individuals within species (colored orange in Figure 1). In contrast to varying proportions of gene trees matching species tree relationships in the ant hosts, a majority of *Blochmannia* gene trees matched relationships of the species tree (67%–99% support for each node). We measured the KF94 distance between each *Blochmannia* gene tree and the host species tree to identify if any regions of the *Blochmannia* genome were relatively discordant from the host phylogenomic signal. In general, the phylogenetic concordance, as measured by the KF94 metric, was consistent across the entire *Blochmannia* genome (Figure 3a).

### 3.3 *Blochmannia* genome sizes, gene composition, rates of molecular evolution

The *Blochmannia* genomes assembled here largely varied per subgenus. In the subgenus *Camponotus*, the genomes varied in size from ~783 to 792 kbp (Figure 3e). This is consistent with *Blochmannia* genomes from this subgenus already on GenBank [B. pennsylvanicus (NC_007292.1) = 791 kbp; B. chromaiodes (NC_020075.1) = 791 kbp]. In contrast, individuals in the subgenus *Tanaemyrmex* had highly variable *Blochmannia* genome sizes. Five of the genomes ranged in size from 775 kbp to 781 kbp, while B. ocreatus was ~746 kbp and a sequence from GenBank for B. vafer (NC_014909.2) was ~722 kbp (Figure 3e).

![Figure 3](image-url)

**Figure 3** Variation in molecular evolution across the *Blochmannia* genomes. (a) The KF94 distance between the host species tree and *Blochmannia* genes. The KF94 distance measures differences between phylogenetic topologies including branch lengths, where a value of zero is an identical tree. (b) Rates of molecular evolution in *Blochmannia* genes relative to the host species tree. (c) Gene identity percentage in *Blochmannia* genes for alignments with indels removed. For all statistics, lines indicate mean values across windows of 10 genes. (d) Relationship of evolutionary rates between hosts and endosymbionts. PIC = phylogenetic independent contrasts. (e) *Blochmannia* genome assembly sizes for all new assemblies in this study as well as those published on GenBank for these *Camponotus* subgenera. GenBank sequences: *Blochmannia pennsylvanicus* (NC_007292.1), B. vafer (NC_014909.2), B. chromaiodes (NC_020075.1)
The Blochmannia genomes contained between 576 and 601 genes, and in total across all genomes, 607 unique coding genes were annotated. In total, 65 genes exhibited variable presence/absence among the samples sequenced here (Figure 4). Of those 65 genes, 37 exhibited phylogenetic signal of gene loss.

The Blochmannia genes evolved at rates ~20–30× faster than the rate of evolution of the ant hosts, with slight variation across the Blochmannia genome (Figure 3b). Additionally, it appeared that Blochmannia genes in ant hosts of the subgenus Camponotus had a slightly slower rate of evolution than those of the ant host subgenus Tanaemyrmex (Figure 3b). If we use the Camponotus rate of molecular evolution to put Blochmannia rates in a timed absolute context, the mean Blochmannia gene evolution rate is about 5.474 × 10⁻⁸ substitutions/site/year (range = 1.454 × 10⁻⁸ to 1.256 × 10⁻⁷ substitutions/site/year). Species tree root-to-tip distances for host and endosymbionts (trees in Figure 1) showed a significant positive correlation (Figure 3D), suggestive of a genome-wide molecular evolution association between hosts and their endosymbionts.

Blochmannia gene identity within host subgenera was generally consistent across the endosymbiont genome, suggestive of similar evolutionary forces acting across most of the genome at the taxonomic scale of host clades (Figure 3C). We also tried to identify relative rates of evolution and percent sequence identity in intergenic regions. To do this, we performed whole-genome alignments using progressiveMauve (Darling et al., 2010). However, endosymbiont intergenic sequences were so divergent between host subgenera, and in some cases, between host species, that we were unable to recover any high-quality alignments in these regions (e.g., large nonoverlapping sections in these regions of the alignments). Needless to say, the rates of evolution in these intergenic regions are likely much higher than the genic rates in Figure 3b.

We found a strong positive association between Blochmannia genome-wide \(K_s/K_a\) ratios and number of Blochmannia genes with relaxed selection strength \((r = 0.93, p = 0.020)\) (Figure 5a). Blochmannia genome sizes were associated with both \(K_s/K_a\) ratios \((r = -0.90, p = 0.036)\) (Figure 5b) and with endosymbiont genetic drift \((r = -0.72, p = 0.167)\) (Figure 5c).

**FIGURE 4** Blochmannia gene presence/absence phylogenetic heatmap. Of 607 total genes annotated, 65 varied in the presence/absence among samples. 37 of 65 genes lost in at least one individual exhibit phylogenetic signal. Dark green indicates presence, while light yellow indicates absence.

**FIGURE 5** Genome evolution associations in Blochmannia. PIC = phylogenetic independent contrast. Shifts in selection strength were obtained using the program RELAX.
$p = .036$ (Figure 5b) and number of *Blochmannia* genes with relaxed selection strength ($r = -0.72, p = .167$) (Figure 5c).

### 3.4 Impacts of host demography on endosymbiont evolution

Contemporary estimates of host effective population sizes ranged from ~5000 to 50,000 and, with a couple of exceptions, were largely consistent within species (Figure 6). Variation in demographic histories within species may be indicative of variation in among population gene flow (i.e., a lack of panmixia), and therefore, the overall demographic trends for each individual should be interpreted with this in mind. Overall, however, harmonic mean population size through the last 200,000 years was highly correlated with observed heterozygosity for each individual ($r = 0.850$, $p < .001$), and suggests that the MSMC population size estimates reflect population history, even if not simply population size trends (e.g., variance in estimates due to differential population structure). As such, we looked for correlations between endosymbiont traits and host population sizes using these harmonic mean population size estimates.

In positive selection tests, 19 *Blochmannia* genes showed evidence for selection. These signatures of positive selection appeared somewhat randomly in the phylogeny (not shown). We also tested for shifts in selection strength (i.e., intensified or relaxed) among the host lineages for all *Blochmannia* genes. We found a positive relationship between host population size estimates and number of endosymbiont genes with shifts toward intensified selection pressures (Figure 7). In contrast, we found no relationship between number of genes with relaxed selection strength and host demography (not shown). As previously mentioned, some gene loss in endosymbiont genomes exhibited phylogenetic signal (Figure 4). Despite this, we found no evidence for a relationship between host population size and gene loss in endosymbiont genomes (Figure 7c). Additionally, we found no significant associations between host population sizes and either *Blochmannia* genome-wide $K_a/K_s$ ratios (Figure 7b) or *Blochmannia* genome size (Figure 7d).

### 4 DISCUSSION

We sequenced 17 carpenter ant hosts and their *Blochmannia* endosymbionts to address questions about host demography impacts on endosymbiont evolution. We added a whole-genome resource for one species in the subgenus *Camponotus* and more than doubled the number of publicly available *Blochmannia* full-genome sequences. With these resources, we investigated questions related to the (1) codiversification of hosts and endosymbionts, (2) molecular evolution of endosymbionts, and (3) effects of host demography on endosymbiont genome patterns of natural selection, drift, size evolution, and gene loss.

![Figure 6](image_url) Demographic history estimated with MSMC2. Different individuals are represented with differently colored lines and bootstraps are shown with thin lines.

![Figure 7](image_url) Relationships of host demography and *Blochmannia* molecular evolution. Shifts in selection strength were obtained using the program RELAX. Associations in panels B–D were not significant. PIC = phylogenetic independent contrast.
4.1 | Codiversification of carpenter ant hosts and Blochmannia endosymbionts

Using whole-genome sequencing of both carpenter ant hosts and their bacterial endosymbionts, we identified generally strict codiversification (Figure 1). There was some phylogenetic incongruence between host and endosymbiont trees among individuals within species, but all species-level relationships were completely congruent. These patterns are consistent with expectations of co-speciation between hosts and vertically transmitted endosymbionts; similar evidence of codiversification between hosts and endosymbionts has been found in bivalves (Distel et al., 1994), weevils (Toju et al., 2013), flies (Chen et al., 1999; Hosokawa et al., 2012), cockroaches (Clark et al., 2001; Lo et al., 2003), aphids (Clark et al., 2000), psyllids (Thao et al., 2000), and previous studies in carpenter ants (Degnan et al., 2004; Sauer et al., 2000). Generally, previous studies investigating codiversification have inferred phylogenies using one or a few molecular markers; in contrast, by sequencing full genomes for both hosts and endosymbionts, we were able to obtain strongly supported species trees as well as estimate variation in lineage sorting across Blochmannia genes (Figure 3a) with phylogenetic statistics. Here, we expected one of two patterns: (1) consistent signal across the genome with relatively similar overall rates of evolution across Blochmannia genes, or (2) a highly variable landscape of phylogenetic congruence and incongruence caused by variable selective pressures across the genome. Indeed, we found a generally consistent pattern of lineage sorting across the genome as evidenced by a stable estimate of the KF94 statistic across the Blochmannia genome.

4.2 | Rates of molecular evolution in Blochmannia endosymbionts

We found that Blochmannia genes evolved at a rate ~30x faster than the host genome (Figure 3). In addition, intergenic regions were so divergent across lineages that we were not able to align them properly. This endosymbiont–host relative evolution rate is similar to the level reported in Buchnera bacterial endosymbionts of aphids (~36x) by Moran et al. (1995). On an absolute scale, we estimated Blochmannia genes evolve at ~5.474 × 10⁻⁸ substitutions/site/year. Degnan et al. (2004) estimated substitution rates at synonymous sites for four Blochmannia genes and measured rates (~1.094 × 10⁻⁷ substitutions/site/year) about twice as fast as our estimates (from all genes from the entire genome). This difference makes sense because we should expect substitution rates that do not lead to amino acid changes to be faster relative to substitution rates at all genetic sites. Additionally, the absolute rates of molecular evolution identified here in Blochmannia are about an order of magnitude faster than those reported in Buchnera (Clark et al., 1999).

Relatively, fast evolution rates are expected in endosymbionts because of their life histories; insect endosymbionts’ asexuality and propensity to undergo regular bottlenecks because of their mode of inheritance lead to small effective populations sizes and relatively fast evolution (Mira & Moran, 2002; Wernegreen, 2002). As such, endosymbionts also have faster evolutionary rates compared with their free-living relatives, including increased rates of evolution at nonsynonymous coding sites (Brown & Wernegreen, 2016; Degnan et al., 2004; Moran, 1996). Because Blochmannia endosymbionts are asexual and likely to have small population sizes, they may undergo rapid genetic drift and experience accelerated molecular evolution (Pettersson & Berg, 2007; Rispe & Moran, 2000; Woolfit & Bromham, 2003). Additionally, even with small population sizes, obligate endosymbionts may still be under very strong within-host selective pressures, further accelerating their molecular evolution (Perreau et al., 2021).

Endosymbiont molecular evolution rates may vary somewhat across the genome and may have host-lineage-specific rates of molecular evolution (Kuo & Ochman, 2009). Indeed, we found that relative rates of molecular evolution varied somewhat across Blochmannia genomes (Figures 3b,c). Additionally, we found that lineage-specific rates of Blochmannia molecular evolution were correlated with host rates of evolution (Figure 3d). These results are similar to those found in Camponotus and Blochmannia using a small genetic dataset (two host genetic loci and four Blochmannia genetic loci) (Degnan et al., 2004) and suggest that molecular evolution rates—while much faster in Blochmannia—are correlated between carpenter ant hosts and Blochmannia endosymbionts at genome-wide scales. Correlated rates of molecular evolution between hosts and endosymbionts have also been demonstrated in (1) aphids and their Buchnera endosymbionts (Arab & Lo, 2021) and (2) cockroaches and their Blattabacterium endosymbionts (Arab et al., 2020). Overall, our results corroborate previous evidence that endosymbionts have faster rates of evolution relative to both their hosts and to their free-living bacterial relatives, even when evolutionary rates are correlated between hosts and their endosymbionts.

4.3 | Does host demography shape endosymbiont evolution?

Because population genomic processes are influenced by effective population size, and endosymbiont effective population size is intrinsically linked with host effective population sizes (Mira & Moran, 2002; Wernegreen, 2002), we may have the expectation that host demographic patterns partially influence endosymbiont molecular evolution. Here, we investigated whether host demography influenced four factors of endosymbiont genome evolution: (1) natural selection, (2) genetic drift, (3) genome size, and (4) patterns of gene loss.

We found no relationship between host demography and both signatures of positive selection and relaxation of selection strength in Blochmannia genes. In contrast, we found a positive relationship between host population sizes and shifts toward intensified selection pressures in Blochmannia genes (Figure 7a). In endosymbionts in general, we may expect relaxed selection relative to patterns in
free-living bacteria (Wernegreen, 2002). Indeed, selection is often identified in insect endosymbionts, but generally, only in a small fraction of genes (Alleman et al., 2018; Chong et al., 2019; Williams & Wernegreen, 2012). Based on our results (Figure 7a), it appears that shifts in selection pressures may at least in part be influenced by host demographic processes.

We also tested for an effect of host demography on patterns of endosymbiont genetic drift, gene loss, and overall genome size. Here, we found no relationship between host population sizes and these characteristics of endosymbiont genome evolution (Figure 7). We initially anticipated that endosymbiont genetic drift, and associated gene loss and genome size evolution, would occur faster in endosymbionts with small host effective population sizes. While this was not the case with the entire dataset (Figure 7), the host species with the smallest estimated population sizes—C. laevisimus Mackay, 2019—showed the (1) highest rate of endosymbiont genetic drift as measured by genome-wide K_s/K_a ratios, (2) most endosymbiont genes with shifts toward relaxed selective pressures relative to other endosymbiont lineages, and (3) lowest Blochmannia gene count (Table 1).

Additionally, we found that about half of the gene loss was phylogenetically informative (Figure 4). This suggests relatively random patterns of gene loss in the phylogeny; most gene losses lacking phylogenetic signal were singleton gene losses (Figure 4). This is consistent with previous research in Blochmannia endosymbionts identifying lineage-specific gene loss largely due to relaxed selection constraints and genetic drift (Williams & Wernegreen, 2015). Similarly, in cockroach Blattabacterium endosymbionts, Kinjo et al. (2021) found that gene loss was lineage-specific, and that some genes showed parallel gene loss across multiple Blattabacterium lineages.

While we did not find a relationship between host demography and the evolution of Blochmannia genome sizes, we identified associations between a combination of relaxed selection strength, genome-wide genetic drift, and Blochmannia genome sizes (Figure 5). Higher genome-wide K_s/K_a ratios were strongly associated with smaller Blochmannia genome sizes (Figure 5b). These results suggest that genetic drift, or possibly a combination of genetic drift and relaxed selection strength, is shaping genome size reduction in the Blochmannia genomes sampled here. This negative association between genome-wide K_s/K_a ratios and genome size is like that identified by Kuo et al. (2009) across 42 free-living and symbiotic pairs of bacteria and suggests a general relationship between the relative rate of nonsynonymous genetic changes and genome size reduction.

Overall, we found that host demography is associated with shifts in selection strength in Blochmannia genomes, but not associated with several other aspects of Blochmannia molecular evolution. As such, we may infer that either (1) our small sample sizes (number of species) may be precluding us from identifying weak correlations between host demography and Blochmannia molecular evolution, or (2) host effective population sizes may not directly reflect endosymbiont effective population sizes, leading to a lack of or weak relationship between host population sizes and endosymbiont molecular evolution. If different host species or populations have varied patterns of endosymbiont transmission population bottlenecks (Mira & Moran, 2002) or endosymbiont within-host selection (Perreaud et al., 2021), we may expect a decoupling of host and endosymbiont effective population sizes.

5  |  CONCLUSIONS

We used whole-genome sequencing of both carpenter ant hosts and their Blochmannia endosymbionts to investigate the influence of host demography on symbiont molecular evolution. We identified strict codiversification of Camponotus hosts and their Blochmannia endosymbionts. Blochmannia genes are evolving about 30x faster than host genomes, with relatively consistent evolutionary rates across the Blochmannia genome. We found that some, but not all, patterns of natural selection in Blochmannia genomes were in part shaped by host demographic history. Blochmannia genome size evolution was not associated with host demography but was associated with genome-wide estimates of genetic drift and number of genes with relaxed selection pressures.

AUTHOR CONTRIBUTIONS

Joseph D Manthey: Conceptualization (equal); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (equal); writing – original draft (lead); writing – review and editing (equal). Jennifer C Giron: Conceptualization (equal); resources (equal); writing – original draft (supporting); writing – review and editing (equal). Jack Hruska: Conceptualization (equal); resources (equal); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST

None declared.

DATA AVAILABILITY STATEMENT

Data associated with this manuscript may be found in the following repositories: • Raw sequencing data (NCBI BioProject PRJNA839641) • Blochmannia genome assemblies (NCBI BioProject PRJNA839441; NCBI Genome accessions CP097749-CP097765) • Camponotus reference genome (Dryad: to be added following manuscript acceptance in accordance with Ecology and Evolution Dryad submissions) • Code for analyses (GitHub: https://github.com/jdmanthey/camponotus_genomes1).

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REFERENCES

Alleman, A., Hertweck, K. L., & Kambhampati, S. (2018). Random genetic drift and selective pressures shaping the Blattabacterium genome. Scientific Reports, 8(1), 1–12.

Anbutsu, H., Moriya, M., Nikoh, N., Hosokawa, T., Futahashi, R., Tanahashi, M., Meng, X. Y., Kuriwada, T., Mori, N., Oshima, K., & Hattori, M. (2017). Small genome symbiont underlies cuticle hardness in beetles. Proceedings of the National Academy of Sciences, USA, 114(40), E8382–E8391.

André, E. (1881). Catalogue raisonné des Formicides provenant du voyage en Orient de M. Abeille de Perrin et description des espèces nouvelles. Annales de la Société Entomologique de France, 6(1), 53–78.

AntWeb. (2022). AntWeb. Version 8.73. California Academy of Science. Available at: https://www.antweb.org

Arab, D. A., Bourguignon, T., Wang, Z., Ho, S. Y., & Lo, N. (2020). Evolutionary rates are correlated between cockroach symbionts and mitochondrial genomes. Biology Letters, 16(1), 20190702.

Arab, D. A., & Lo, N. (2021). Evolutionary rates are correlated between Buchnera Endosymbionts and the mitochondrial genomes of their aphid hosts. Journal of Molecular Evolution, 89(4), 238–248.

Ashmead, W. (1905). A skeleton of a new arrangement of the families, subfamilies, tribes and genera of the ants, or the superfamily Formicoidea. The Canadian Entomologist, 37(11), 381–384.

Bao, Z., & Eddy, S. R. (2002). Automated de novo identification of repeat sequence families in sequenced genomes. Genome Research, 12(8), 1269–1276.

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the royal statistical society. Series B (Methodological), 57, 289–300.

Benson, G. (1999). Tandem repeats finder: A program to analyze DNA sequences. Nucleic Acids Research, 27(2), 573–580.

Blaimer, B. B., Brady, S. G., Schultz, T. R., Lloyd, M. W., Fisher, B. L., & Ward, P. S. (2015). Phylogenomic methods outperform traditional multi-locus approaches in resolving deep evolutionary history: A case study of formicine ants. BMC Evolutionary Biology, 15(1), 271.

Blochmann, F. (1892). Über das Vorkommen von bakterienähnlichen Gebilden in den Geweben und Eiern verschiedener Insekten. Centralblatt für Bakteriologie und Parasitenkunde, 11(8), 234–240.

Boman, J., Frankl-Vilches, C., da Silva dos Santos, M., de Oliveira, E. H., Gahr, M., & Suh, A. (2019). The genome of blue-capped cordon-bleu uncovers higher diversity of LTR retrotransposons in zebra finch. Genes, 10(4), 301.

Bonasio, R., Zhang, G., Ye, C., Mutti, N. S., Fang, X., Qin, N., Donahue, G., Yang, P., Li, Q., Li, C., Zhang, P., Huang, Z., Berger, S. L., Reinberg, D., Wang, J., & Liebig, J. (2010). Genomic comparison of the ants Camponotus floridanus and Harpegnathos saltator. Science, 329(5995), 1068–1071.

Bondroit, J. (1918). Les fourmis de France et du Belgique. Annales de la Société Entomologique de France, 87, 1–174. https://www.biodiversitylibrary.org/page/928153

Breitbart, A., Purcell, J., Avil, A., Tran van, P., Zhang, J., Brütsch, T., Sundström, L., Helanterä, H., & Chapuisat, M. (2020). An ancient and eroded social supergene is widespread across Formica ants. Current Biology, 30(2), 304–311 e304.

Bright, M., & Bulgheresi, S. (2010). A complex journey: Transmission of microbial symbionts. Nature Reviews Microbiology, 8(3), 218–230.

Brown, B. P., & Wernegreem, J. J. (2016). Deep divergence and rapid evolutionary rates in gut-associated Acetobacteraceae of ants. BMC Microbiology, 16(1), 1–17.

Brownlie, J. C., & Johnson, K. N. (2009). Symbiont-mediated protection in insect hosts. Trends in Microbiology, 17(8), 348–354.

Buckley, S. (1866). Descriptions of new species of north American Formicidae. Proceedings of the Entomological Society of Philadelphia, 6, 152–172.

Bushnell, B. (2014). BBMap: A Fast, Accurate, Splice-Aware Aligner. https://sourceforge.net/projects/bbmap/
Distel, D., Felbeck, H., & Cavanaugh, C. (1994). Evidence for phylogenetic congruence among sulfur-oxidizing chemotrophic bacterial endosymbionts and their bivalve hosts. *Journal of Molecular Evolution*, 38(5), 533–542.

Donisthorpe, H. (1946). New species of ants (Hym. Formicidae) from the Island of Mauritius. *Annals and magazine of Natural History*, 11(12), 776–782.

Dudchenko, O., Batra, S. S., Omer, A. D., Nyquist, S. K., Hoeger, M., Durand, N. C., Shamim, M. S., Machol, I., Lander, E. S., Aiden, A. P., & Aiden, E. L. (2017). De novo assembly of the Aedes aegypti genome using hi-C yields chromosome-length scaffolds. *Science*, 356(6333), 92–95.

Durand, N. C., Robinson, J. T., Shamim, M. S., Machol, I., Mesirov, J. P., Lander, E. S., & Aiden, E. L. (2016). Juicebox provides a visualization system for hi-C contact maps with unlimited zoom. *Cell Systems*, 3(1), 99–101.

Emery, C. (1893). Beiträge zur Kenntniss der nordamerikanischen Ameisenfauna. *Zoologische Jahrbücher. Abteilung für Systematik, Geographie und Biologie der Tiere*, 7, 633–682.

Faulk, C. (2022). De novo sequencing, diploid assembly, and annotation of the black carpenter ant, *Camponotus pennsylvanicus*, and its symbionts by one person for $1000, using nanopore sequencing. *bioRxiv*. https://doi.org/10.1101/2022.03.31.486652

Feldhaar, H., Straka, J., Krischke, M., Berthold, K., Stoll, S., Mueller, M. J., & Gross, R. (2007). Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. *BMC Biology*, 5(1), 48.

Felsenstein, J. (1985). Phylogenies and the comparative method. *The American Naturalist*, 125(1), 1–15.

Fowler, H. G. (1986). Polymorphism and colony ontogeny in north American carpenter ants (Hymenoptera: Formicidae: Camponotus pennsylvanicus and Camponotus ferrugineus). *Zoologische Jahrbücher. Abteilung für allgemeine Zoologie und Physiologie der Tiere*, 90(2), 297–316.

Gil, R., Latorre, A., & Moya, A. (2004). Bacterial endosymbionts of insects: Insights from comparative genomics. *Environmental Microbiology*, 6(11), 1109–1122.

Gil, R., Silva, F. J., Zientz, E., Delmotte, F., González-Candelas, F., Latorre, A., van Ham, R. C. H. J., Gross, R., & Moya, A. (2003). The genome sequence of Blochmannia floridanus: Comparative analysis of reduced genomes. *Proceedings of the National Academy of Sciences, USA*, 100(16), 9388–9393.

Gueguen, G., Vavre, F., Gnankine, O., Peterschmitt, M., Charif, D., Chiel, E., Gottlieb, Y., Ghanim, M., Zchori-Fein, E., & Fleury, A. (2010). Endosymbiotic metacommunities, mtDNA diversity and the evolution of the Bemisia tabaci (Hemiptera: Aleyrodidae) species complex. *Molecular Ecology*, 19(19), 4365–4376.

Guindon, S., Dufayard, J.-F., Lefort, V., Anisimova, M., Hordijk, W., & Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology*, 59(3), 307–321.

Guyomar, C., Delage, W., Legeai, F., Mougel, C., Simon, J.-C., & Lemaitre, C. (2020). MinYS: Mine your Symbiont by targeted genome assembly in symbiotic communities. *NAR Genomics and Bioinformatics*, 2(3), lqa047.

Hauschteck, J. (1983). Ant chromosomes II. Karyotypes of Western Palearctic species. *Insectes Sociaux*, 30, 149–164.

Heddi, A., Grenier, A.-M., Khatchadourian, C., Charles, H., & Nardon, P. (1999). Four intracellular genomes direct weevil biology: Nuclear, mitochondrial, principal endosymbiont, and Wolbachia. *Proceedings of the National Academy of Sciences, USA*, 96(12), 6814–6819.

Hosokawa, T., Nikoh, N., Koga, R., Satō, M., Tanashashi, M., Meng, X.-Y., & Fukatsu, T. (2012). Reductive genome evolution, host–symbiont co-specialisation and uterine transmission of endosymbiotic bacteria in bat flies. *The ISME Journal*, 6(3), 577–587.

Imai, H. T. (1969). Karyological studies of Japanese ants. I. Chromosome evolution and species differentiation in ants. *Science Report Tokyo Kyoiku Daigaku Section B*, 14, 27–46.

Imai, H. T., & Yoshida, T. (1964). Chromosome observations in Japanese ants. *Annual report of the National Institute of Genetics (Japan)*, 15, 64–66.

Ingram, K. K., Pilko, A., Heer, J., & Gordon, D. M. (2013). Colony life history and lifetime reproductive success of red harvester ant colonies. *Journal of Animal Ecology*, 82(3), 540–550.

Jurka, J., Kapitonov, V. V., Pavlicek, A., Klonowski, P., Kohny, O., & Walichiewicz, J. (2005). Repbase update, a database of eukaryotic repetitive elements. *Cytogenet and Genome Research*, 110(1–4), 462–467.

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), 772–780.

Kikuchi, Y. (2009). Endosymbiotic bacteria in insects: Their diversity and culturability. *Microbes and Environments*, 24, 195–204.

Kinjo, Y., Lo, N., Martin, P. V., Tokuda, G., Pigolotti, S., & Bourguignon, T. (2021). Enhanced mutation rate, relaxed selection, and the "domino effect" are associated with gene loss in Blattabacterium, a cockroach endosymbiont. *Molecular Biology and Evolution*, 38(9), 3820–3831.

Koren, S., Walenz, B. P., Berlin, K., Miller, J. R., Bergman, N. H., & Phillippy, A. M. (2017). Canu: Scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. *Genome Research*, 27, 215087–215116.

Korf, I. (2004). Gene finding in novel genomes. *BMC Bioinformatics*, 5(1), 59.

Kuhner, M. K., & Felsenstein, J. (1994). A simulation comparison of phylogeny algorithms under equal and unequal evolutionary rates. *Molecular Biology and Evolution*, 11(3), 459–468.

Kuo, C.-H., Moran, N. A., & Ochman, H. (2009). The consequences of genetic drift for bacterial genome complexity. *Genome Research*, 19(8), 1450–1454.

Kuo, C.-H., & Ochman, H. (2009). Inferring clocks when lacking rocks: The variable rates of molecular evolution in bacteria. *Biological Direct*, 4(1), 35.

Kumper, M., Stigloher, C., Feldhaar, H., & Gross, R. (2016). Distribution of the obligate endosymbiont *Blochmannia floridanus* and expression analysis of putative immune genes in ovaries of the carpenter ant *Camponotus floridanus*. *Arthropod Structure & Development*, 45(5), 475–487.

Laetsch, D. R., & Blaxter, M. L. (2017). BlobTools: Interrogation of genome assemblies. *F1000Research*, 6(1287), 1287.

Latreille, P. (1802). In T. Barrois (Ed.), *Histoire naturelle des fourmis*, et recueil de mémoires et d’observations sur les abeilles, les araignées, les faucheurs, et autres insectes. https://doi.org/10.5962/bhl.title.11138

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with burrows–wheeler transform. *Bioinformatics*, 25(14), 1754–1760.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., & 1000 Genome Project Data Processing Subgroup. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics*, 25(16), 2078–2079.

Linnaeus, C. v. (1758). I Systema Natura per regna Tria Naturae: Secundum classdes, Ordines, genera, species, cum Charactetibus, Differentiis, Synonymis, Locis. *Impensis Direct. Laurentii Salvii*, Holmiae, 824 pp. https://doi.org/10.5962/bhl.title.542

Lo, N., Bandi, C., Watanabe, H., Nalepa, C., & Beninati, T. (2003). Evidence for cocladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. *Molecular Biology and Evolution*, 20(6), 907–913.

Mackay, W. (2019). *New World carpenter ants of the Hyperdiveerse genus Camponotus. Volume 1: Introduction, keys to the subgenera and species complexes and the subgenus Camponotus*. Lambert Academic Publishing.

Mayr, G. L. (1861). *Die Europäischen Formiciden: nach der analytischen Methode bearbeitet* (p. 32). Gerold. https://www.biodiversitylibrary.org/page/13950352
Sukumaran, J., & Holder, M. T. (2010). DendroPy: A python library for phylogenetic computing. *Bioinformatics*, 26(12), 1569–1571.

Tatusova, T., DiCuccio, M., Badretdin, A., Chetvernin, V., Nawrocki, E. P., Zaslavsky, L., Lomsadze, A., Pruitt, K. D., Borodovsky, M., & Ostell, J. (2016). NCBI prokaryotic genome annotation pipeline. *Nucleic Acids Research*, 44(14), 6614–6624.

Thao, M. L., Moran, N. A., Abbot, P., Brennan, E. B., Burckhardt, D. H., & Baumann, P. (2000). Cospeciation of psyllids and their primary prokaryotic endosymbionts. *Applied and Environmental Microbiology*, 66(7), 2898–2905.

Toju, H., Tanabe, A. S., Notsu, Y., Sota, T., & Fukatsu, T. (2013). Diversification of endosymbiosis: Replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. *The ISME Journal*, 7(7), 1378–1390.

Viereck, H. (1903). *Hymenoptera of Beulah, New Mexico*. [part]. *Transactions of the American Entomological Society*, 29, 56–87.

Ward, P. S., Blaimer, B. B., & Fisher, B. L. (2016). A revised phylogenetic classification of the ant subfamily Formicinae (hymenoptera: Formicidae), with resurrection of the genera Colobopsis and Dinomyrmex. *Zootaxa*, 4072(3), 343–357.

Wernegreen, J. J. (2002). Genome evolution in bacterial endosymbionts of insects. *Nature Reviews Genetics*, 3(11), 850–861.

Wernegreen, J. J., Kauppinen, S. N., Brady, S. G., & Ward, P. S. (2009). One nutritional symbiosis begat another: Phylogenetic evidence that the ant tribe Camponotini acquired Blochmannia by tending sap-feeding insects. * BMC Evolutionary Biology*, 9(1), 292.

Wertheim, J. O., Murrell, B., Smith, M. D., Kosakovsky Pond, S. L., & Scheffler, K. (2015). RELAX: Detecting relaxed selection in a phylogenetic framework. *Molecular Biology and Evolution*, 32(3), 820–832.

Wick, R. R., Schultz, M. B., Zobel, J., & Holt, K. E. (2015). Bandage: Interactive visualization of de novo genome assemblies. *Bioinformatics*, 31(20), 3350–3352.

Williams, L. E., & Wernegreen, J. J. (2012). Purifying selection, sequence composition, and context-specific indel mutations shape intraspecific variation in a bacterial endosymbiont. *Genome Biology and Evolution*, 4(1), 44–51.

Williams, L. E., & Wernegreen, J. J. (2015). Genome evolution in an ancient bacteria-ant symbiosis: Parallel gene loss among Blochmannia spanning the origin of the ant tribe Camponotini. *PeerJ*, 3, e881.

Wilson, E. O. (1976). Which are the most prevalent ant genera. *Studia Entomologica*, 19(1–4), 187–200.

Wolschin, F., Hölldobler, B., Gross, R., & Zientz, E. (2004). Replication of the endosymbiotic bacterium *Blochmannia floridanus* is correlated with the developmental and reproductive stages of its ant host. *Applied and Environmental Microbiology*, 70(7), 4096–4102.

Woolfit, M., & Bromham, L. (2003). Increased rates of sequence evolution in endosymbiotic bacteria and fungi with small effective population sizes. *Molecular Biology and Evolution*, 20(9), 1545–1555.

Yang, Z. (1997). PAML: A program package for phylogenetic analysis by maximum likelihood. *Bioinformatics*, 13(5), 555–556.

Zhang, C., Rabiee, M., Sayyari, E., & Mirarab, S. (2018). ASTRAL-III: Polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinformatics*, 19(Suppl 6), 153. https://doi.org/10.1186/s12859-018-2129-y

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