Lack of host phylogenetic structure in the gut bacterial communities of New Zealand cicadas and their interspecific hybrids

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Host-microbe interactions are intimately linked to eukaryotic evolution, particularly in sap-sucking insects that often rely on obligate microbial symbionts for nutrient provisioning. Cicadas (Cicadidae: Auchenorrhyncha) specialize on xylem fluid and derive many essential amino acids and vitamins from intracellular bacteria or fungi (Hodgkinia, Sulcia, and Ophiocordyceps) that are propagated via transmission from mothers to offspring. Despite the beneficial role of these non-gut symbionts in nutrient provisioning, the role of beneficial microbiota within the gut remains unclear. Here, we investigate the relative abundance and impact of host phylogeny and ecology on gut microbial diversity in cicadas using 16S ribosomal RNA gene amplicon sequencing data from 197 wild-collected cicadas and new mitochondrial genomes across 38 New Zealand cicada species, including natural hybrids between one pair of two species. We find low abundance and a lack of phylogenetic structure and hybrid effects but a significant role of elevation in explaining variation in gut microbiota.

The study of patterns of associations between microbes and their hosts have provided many evolutionary insights, from the endosymbiotic origins of the eukaryotes to the microbial basis of adaptations that are not directly encoded by the eukaryotic genome itself. The reliance of hosts on functions provided by their microbes can lead to selection for specificity in host-microbe relationships when microbes are inherited through vertical transmission from mothers to offspring or selected by hosts from the environment every generation. Often, such microbes are required for host development and are spatially segregated into specific cells and tissues within the body of a host individual.

Cicadas (Hemiptera: Auchenorrhyncha: Cicadidae) represent a well-studied example of obligate and specific associations between animals and microbes. They obtain essential amino acids and vitamins absent in their diets from two obligate symbionts, "Candidatus Sulcia muelleri" (hereafter Sulcia) and "Candidatus Hodgkinia cicadica" (hereafter Hodgkinia). These microbes have largely non-overlapping metabolic functions required by their hosts for development and are housed in bacteriomes outside of the gut but within the cicada abdomen. As previously predicted by early microscopy, recent molecular work shows that the loss of Hodgkinia is coincident with replacements by yeast-like Ophiocordyceps fungi.

Although these obligate symbionts—Sulcia, Hodgkinia, and Ophiocordyceps—are important in supplying nutrients to cicada hosts, they are not known to reside in the cicada gut. Comparatively little is known about cicada gut-associated microbes despite the increasingly recognized nutritional role of animal gut microbiota. Previous studies on cicada gut-associated microbiota have focused on Meimuna mongolica and Platypleura kaempferi. As opposed to the specificity of obligate symbionts, gut microbiota may be facultative or transient with little specificity and involve complex microbial communities with varying functions across fluctuating ecological conditions, but may nonetheless be essential for host development.

Variation in microbial communities among animal hosts may be explained by many factors, including the host phylogeny itself, particularly when microbes are inherited. However, many ecological correlates have also been shown to impact host-associated microbial communities, particularly the conditions of the gut niche which experiences continuous input of environmental microbes. It remains unclear, however, to what extent...
microbial community assembly within many host species is influenced by fluctuating ecological conditions as compared to host evolution. Indeed, transient microbes may not have functions relevant to host fitness\textsuperscript{36,37}. Yet these microbes may be a source of adaptive functions of novel microbial metabolism as hosts diversify\textsuperscript{38}. Broader surveys are needed to enhance our understanding of the causes and consequences of variation in host-associated gut microbiota and whether they play important roles in organisms with other symbiotic associations such as cicadas.

In this study, we generate 16S rRNA gene amplicons of microbial communities and newly assembled mitochondrial genomes across 38 New Zealand (NZ) cicada species and hybrids between one pair of two species to understand the relative contributions of host phylogeny and ecology to variation in gut microbiota. These 38 species comprise three genera and represent a radiation of species from a single colonizing ancestor which now occupy a wide variety of habitats and elevations. We show that phylogenetic distances among cicada hosts are poor predictors of gut microbial communities compared to elevational differences, suggesting little host specificity and a role for ecological heterogeneity. Further supporting this lack of host specificity, we found no evidence of significant differences in gut microbial communities between interspecific hybrids and their parental species. These results point to the stochasticity of bacterial communities across an island landscape and may be related to the fact that most essential nutrients are supplied by specialized bacteriome-associated symbionts.

Methods

Sample collection and processing. Collection and outgroup choice. We collected New Zealand cicadas in 95% ETOH from 1995 to 2018 and stored them at −80 °C until processing (see Table S1 for specimen details). Based on previous work\textsuperscript{39}, we included several Caledopsalta (Cicadettini, Cicadettinae) cicada specimens from New Caledonia as an outgroup comparison to the New Zealand cicada clade comprising Kikihia, Maoricicada, and Rhodopsalta. We also included several Magvicicada (Lamotiallnini, Cicadettinae) from North America. Although we focus on the aforementioned groups, our dataset also includes, for comparative purposes, Neotibicen (North American, Cryptoptamuspanini, Cicadinae) and Platypedia (North America, Tibicininae) specimens but these are not discussed as part of our main findings. In summary, we caught cicadas by net or lured them using manually produced mating clicks, taking advantage of the attraction that male cicadas have for wing flapping sounds and movements associated with conspecific females\textsuperscript{40,41}. We identified each specimen to species in the field using a combination of song, morphology, and knowledge of their evolutionary history and distribution\textsuperscript{42–44}. One hybrid-descended lineage, K. “muta × tua”, was sampled. This lineage possesses K. muta nuclear DNA, song, and morphology, but K. “tua” mtDNA as a result of hybridization and introgression of the mitochondrion\textsuperscript{44}. The identity of K. “muta × tua” samples were inferred based on the above criteria and the geographic distribution of the K. muta and K. “muta × tua” lineages. For specimens sampled from localities known to have both K. muta and K. “muta × tua” lineages, we sequenced the mitochondrial COI gene and treated 100% matches to K. “tua” sequences in GenBank as confirmation of K. “muta × tua” identity.

Dissection. We sterilized specimens by submerging them in 2% bleach, letting them sit for 1–2 min and then washing them in both 50–70% alcohol and sterile water. We then dissected specimens using small scissors, forceps, and pins to access gut tissue ventrally. The complex structure of the cicada gut required prolonged (15–30 min) and relatively tedious dissection compared to insects with relatively simpler guts (Fig. S1). We either extracted both gut and reproductive tissue or only gut tissue depending on the specific dataset produced (dataset specifications provided below). Tissue samples were either directly placed into Powersoil bead tubes or stored in sterile cryotubes and kept frozen until DNA extraction. All dissection equipment was sterilized with 10% bleach and then treated with UV light in a crosslinker for at least one minute prior to dissection. We carried out dissections over the course of several months, with 2–15 dissections on any given day.

We binned processed samples into three sample batches based on the timing and methodological differences in processing, the workers who processed them, and sampling design: B1, B2, B3 (Table S1). Dataset-specific variations are described as follows:

B1 dataset: Combined gut and male reproductive tissue from Kikihia muta and Kikihia “tuta” representing nine parental populations and six previously identified hybrid populations extracted using a Powersoil DNA Isolation kit (MO BIO Laboratories) under the standard protocol. The entire purification process was performed using the Powersoil DNA Isolation protocol (not DNeasy).

B2 dataset: Gut tissue from a broad sampling of New Zealand cicada species mostly within Kikihia extracted by mechanical lysing within Powersoil bead tubes containing Powersoil lysis buffer and subsequent DNeasy 96-well plate extraction under standard protocols (beginning after Proteinase-K treatment and incubation).

B3 dataset: Gut tissue from a broad sampling of New Zealand cicada species including outgroup species from New Caledonia and various North American cicadas extracted by mechanical lysing within Powersoil bead tubes containing Powersoil lysis buffer and subsequent DNeasy 96-well plate extraction under standard protocols (beginning after Proteinase-K treatment and incubation).

Amplicon sequencing of 16S V4 rRNA. We amplified the V4 region of bacterial 16S rRNA using universal barcoded primers 515F (5′-GTGCCAGCMGCGGTAA-3′) and 806R (5′-GACTACHVGGGTWT CTAAT-3′) with attached Illumina-compatible adapters and indices (Microbial Analysis, Resources, and Services Facility, University of Connecticut) under the following PCR conditions: 95 °C for 2 min, 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and then a final extension at 72 °C for 5 min. All libraries were quantified with QIAxcel and manually inspected for proper marker alignment. We normalized libraries by pooling to the lowest concentration for each of the sample sets. Samples that could not be quantified due to low concentra-
tion were pooled using the maximum product available. Pooled samples were cleaned and size-selected using a bead-based approach. Final 16S amplicons were sequenced paired end on Illumina MiSeq. Dataset-specific variations are described as follows:

- **B1 dataset**: Amplified with V4 16S rRNA primers as above using EmeraldAmp GT PCR Master Mix (TAKARA BIO).
- **B2 dataset**: Amplified in a separate facility (Microbial Analysis, Resources, and Services Facility, University of Connecticut) with V4 16S rRNA primers as above using GoTaq DNA Polymerase (PRIMEGA).
- **B3 dataset**: First amplified with primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGT TACCTTGTTACGACTT-3') using EmeraldAmp GT PCR Master Mix (TAKARA BIO) to minimize the amplification of non-bacterial taxa under the following cycling conditions: 94 °C for 5 min, then 5 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 1.5 min, and then a final extension at 72 °C for 10 min. The product was used as a template for amplification with V4 16S rRNA primers as in dataset B1.

**Negative controls.** We took various controls when processing the B2 and B3 datasets, most of which were taken as part of the B3 dataset. Six PCR controls across the two datasets were taken (two controls in the B2 dataset and four controls in B3 dataset) during library amplification using V4 16S rRNA primers (Fig. S2, PCR and control). The remaining controls are associated with the B3 dataset and are as follows: Six dissection reagent controls of fluid used prior to dissection (Fig. S2, dissection), 10 controls of surface contents of forceps after transferring tissue from dissection plates to extraction tubes and sterilizing (Fig. S2, transfer), five surface sterilization controls of the fluid used to wash specimens prior to dissection (Fig. S2, wash), and six extraction kit controls from both the DNeasy and powersoil kits (Fig. S2, dneasy and powersoil).

**16S rRNA amplicon data processing.** We denoised and merged reverse and forward reads in QIIME 2⁴⁵ using the DADA2 pipeline⁴⁶ separately for each dataset, with the exception of the B3 dataset in which we only considered forward reads due to high error rates in the reverse reads. We aligned the denoised sequences in MAFFT⁴⁷, filtered the alignments, and constructed a midpoint-rooted phylogeny using the “align-to-tree-only” method⁴⁸ with the MAFFT v. 7 E-INS-i algorithm and manually edited in Geneious v. 10.1.3 for a final mitochondrial genome alignment. We designed a partitioning scheme that included combined 1st and 2nd codon positions and the first 1.25 codons of the V4 region of 16S rRNA extracted from SILVA 99% OTUs (Release 132) database. The resulting feature tables, phylogenies, classifications, and sample metadata were primarily processed using the R package phyloseq⁴⁸. We removed ASVs classified to mitochondria, chloroplast, cicada bacterial endosymbiont (Hodgkinia and Sulcia), eukaryote, and archaea. To minimize noise introduced by sequencing errors, unclassified taxa, and low sequencing coverage, we removed ASVs that could not be classified at the Kingdom, Phylum, or Class taxonomic levels and ASVs with a total abundance across all samples within a dataset of less than three. We included only samples with a total abundance greater than 100. The remaining ASVs per dataset were further collapsed using the “tip_glom” function in phyloseq to cluster ASVs based on cophenetic distances with a tree height of 0.03. We then used the R package decontam⁴⁹ to identify putative contaminants. However, we were unsuccessful in identifying contaminants with our post-PCR DNA concentration data using the frequency-based filters under reasonable thresholds due to large inter-sample variability in the presence and abundance of different ASVs. Instead, we relied on control samples in the B2 and B3 datasets. We used the prevalence-based filter with a threshold of 0.3 to remove ASVs that were enriched in the controls and then we subsequently removed all ASVs in all datasets that were classified to these putative contaminant genera (Fig. S2).

**Quantitative PCR (qPCR) of 16S rRNA amplicons.** To estimate how initial copy numbers of target molecules may differ across our samples, we submitted a subset of samples for qPCR using universal V4 region primers and a CFX Opus 384 Real-Time PCR System. We sought to assess whether quantification of samples after PCR but before pooling would be predictive of the number of target molecules initially present. Thus we analyzed DNA extracts of 24 samples including those of six negative controls and other samples with a range of expected amplicon copy numbers based upon initial quantification as well as sequencing results (Fig. S3 and Table S4). Samples were quantified in triplicate and average initial amplicon copy number estimated based upon correlation to curve calculated with standard.

**Cicada mitochondrial genome phylogeny.** We assembled host mitochondrial genomes using off-target capture data from an anchored hybrid enrichment dataset of worldwide cicada lineages⁵⁰-⁵². We first duplicated merged and unmerged reads using the function clampify in BBMap⁵³, trimmed adapters and reads with Q < 20 using Trimmomatic⁵⁴, and then assembled reads using both merged and unmerged data in SPAdes v. 3.12.0⁵⁵,⁵⁶. We extracted mitochondrial contigs from the resulting assembly using blastn with a published partial K. muta reference mitochondrial genome—Genbank MG737773—used BWA v. 0.7.5a⁵⁶ in a second processing step, and then reassembled the resulting reads with SPAdes 3.12.0. The final sets of mitochondrial contigs for each sample were aligned to various published and nearly complete mitochondrial genomes⁵⁷ using the MAFFT v. 7 E-INS-i algorithm⁵⁸, combined into single mitochondrial genome sequences, and manually edited to exclude misassembled regions in Geneious v. 10.1.1⁵⁹. We used these mitochondrial sequences as sample-specific baits in MITObim v. 1.9.1⁶⁰ to assemble improved, higher-quality mitochondrial genomes that were aligned with the MAFFT v. 7 E-INS-i algorithm and manually edited in Geneious v. 10.1.3 for a final mitochondrial genome alignment. We designed a partitioning scheme that included combined 1st and 2nd codon positions and the 3rd codon position for each protein-coding gene, partitions for each of the 12S rRNA and 16S rRNA loci, and a
Results

Patterns of bacterial communities of New Zealand cicadas suggest a low abundance and highly variable microbiota not present in eggs. Prior to filtering each dataset, some samples contained low total ASV abundances (Fig. 1, top), suggesting that cicada gut tissues sometimes contain low amounts of bacterial cells or that the dissection procedure failed to isolate many microbial cells. Our qPCR data of 16S rRNA amplicons from a subset of our samples corroborate these patterns (some of which were quantified as possessing fewer than 100 initial copies of the 16S ribosomal rRNA) and show that the absolute quantity of 16S rRNA amplicons inferred from qPCR is positively correlated with the DNA concentration data we collected post-PCR for a subset of samples (Fig. S3, R² = 0.5375). Our results also showed that controls were consistently quantified as having very few initial target molecule copies (fewer than 0.5 in all cases). Herein, we use our DNA concentration data to inform our filtering procedure.

After filtering samples (see “16S rRNA amplicon data processing” in “Methods”), we identified 18 major bacterial families with high relative abundance and prevalence across samples and datasets, with Burkholderiaceae, Micrococcaceae, Rhizobiaceae (non-Hodgkinia), and Enterobacteriaceae among the top four groups in terms of relative abundance (Fig. 2A heatmap, Figs. S4, S5). We could not detect a qualitative structuring of these major bacterial families among clades defined by the host mitochondrial genome phylogeny. We used principal coordinates analysis (PCoA) ordinations of Bray distances derived from relative abundances of ASVs belonging to these major bacterial groups and averaged across samples within species (Fig. 2B) to show that variation in bacterial communities surveyed in this study are primarily explained by differences in datasets (permanova, R² = 0.17, p < 0.01**) rather than differences in genera or species sampled (permanova, p > 0.05). In addition, we could not find significant contributions of host genera or species in structuring communities within datasets using either weighted and unweighted UniFrac distances of unmerged samples (see Fig. S6). Although specimens used in this study were collected over many years, we do not find a qualitative effect of collection date in structuring communities (see Fig. S7) and the effect of collection year was insignificant in explaining microbial community differences when considering each dataset separately (permanova, p > 0.05). After filtering, we were left with one nymphal sample with a similar microbial composition as adult samples (see Fig. 2B), but we are unable to make conclusions about the nymphal microbiome with these data alone.

Mitochondrial genome phylogeny improves resolution of relationships among New Zealand cicadas. Our host phylogeny is based on a whole mitogenome data, nearly seven-fold increase in alignment length (~14,000 bp) from previous phylogenies4,5, which were based on approximately 2000 bp of sequence length.
data. Our phylogeny (Fig. 2A, Fig. S8) recovers *Rhodopsalta microdora* as sister to *R. leptomera + R. cruentata* with 100% bootstrap support, confirming results with fewer genes66, and groups *Rhodopsalta* with *Maoricicada* with 100% bootstrap support. The *Maoricicada* taxon sampling is reduced compared to the previous *Maorici- cada* mitochondrial tree, but despite the missing taxa the mitogenome phylogeny is congruent with the previous tree64. For the *Kikihia* taxa, the mitogenome phylogeny recovers the same major well-supported clades previously identified65.

**Elevational differences explain bacterial community variation better than host phylogeny.** We used various statistical tests to assess the effect of host phylogeny on bacterial community variation and found no evidence of host phylogenetic structure. Mantel tests between unweighted and weighted UniFrac distances among gut microbial communities and cophenetic distances among host mitochondrial genomes were insignificant based on permutation tests for both B2 and B3 datasets (p > 0.05). Tests of phylogenetic signal (Pagel’s lambda and K statistic) using the first axis positions of either unweighted or weighted UniFrac PCoA ordinations as phylogenetically distributed traits were insignificant for each dataset as well (p > 0.05). In addition, ordinations of both weighted and unweighted UniFrac distances did not produce clustering that corresponded to host taxonomy at either the genus or species levels (Fig. 2B and Fig. S6).

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**Figure 2.** Distribution of major taxonomic groups using 16S V4 rRNA classification of amplicon sequence variants (ASVs) across all datasets. (A) Maximum likelihood phylogeny using nearly complete mitochondrial genomes of the sampled host species. Branches are collapsed at a bootstrap threshold of 90; bootstrap supports for all nodes are shown in Fig. S8. Tip labels contain the number of individuals sampled in parentheses and match specific epithets in Table S1. Branch colors represent different genera. Species not used to construct the phylogeny are added below the tree (*Amphipsalta strepitans, Amphipsalta cingulata, Caledopsalta sp.*, *Magicicada septendecim*). (Right) Heatmap of relative abundances of major bacterial families averaged across individuals and datasets per species. (B) Ordination using weighted UniFrac distances of filtered bacteria-only samples using relative abundances of major bacterial families averaged across individuals within species. Colors correspond to host genera. Shapes correspond to datasets (square = B1, circle = B2, triangle = B3). The axis labels report the percent variation explained by the corresponding principal components.
compared to the ecological covariates that were also included in these models (Fig. 3D and Table S2). We do not find similarly strong patterns in the B3 dataset, but this dataset was enriched for low-abundance bacteria resulting from nested PCR amplification (see "Amplicon sequencing of 16S V4 rRNA" in "Methods") which likely erased signals of a positive relationship between elevation and weighted bacterial community distances given methodological effects on relative abundances. However, we find that in both the B2 and B3 datasets, the best fitting Beta regression models of weighted UniFrac distances always included elevation as an explanatory variable (Fig. 3D, top) and that elevation was significant in all models using the B2 dataset despite having small effect sizes (Table S2).

Microbial communities in hybrids between Kikihia muta and K. "tuta" resemble those of their parental species. Specimens with evidence of introgression between K. muta and K. "tuta", which we refer to as hybrids, in the B1 dataset did not show qualitative differences in the relative abundance of bacterial ASVs compared to parental species (Fig. 4A). Ordination of gut microbial communities using unweighted and weighted UniFrac distances showed that hybrids cluster with parental species (Fig. 4B) with significant effects of processing date (i.e., time at which samples were purified for DNA) and sample depth (i.e., total abundance of all ASVs). The processing date explained 9% and 11% of variation in unweighted and weighted UniFrac distances (permanova, p < 0.05***), respectively. However, hybrid status did not significantly explain variation in weighted UniFrac distances (permanova, p > 0.05) and explained less variation than processing date in unweighted UniFrac distances (permanova, R² = 7%, p < 0.05***).

Pairwise unweighted and weighted UniFrac distances were elevated in comparisons of samples that included a hybrid specimen (Fig. 4C, hybrid/hybrid and hybrid/parent pairwise comparisons). Although this pattern may suggest that hybrids have a divergent and more variable microbiome compared to parents, these results include...
the effects of processing date. Considering only comparisons of samples processed on the same day (Fig. 4C, same_processing_dates), comparisons between hybrid and parental samples have indistinguishable differences in their bacterial communities. We found that the mean and variance of bacterial community distances are indistinguishable between comparisons that included a hybrid specimen and comparisons that included only parental specimens (Kolmogorov–Smirnov Test, p > 0.05; F-test, p > 0.05).

**Discussion**

**Lack of stable microbial communities in New Zealand cicadas.** Studies of plant sucking bugs with specialized bacteriome-dwelling endosymbionts have with few exceptions not focused on the microbiota outside the bacteriome. Our dataset suggests that adult cicadas have low abundance transient microbial communities in their guts as shown in some other insects. For example, in caterpillars the structural simplicity of the gut niche in combination with the rapid pace at which ingested material moves it is suggested to contribute to the lack of resident microbes (i.e., bacteria that are able to replicate and establish at a higher rate than they are lost due to death or excretion). However, the cicada gut niche is structurally more complex and larger in relative volume and especially surface area than that of most other insects, and includes many separate functional compartments that may be amenable to hosting resident microbes. The paucity of abundant and consistent microbial residents in this study may be explained by similar factors as in caterpillars, including the physiological pace of food processing driven by plant transpiration and osmotic pressure gradients via the cicada filter process.
chamber. These facets of cicada physiology are likely not amenable to microbial colonization as the quick flow of nutritionally poor xylem fluid through the gut may create a harsh environment in which resident bacteria might not be able to establish and proliferate. In addition to being rapidly transported through the cicada alimentary canal, xylem fluid is primarily composed of water with major solutes that include potassium, sodium, calcium, magnesium, chloride, and phosphate. This is analogous to the rapid movement of food in the highly alkaline gut environments of caterpillars, which have been reported to lack appreciable evidence of a resident and functional gut microbiota. We assume that relatively few microbes other than those adapted to living in xylem vessels (i.e., plant pathogens) can live in this cicada gut environment. Second, the presence of obligate bacterial and fungal symbionts outside of the gut circumvents any obvious need for the functional dependence of adult cicadas on microbe-derived essential nutrients in the gut, reducing the likelihood of selection for life-history or physiological traits that maintain either vertical inheritance or faithful horizontal acquisition of gut microbiota.

While the patterns shown in this study suggest the lack of a consistent resident gut microbiota, low-abundance microbial communities are difficult to detect. We were unable to detect differences in total bacterial abundance that correlated with host phylogeny or other abiotic correlates we considered. The lack of a consistent gut microbiota across host taxa is not completely unexpected because it is uncertain what role gut microbial colonization may have in supplementing an already complete cicada diet or providing other transient functions (e.g., immunity to pathogens) as cicadas navigate fluctuating environments. We could not conclude this without looking. Any questions regarding a possible functional role of what we found to be low abundance bacteria would require ecological experiments and tests of fitness. Given the long life of most cicada nymphs compared to mammals, within which gut microbiota are structured by host phylogeny, and the much shorter span of the adult stage, it is unlikely that transient microbes are able to establish within adult cicada guts. A greater sampling of nymphal gut microbial diversity and abundance is needed to understand the distribution of environmentally acquired microbiota in the much longer-lived nymphal stage, as well as the microbes that are transmitted to the adult stage from the nymphal stage. Previous work has shown that *Magicicada* nymphs had gut microbial communities that differed from both the adults and the soil in which they were found. While the lack of microbial diversity in eggs dissected from the abdomens of female cicadas in this study suggests a lack of vertical transmission of non-endosymbiotic bacteria, both the overwhelming relative abundance of cicada endosymbiont cells and the lack of other bacterial cells in these samples may prevent detection of low-abundance microbes.

**New Zealand cicada mitochondrial phylogeny compared to previous studies with much less data.** An added feature of our study is a new phylogeny of the host NZ cicada species using nearly complete mitochondrial genomic data (~14 kb). Support among the NZ cicada species is improved by the inclusion of most mitochondrial genes. Relationships within the *Maorocicada* are better supported but otherwise compatible with that of previous studies based on 2274 bp of mtDNA with slightly larger taxon sampling. Relationships involving *M. campbelli* (Fig. 2A, campbelli-northSI and campbelli-southSI) and *M. iolanthe* (Fig. 2A, iolanthe) remain inconsistent with species trees generated from nuclear loci that group these taxa with the other lower elevation species, *M. hamiltoni* (Fig. 2A, hamiltoni-northSI) and *M. lindsayi* (Fig. 2A, lindsayi), in agreement with similarities in male genitalia, mating songs, and habitat specialization. Low support at the base of the *Maoricicada* alpine clade suggests rapid diversification during a period of intense mountain building. Relationships among *Kikihia* match those of previous mitochondrial phylogenies, including a lack of resolution among some clades within more data. This is not surprising given evidence for rapid radiation and extensive hybridization (including mtDNA capture) in this group. Bacterial communities in other cicada genera—*Magicicada, Caledopsalta, Neotibicen,* and *Platypedius*—cluster haphazardly among the NZ cicadas (Fig. 2).

**Lack of host specificity in New Zealand cicada microbial communities.** Our analysis suggested no host phylogenetic structure among bacterial communities in New Zealand cicadas, even when accounting for variation explained by ecological differences, though several other studies have shown a significant effect of host phylogeny in structuring microbial communities in, for example, corbiculate bees, mammals, tropical birds, ants, deer mice, fruit flies, mosquitoes, and wasps. Small phylogenetic distances among host taxa and the suggested lack of functional importance of gut microbial communities given the presence of specialized endosymbionts likely contributed to these results.

In addition, we could not find significant differences in gut bacterial composition between hybrids and their parental species, reinforcing our conclusions that cicadas lack resident gut microbiota that are structured by host phylogeny. Indeed, if host adaptations that maintain assembly of bacterial communities have evolved, we would expect that these mechanisms in hybrids would deteriorate when genomic variants that have undergone divergent ecological selection for gut microbial community assembly are combined into different genetic backgrounds. Improper regulation of gut bacteria in hybrid hosts may be one source of hybrid lethality, however support for this hypothesis is uncertain. We found little evidence that host genetics influenced microbial community assembly in cicadas, yet other hybrid crosses have shown a host genetic effect in mammals.

Although gut microbial communities in New Zealand cicadas do not seem to be host specific, the composition of these communities may be highly influenced by elevational differences among host individuals. Elevational diversity is a prominent feature of the New Zealand landscape, with dramatically varying ecosystems along elevational gradients. Understanding the many ecological factors contributing to variation in the microbiome is an important next step for understanding host-associated microbial diversity. While our data suggest an association with elevation, we lack sufficient power to identify specific bacterial taxa enriched at high or low elevations. In addition, future studies with broader within-species sampling are needed to investigate the causes of intraspecific variation in the microbiome. Although we do not have sufficient intraspecific sampling of elevationally diverse
species in this study, some Kikihia, in particular, occupy broad elevational ranges and may be good candidates for additional sampling. Nonetheless, elevation is the best predictor of microbial community variation in our analyses.

**Phytoplasma plant pathogens in New Zealand cicadas.** We report the first sequence-based identification, to our knowledge, of the plant pathogen *Candidatus* Phytoplasma in cicadas. Phytoplasmas (Phylum Tenericutes: Class Mollicutes) comprise a group of prokaryotic relatives to mycoplasmas and spiroplasmas and are transmitted among plant hosts by insect vectors through phloem. These insect vectors primarily represent the phloem-feeding members of the hemipteran groups Cicadellidae, Fulgoromorpha, and Pylliidae, but may be transmitted by the families Pentatomidae and Tingidae as well. Despite interactions with both plant host and insect vector, phytoplasma strains do not appear to have insect-vector specificity and many different vectors may transmit a single phytoplasma strain or vice versa. This pattern is recapitulated in other insect-vectored plant pathogens, particularly *Xylella* pathogens which are found in xylem. All cicadas are xylem feeders so far as is known and *Xylella* transmission has been reported in the apple cicada *Diceroprocta apache* into California grapevine and by various Brazilian cicadas into coffee. Rather than *Xylella*, we found that phytoplasmas were in high abundance across six specimens representing *K. paxillae*, *K. murihiika*, *K. aotea*, *K. muta*, *K. nelsonensis*, and *K. ochrina* (see Fig. 2 heatmap, “Candidatus Phytoplasma”). We did not detect phytoplasmas in any New Zealand cicada genus other than *Kikihia*, which tend to occupy lower elevation habitats characterized by grasses and shrubs. Phytoplasmas comprise a large majority of the total rRNA abundance when present in these samples. The high abundance of 16S rRNA amplicons that were classified to phytoplasmas after our filtering procedure suggests that cicadas may be able to vector these phloem-specific plant pathogens despite being xylem feeders. While this is unlikely and requires further investigation, a similar situation has been found in some phloem-feeding hemipterans which acquire and transmit xylem-specific *Xylella* pathogens.

**Conclusion**

In summary, we find that gut microbial communities in NZ cicadas are low abundance and not strongly structured by host phylogeny and that hybrid cicadas resemble parental species, suggesting a lack of host specificity. Instead, we find that elevation differences among individuals explain variation in gut microbiota better than phylogenetic relatedness among hosts. Our new and nearly complete mitochondrial genomes improved phylogenetic relatedness among hosts. The high abundance of 16S rRNA amplicons that were classified to phytoplasmas after our filtering procedure suggests that cicadas may be able to vector these phloem-specific plant pathogens despite being xylem feeders. While this is unlikely and requires further investigation, a similar situation has been found in some phloem-feeding hemipterans which acquire and transmit xylem-specific *Xylella* pathogens.

**Data Availability:**

The datasets generated and/or analyzed during the current study are available in the GenBank repository (Accession: PRJNA879614, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA879614).

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**References**

1. McFall-Ngai, M. et al. Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. USA* **110**, 3229–3236 (2013).
2. Archibald, J. M. Endosymbiosis and eukaryotic cell evolution. *Curr. Biol.* **25**, R911–R921 (2015).
3. Moran, N. A. Symbiosis as an adaptive process and source of phenotypic complexity. *Proc. Natl. Acad. Sci. USA* **104**(Suppl 1), 8627–8633 (2007).
4. Hurst, G. D. D. Extended genomes: Symbiosis and evolution. *Interface Focus.* https://doi.org/10.1098/rsfs.2017.0001 (2017).
5. Moran, N. A., McCutcheon, J. P. & Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**, 165–190 (2008).
6. Kikuchi, Y., Hosokawa, T. & Fukatsu, T. Insect-microbe mutualism without vertical transmission: A stinkbug acquires a beneficial gut symbiont from the environment every generation. *Appl. Environ. Microbiol.* **73**, 4308–4316 (2007).
7. Kikuchi, Y., Hosokawa, T. & Fukatsu, T. An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. *ISME J.* **5**, 446–460 (2011).
8. Hu, Y. et al. Herbivorous turtle ants obtain essential nutrients from a conserved nitrogen-recycling gut microbiome. *Nat. Commun.* **9**, 2440. https://doi.org/10.1038/s41467-018-03357-y (2018).
9. Salem, H. et al. Drastic genome reduction in an herbivore’s pectinolytic symbiont. *Cell* **171**, 1520–1531 (2017).
10. Bennett, G. M. & Moran, N. A. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci. USA* **112**, 10169–10176 (2015).
11. Campbell, M. A. et al. Changes in endosymbiont complexity drive host-level compensatory adaptations in cicadas. *MBio* **9**, e02104-18 (2018).
12. Buchner, P. Symbiosis in animals which suck plant juices. In *Endosymbiosis of Animals with Plant Microorganisms* 210–432 (Interscience, 1965).
13. McCutcheon, J. P., McDonald, B. R. & Moran, N. A. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proc. Natl. Acad. Sci. USA* **106**, 15394–15399 (2009).
14. Christensen, H. & Fogel, M. L. Feeding ecology and evidence for amino acid synthesis in the periodical cicada (*Magicicada*). *J. Insect Physiol.* **57**, 211–219 (2011).
15. McCutcheon, J. P., McDonald, B. R. & Moran, N. A. Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. *PLoS Genet.* **5**, e1000565 (2009).
16. Campbell, M. A. et al. Genome expansion via lineage splitting and genome reduction in the cicada endosymbiont *Hodgkinia*. *Proc. Natl. Acad. Sci. USA* **112**, 10192–10199 (2015).
17. Müller, H. J. Neure vorstellungen über verbreitung und phylogenie der endosymbionsen der zikaden. Z. Morphol. Oekol. Tierw. 61, 190–210 (1962).
18. Müller, H. J. Zur systematik und phylogenie der zikaden-endosymbionsen. Biol. Zent. 68, 343–368 (1949).
19. Matsura, Y. et al. Recurrent symbiont recruitment from fungal parasites in cicadas. Proc. Natl. Acad. Sci. 115, E5970–E5979 (2018).
20. Zhou, W. et al. Analysis of inter-individual bacterial variation in gut of cicada Meimuna mongolica (Hemiptera: Cicadidae). J. Insect Sci. 15, 1–6 (2015).
21. Zheng, Z., Wang, D., He, H. & Wei, C. Bacterial diversity of bacteriomes and organs of reproductive, digestive and excretory systems in two cicada species (Hemiptera: Cicadidae). PLoS One 12, 1–21 (2017).
22. Wang, D., Huang, Z., He, H. & Wei, C. Comparative analysis of microbial communities associated with bacteriomes, reproductive organs and eggs of the cicada Subpallidula yangi. Arch. Microbiol. 200, 227–235 (2018).
23. Dillón, R. J. & Dillón, V. M. The gut bacteria of insects: Non-pathogenic interactions. Annu. Rev. Entomol. 49, 71–92 (2004).
24. Ng, S. H., Stat., M., Bunce, M. & Simmons, L. W. The influence of diet and environment on the gut microbial community of field crickets. Ecol. Evol. 8, 4704–4720 (2018).
25. Nishida, A. H. & Ochman, H. Rates of gut microbiome divergence in mammals. Mol. Ecol. 27, 1884–1897 (2018).
26. Douglas, A. E. & Werren, J. H. Holes in the hologenome: Why host–microbe symbioses are not holobionts. Proc. Natl. Acad. Sci. 114, 12–19 (2016).
27. Gruenenberg, J., Engelen, A. H., Costa, R. & Wichard, T. Macroalgald morphogenesis induced by waterborne compounds and bacteria in coastal seawater. PLoS One 9, e0146307 (2016).
28. Lin, J. D., Lemay, M. A. & Parfrey, L. W. Diverse bacteria utilize alginate within the microbiome of the giant kelp Macrocystis pyrifera. Front. Microbiol. 9, 1914 (2018).
29. Coon, K. L., Vogel, K. J., Brown, M. R. & Strand, M. R. Mosquitoes rely on their gut microbiota for development. Mol. Ecol. 23, 2727–2739 (2014).
30. Coon, K. L., Brown, M. R. & Strand, M. R. Mosquitoes host communities of bacteria that are essential for development but vary greatly between local habitats. Mol. Ecol. 25, 5808–5826 (2016).
31. Kwong, W. K. et al. Dynamic microbiome evolution in social bees. Sci. Adv. 3, 1–17 (2017).
32. Brooks, A. W., Kehl, K. D., Brucker, R. M., van Opstal, E. J. & Bordenstein, S. R. Phyllosymbiosis: Relationships and functional effects of microbial communities across host evolutionary history. PLoS Pathog. 14, 1–12 (2016).
33. Kropáčková, L. et al. Codiversification of gastrointestinal microbiota and phylogeny in passerines is not explained by ecological divergence. Mol. Ecol. 26, 5292–5304 (2017).
34. Hird, S. M., Sánchez, C., Carstens, B. C. & Brumfield, R. Comparative gut microbiota of 59 neotropical bird species. Front. Microbiol. 6, 1403. https://doi.org/10.3389/fmicb.2015.01403 (2015).
35. Hu, Y., Lukasik, P., Moreau, C. S. & Russell, J. A. Correlates of gut community composition across an ant species (Cephalotes var) elucidated causes and consequences of symbiotic variability. Mol. Ecol. 23, 1284–1300 (2014).
36. Hammer, T. J., Sanders, J. G. & Fierer, N. Not all animals need a microbiome. Trends Ecol. Evol. 6, 455–477 (2012).
37. Hammer, T. J., Janzen, D. H., Hallwachs, W., Jaffe, S. P. & Fierer, N. Caterpillars lack a resident gut microbiome. Proc. Natl. Acad. Sci. 114, 9641–9646 (2017).
38. Shapira, M. Gut microbiotas and host evolution: Scaling up symbiosis. Trends Ecol. Evol. 31, 539–549 (2016).
39. Marshall, D. C. et al. Inflation of molecular clock rates and dates: Molecular phylogenetics, biogeography, and diversification of a global cicada radiation from Australasia (Hemiptera: Cicadidae). Syst. Biol. 65, 16–34 (2016).
40. Lane, D. H. The recognition concept of speciation applied in an analysis of putative hybridization in New Zealand cicadas of the genus Kikihia (Insects: Hemiptera: Cicadidae). Speciation and the Recognition Concept: Theory and Application (The Johns Hopkins Univ Press, 1995).
41. Cooley, J. R. & Marshall, D. C. Sexual signaling in periodical cicadas, Magicicada spp. (Hemiptera: Cicadidae). Behaviour 138, 827–855 (2001).
42. Fleming, C. A. Adaptive Radiation in New Zealand Cicadas (American Philosophical Society, 1975).
43. Dugdale, J. S. & Fleming, C. A. New Zealand cicadas of the genus Maoricicada (Homoptera: Cicadidae). N. Z. J. Zool. 5, 295–340 (1978).
44. Marshall, D. C., Hill, K. B. R., Cooley, J. R. & Simon, C. Hybridization, mitochondrial DNA phylogeny, and prediction of the early stages of reproductive isolation: Lessons from New Zealand cicadas (genus Kikihia). Syst. Biol. 60, 482–502 (2011).
45. Bolyen, E. et al. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. Report No.: e27295v2. PeerJ https://doi.org/10.7287/peerj.preprints.27295v2 (2018).
46. Callahan, B. J. et al. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–586 (2016).
47. Katoh, K. & Standley, D. M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 30, 772–780 (2013).
48. McMurdie, P. J. & Holmes, S. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8, e61217 (2013).
49. Davis, M., Proctor, D., Holmes, S. P., Relman, D. A. & Callahan, B. J. Simple statistical identification and removal of contaminant sequences in marker-genetype metagenomics data. Microbiome 6, 226. https://doi.org/10.1186/s40168-018-0514-2 (2018).
50. Lemmon, A. R., Emme, S. A. & Lemmon, E. M. Anchored hybrid enrichment for massively high-throughput phylogenomics. Syst. Biol. 61, 727–744 (2012).
51. Simon, C. et al. Off-target capture data, endosymbiont genes and morphology reveal a relict lineage that is sister to all other singing cicadas. Biol. J. Linn. Soc. Lond. https://doi.org/10.1111/bib.12109 (2019).
52. Owen, C. L. et al. Detecting and removing sample contamination in phylogenomic data: An example and its implications for Cicadidae phylogeny (Insecta: Hemiptera). Syst. Biol. 71, 1504–1523 (2022).
53. Bushnell, B. BMAp: A Fast, Accurate, Splice-Aware Aligner. Report No.: LBNL-70652. https://wwwosti.gov/biblio/1241166-bmap-fast-accurate-splice-aware-aligner (Lawrence Berkeley National Lab. (LBNL), 2014).
54. Bolger, A. M., Lobe, M. & Usadel, B. Trimomatic: A flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120 (2014).
55. Bankevich, A. et al. SPAdes: A new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477 (2012).
56. Nyrk, S. et al. Assembling genomes and mini-metagenomes from highly chimeric reads. In Research in Computational Molecular Biology 158–170 (Springer, 2013).
57. Lukasik, P. et al. One hundred mitochondrial genomes of cicadas. J. Hered. 110, 247–256 (2019).
58. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25, 1754–1760 (2009).
59. Katoh, K., Rozewicki, J. & Yamada, K. D. MAFFT online service: Multiple sequence alignment, interactive sequence choice and visualization. Brief. Bioinform. https://doi.org/10.1093/bib/bbx108 (2017).
60. Kearse, M. et al. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649 (2012).
61. Hahn, C., Bachmann, L. & Chevreur, B. Reconstructing mitochondrial genomes directly from genomic next-generation sequencing reads—A baiting and iterative mapping approach. Nucleic Acids Res. 41, e129 (2013).
62. Stamatakis, A. RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313 (2014).
63. Miller, M. A., Pfeiffer, W., Schwartz, T. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. 2010 Gateway Computing Environments Workshop (GCE) 1–8 (2010).
64. Buckley, T. R., Cordeiro, M., Marshall, D. C. & Simon, C. Differentiating between hypotheses of lineage sorting and introgression in New Zealand alpine cicadas (Maoricicada Dugdale). Syst. Biol. 55, 411–425 (2006).
65. Marshall, D. C., Slon, K., Cooley, J. R., Hill, K. B. R. & Simon, C. Steady State Pleistocene diversification and a 2-million-year sympatry threshold in a New Zealand cicada radiation. Mol. Phylogenet. Evol. 48, 1054–1066 (2008).
66. Bator, J., Marshall, D. C., Leston, A., Cooley, J. & Simon, C. Phylogeography of the endemic red-tailed cicadas of New Zealand (Hemiptera: Cicadidae: Rhodopsalta): Molecular, morphological and bioacoustical confirmation of the existence of Hudson's Rhodopsalta microdora. Zool. J. Linn. Soc. 195, 1219–1244 (2022).
67. Brumfield, K. D. et al. Gut microbiome insights from 16S rRNA analysis of 17-year periodical cicadas (Hemiptera: Magicicada spp.) Brooks II, VI, and X. Sci. Rep. 12, 16967, https://doi.org/10.1038/s41598-022-20527-7 (2022).
68. Rakitov, R. A. Structure and function of the Malpighian tubules, and related behaviors in juvenile cicadas: Evidence of homology with spittlebugs (Hemiptera: Cicadidae & Cercopoidea). Zool. Anz. 241, 117–130 (2002).
69. Andersen, P. C., Brodbeck, B. V. & Mizell, R. F. Feeding by the leafhopper, Homalodisca coagulata, in relation to xylem fluid chemistry and tension. J. Insect Physiol. 38, 611–622 (1992).
70. Cheung, W. W. K. & Marshall, A. T. Water and ion regulation in cicadas in relation to xylem feeding. J. Insect Physiol. 19, 1801–1816 (1973).
71. Williams, K. S. & Simon, C. The ecology, behavior, and evolution of periodical cicadas. Annu. Rev. Entomol. 40, 269–295 (1995).
72. Logan, D. P., Rowe, C. A. & Maher, B. J. Life history of chorus cicada, an endemic pest of kiwifruit (Cicadidae: Homoptera). N. Z. Entomol. 37, 96–106 (2014).
73. Buckley, T. R. & Simon, C. Evolutionary radiation of the cicada genus Maoricicada Dugdale (Hemiptera: Cicadidae) and the origins of the New Zealand alpine biota. Biol. J. Linn. Soc. Lond. 91, 419–435 (2007).
74. Banker, S. E., Wade, E. J. & Simon, C. The confounding effects of hybridization on phylogenetic estimation in the New Zealand cicada genus Kikihia. Mol. Phylogenet. Evol. 116, 172–181 (2017).
75. Geoussis, M. et al. Unraveling the processes shaping mammalian gut microbiomes over evolutionary time. Nat. Commun. 8, 14319 (2017).
76. Sanders, J. G. et al. Stability and phylogenetic correlation in gut microbiota: Lessons from ants and apes. Mol. Ecol. 23, 1268–1283 (2014).
77. Wang, J. et al. Analysis of intestinal microbiota in hybrid house mice reveals evolutionary divergence in a vertebrate hologenome. Nat. Commun. 6, 6440 (2015).
78. Brucker, R. M. & Bordenstein, S. R. The hologenomic basis of speciation. Science 346, 667–669 (2013).
79. Chandler, I. A. & Turelli, M. Comment on “The hologenomic basis of speciation: Gut bacteria cause hybrid lethality in the genus Nasonia”. Science 345, 1011 (2014).
80. Li, Z. et al. Changes in the rumen microbiome and metabolites reveal the effect of host genetics on hybrid crosses. Environ. Microbiol. Rep. 8, 1016–1023 (2016).
81. Weir, A. H. & Cowley, L. P. Iconic wing patterns of the cicada predator, Xylena fastidiosa. J. Color. 2010:64 (2010).
82. Hopkins, D. L. Xylella fastidiosa: Xylem-limited bacterial pathogen of plants. Annu. Rev. Phytopathol. 27, 271–290 (1989).
83. Karban, R. Why cicadas (Hemiptera: Cicadidae) develop so slowly. Biol. J. Linn. Soc. Lond. 135, 291–298 (2021).
84. Krell, R. K., Boyd, E. A., Nay, J. E., Park, Y.-L. & Perring, T. M. Mechanical and insect transmission of Xylella fastidiosa to Vitis vinifera. Am. J. Enol. Vitic. 58, 211–216 (2007).
85. Paiao, F., Meneguim, A. M., Casagrande, E. C., Lovato, L. & Leite, R. P. Levantamento de espécies de cigarras e transmissão de Xylella fastidiosa em cafeeiro. http://www.sibcafe.ufv.br/handle/123456789/1457 (2003).
86. Elbeaino, T. et al. Identification of three potential insect vectors of Xylella fastidiosa in southern Italy. Phytopathol. Mediterr. 53, 328–332 (2014).

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Author contributions
D.H., E.G., and C.S. collected specimens. D.H., J.V., E.G., E.L., A.L., and C.S. contributed to experimental design. D.H., J.V., E.L., A.L., E.M.L. generated data. D.H., J.V., and M.S. analyzed data. D.H., J.V., M.S., E.G., and C.S. contributed to the writing and editing of the manuscript.

Competing interests
The authors declare no competing interests.

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