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Research Article

RESEARCH ARTICLE

No evidence for phylosymbiosis in western chipmunk species

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One sentence summary: Gut microbiomes of western chipmunks species did not show the same pattern as would be expected based on their species phylogeny

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ABSTRACT

Phylosymbiosis refers to a congruent pattern between the similarity of microbiomes of different species and the branching pattern of the host phylogeny. Phylosymbiosis has been detected in a variety of vertebrate and invertebrate hosts, but has only been assessed in geographically isolated populations. We tested for phylosymbiosis in eight (sub)species of western chipmunks with overlapping ranges and ecological niches; we used a nuclear (Acrosin) and a mitochondrial (CYTB) phylogenetic marker because there are many instances of mitochondrial introgression in chipmunks. We predicted that similarity among microbiomes increases with: (1) increasing host mitochondrial relatedness, (2) increasing host nuclear genome relatedness and (3) decreasing geographic distance among hosts. We did not find statistical evidence supporting phylosymbiosis in western chipmunks. Furthermore, in contrast to studies of other mammalian microbiomes, similarity of chipmunk microbiomes is not predominantly determined by host species. Sampling site explained most variation in microbiome composition, indicating an important role of local environment in shaping microbiomes. Fecal microbiomes of chipmunks were dominated by Bacteroidetes (72.2%), followed by Firmicutes (24.5%), which is one of the highest abundances of Bacteroidetes detected in wild mammals. Future work will need to elucidate the effects of habitat, ecology and host genomics on chipmunk microbiomes.

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INTRODUCTION

The complex microbial communities that inhabit vertebrate hosts—the microbiome—are involved in many aspects of host biology. Besides digestive functions, microbiomes are involved in immune function, hormone regulation and development—reviewed in Cryan and O'Mahony (2011); Zeevi, Korem and Segal (2016) and Clavijo and Flórez (2018). Microbiome traits often correlate with host genetics (Benson *et al.* 2010; Goodrich *et al.* 2014; Blekhman *et al.* 2015) and taxonomy (e.g. Hird *et al.* 2015), which together could support a link between the process of host speciation and microbiome composition (but see Moran, Ochman and Hammer (2019)). Such links can lead to phylosymbiosis, the congruence between the ecological similarity of microbiomes and the branching pattern of the host phylogeny (Brooks *et al.* 2016). Because inference of phylosymbiosis is one of the pattern detection, phylosymbiosis is agnostic to mechanism; significant phylosymbiosis can be the result of shared ecology or evolutionary history and, thus, is a preliminary way to assess the strength of the eco-evolutionary signal between hosts and microbiomes. When phylosymbiosis is high, traits of the host that are most similar between closely related species, or individuals (i.e. that exhibit phylogenetic signal), are assumed to shape the microbiome. This process is also referred to as host-filtering and has been hypothesized to result in phylosymbiosis (Brooks *et al.* 2016; Douglas and Werren 2016; Mazel *et al.* 2018). Mazel *et al.* (2018) showed that phylosymbiosis can occur through host-filtering, and does not necessarily rely on long-term host-microbiome co-evolution. In addition to shared traits, vertical transmission of the microbiome could result in phylosymbiosis due to the direct microbial transfer between related individuals (Moran, Ochman and Hammer 2019).

The absence of phylosymbiosis may be a signal that the microbiome is influenced by external factors, such as rapid environmental change, relative to evolutionary changes in the host, or that the microbiome is influenced by traits that do not show a phylogenetic signal. Microbiome patterns of similarity would, therefore, not mirror the host's phylogenetic relationships.

Phylosymbiosis has been detected across many taxa and time scales (Brooks *et al.* 2016; Kohl *et al.* 2018; Kohl, Dearing and Bordenstein 2018; Mazel *et al.* 2018; Lim and Bordenstein 2019). Even within the gastrointestinal tract, different sections can reflect host phylogenetic divergence (Kohl, Dearing and Bordenstein 2018). In mammals, the American pika (*Ochotona princeps*) showed strong evidence for phylosymbiosis among populations inhabiting isolated mountaintops (Kohl *et al.* 2018). Similarly, different species of captive-raised deer mice (*Peromyscus*) were shown to carry species-specific microbiomes (Brooks *et al.* 2016).

The degree of phylosymbiosis in rapidly diverged wild species with overlapping ecological niches is unknown. Chipmunks (genus *Tamias*) of western North America (subgenus *Neotamias*) are a rapid radiation, where 23 species have evolved within the last ∼1.5–3 MY (Reid, Demboski and Sullivan 2012; Sullivan *et al.* 2014). Species of western chipmunks can be widely distributed or geographically restricted, often co-occur (e.g. 2–4 species), can exhibit overlapping morphologies (e.g. cranial morphologies; Sutton and Nadler 1974) and, in many cases, represent classic examples of niche partitioning. Some species are specialists (e.g. *Tamias ruficaudus, Tamias dorsalis*), whereas others have broader niches (e.g. *Tamias minimus, Tamias amoenus*). Where ranges overlap, the specialist often excludes the generalist from areas where it could otherwise occur. Multiple examples of ancient (e.g. Good *et al.* 2008) and recent mitochondrial introgressions (e.g. 27) also occur across the clade—summarized in (Sullivan *et al.* 2014)—due to low levels of gene flow between diverging groups.

Little is known about the chipmunk microbiome, including the degree of phylosymbiosis. Here, we characterize the fecal microbiomes of 46 wild caught western chipmunks, belonging to eight subspecies. We quantify phylosymbiosis using two different host genetic markers, acrosin and CYTB; mitochondrial haplotype can affect the microbiome (Ma *et al.* 2014) and the discordance between mitochondrial and nuclear phylogenies in western chipmunks led to the hypothesis that mitochondrial clade may be more strongly related to the microbiome than nuclear data. We predicted that microbiome similarity would generally increase with: (1) increasing host mitochondrial relatedness, (2) increasing host nuclear genome relatedness and (3) decreasing geographic distance among hosts.

METHODS

Study species and sites

We tested for phylosymbiosis in 46 individuals belonging to 8 subspecies representing 6 species of chipmunk (genus: *Tamias*, subgenus: *Neotamias*) collected from the wild at 15 sites in 4 states in the western United States (Fig. 1; For detailed sampling information see Table S1, Supporting Information). The subgenus *Neotamias* contains 23 species of chipmunk that are predominantly found in western North America. Taxonomic assignment of (sub)species was based on phylogenetic data, geographic distribution and morphology of the baculum (*os penis*).

Sample collection

During July 2016 and May–July 2017, fecal pellets were collected directly from the lower large intestine during dissections and placed in sterile tubes. Fecal samples were stored between −86 and −196°C until shipped on dry ice to the University of Connecticut. Chipmunk specimens were deposited in the Denver Museum of Nature & Science (DMNS), and the National Museum of Natural History (USNM), which supplied liver tissues for host phylogenetic analysis. Collection of chipmunks was conducted under state scientific collection permits (Colorado: 17TR2373; Idaho: 120318; Nevada: 396754; New Mexico: 3660). Methods were approved by the Smithsonian Institution National Museum of Natural History Animal Care and Use Committee (proposal no. 2017-03) and followed the guidelines of Sikes (2016).

Extraction and sequencing: microbial DNA

Total DNA was extracted from fecal pellets using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) following manufacturer's instructions, with a final volume of 100 uL. The V4 region of 16S rRNA genes were amplified and sequenced at the Microbial Analyses, Resources and Service (MARS) facility at the University of Connecticut, Storrs, CT, on the Illumina MiSeq platform. Quant-iT PicoGreen kit was used to quantify DNA concentrations. The V4 region of the 16S rRNA gene was amplified using 30 ng of extracted DNA as template. The V4 region was amplified

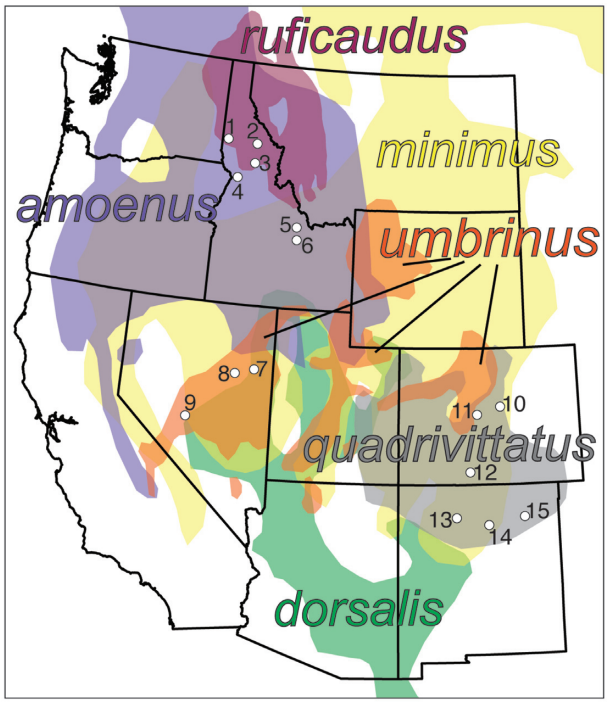

Figure 1. Range maps of the chipmunk species and sampling sites investigated in this study. Sampling site were: Moscow Mountain (1), Weitas Creek (2), Rackliff Creek (3), Slate Creek (4), Lehmi Range (5), Lost River Range (6), Ruby Mountains (7), Cherry Creek Range (8), Toiyabe Range (9), Redskin Creek/Colorado Trail (10), Mosquito Range (11), San Juan Mountains (12), Jemez Mountains (13), Windsor Creek (14) and Mills Canyon (15). All samples were collected in 2017, except for 9 samples that were collected in 2016 from Moscow Mountain, Weitas Creek and Slate Creek in Idaho. For sample size per site, see Table S1 (Supporting Information).

using 515F and 806R with Illumina adapters and dual barcodes (Caporaso and Lauber 2011; Kozich *et al.* 2013). PCR conditions consisted of 95°C for 3.5 min, 30 cycles of 30 s at 95.0°C, 30 s at 50.0°C and 90 s at 72.0°C, followed by final extension at 72.0°C for 10 min. PCR products were normalized based on the concentration of DNA from 250 to 400 bp and pooled. Pooled PCR products were cleaned using the Mag-Bind RxnPure Plus (Omega Biotek) according to the manufacturer's protocol, and the cleaned pool was sequenced on the MiSeq using v4 2 × 250 base pair kit (Illumina, Inc., San Diego, CA).

Extraction and sequencing: chipmunk DNA

Chipmunk host DNA was extracted from liver tissue using the E.Z.N.A Tissue Kit (Omega Biotek, Norcross, GA). Tissue was stored in 95% EtOH and allowed to air-dry before the extraction process to prevent interference with the protocol. DNA was eluted in 200 uL Buffer AE and stored at −20°C. DNA concentration and quality was measured with a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE) and concentrations ranged from 5 to 150 ng/uL.

With some modifications, the PCR procedure from Reid *et al.* (2012) was followed to amplify acrosin (ACR) (1140 bp) and cytochrome B (CYTB) (1117bp; Reid, Demboski and Sullivan (2012)). PCR were performed in 25 uL reactions, as follows: 100–200 ug of DNA, 2.5 uL 10X Taq Reaction Buffer, 1 uL 25mM $MgCl_2$, 0.5uL 10mM dNTPs, 0.5uL 10uM forward and reverse primers, and 0.125uL 10X Taq DNA polymerase. Amplification of CYTB was done using the following PCR conditions: 94°C for 60 sec; 38 cycles of 94°C for 30 sec, 50°C for 30 sec and 72°C for 60 sec; with a final elongation step of 72°C for 6 min. For the amplification of ACR, we used a touchdown procedure to increase PCR specificity as follows: 2 cycles of 94°C for 60 sec, 74–58°C for 90 sec (down cycling 2°C to a final annealing temperature of 58°C), 94°C for 60 sec and 72°C for 60 sec; followed immediately by: 18 cycles of 94°C for 30 sec, 58°C for 30 sec and 72°C for 60 sec; with a final elongation at 72°C for 6 min. PCR products were assessed by gel electrophoresis in a 1% agarose gel to check for successful amplification. All successful PCR products were cleaned with E.Z.N.A Cycle Pure Kit (Omega Biotek, Norcross, GA) and sequenced in both directions (Eurofin Genomics LLC, Louisville, KY). Chromatograms were aligned and edited using CodonCode Aligner (CodonCode Corp., Dedham, MA). Regions of low quality were examined by inspecting chromatograms by eye and coded using the standard nucleotide ambiguity codes.

Microbial sequence analyses

The DADA2 pipeline in R version 3.4.3 was used to process microbial sequence data (Callahan *et al.* 2016; R Development Core Team 2018). DADA2 outputs a high-resolution amplicon sequence variant (ASV) table, which records the number of times each unique sequence is observed in each sample and uses the ASVs as the operational taxonomic unit. After initial quality assessment, sequences were trimmed to remove low quality read areas. Upon assessment of error rates, paired-end sequences were merged and chimeras were removed. Sequences were assigned to taxonomy using RDP's Naïve Bayesian Classifier (Wang *et al.* 2007) with the Silva reference database (v.132; Quast *et al.* 2012). Sequences identified as chloroplast and mitochondrial sequences were removed from the dataset, as well as sequences that did not align to Bacteria. The *DECIPHER* package in R (Wright 2015) performed a multiple-alignment, and the *phangorn* package version 2.4.0 (Schliep 2011) built a phylogenetic tree of the microbial ASVs by constructing a neighbor-joining tree based on a generalized time-reversible (GTR) model with a gamma distribution. The presence and abundance of potential contaminants was assessed using the 'decontam' package in R (Davis *et al.* 2018), which compares sequences in the negative extraction controls to those in the samples. Contaminants were removed from the dataset prior to analyses.

Statistics

We rarefied our data to 23 036 seqs/sample, which was the lowest sequence number in our samples. The rarefied dataset was only used for alpha diversity measures, and all other analyses were performed on non-rarefied data. We calculated richness (observed number of ASVs) and alpha diversity (Shannon diversity index; Shannon and Weaver 1949) of samples using the 'phyloseq' package (McMurdie and Holmes 2013). Because DADA2 removes all singletons from the dataset, diversity estimates are likely biased downwards, as some singletons may represent real, rare microorganisms. Our estimates are comparable within this single dataset, and we note that comparisons of our findings to other results not analyzed with DADA2 should be done with caution. We used Analysis of Variance (ANOVA) to assess differences in richness and Shannon diversity index among chipmunk species and sampling sites. To determine which variables (state, sampling site, species, subspecies, CYTB clade) contributed most to the variation detected in chipmunk fecal microbiomes, we conducted a PerMANOVA using the adonis2 function with by='margin' in the 'vegan' package in R (Oksanen *et al.*

Table 1. Normalized RF scores comparing three distance matrices from chipmunk gut microbiome communities against acrosin (ACR) and CYTB phylogenetic trees derived from the same individuals.

	ACR		CYTB		Species tree
	Individual	Subspecies	Individual	Subspecies	
Bray–Curtis	0.94 ($P > 0.9$)	1 ($P = 1$)	1 ($P = 1$)	1 ($P = 1$)	0.83 ($P > 0.9$)
Unweighted UniFrac	1 ($P = 1$)	1 ($P = 1$)	0.96 ($P > 0.9$)	1 ($P = 1$)	1 ($P = 1$)
Weighted UniFrac	1 ($P = 1$)	1 ($P = 1$)	1 ($P = 1$)	1 ($P = 1$)	1 ($P = 1$)

2018). Non-metric multidimensional scaling (NMDS) plots were constructed for Bray–Curtis and UniFrac distances, and homogeneity of variance was assessed using the betadisper function from the 'vegan' package.

We compared relative abundances of phyla and classes among chipmunk (sub)species and sampling sites. In addition, we compared the average chipmunk microbiome composition to the average microbiome composition of six other mammal species previously described in literature.

Phylogenetic sequence analyses

We conducted separate phylogenetic analyses of ACR and CYTB because of observed mitochondrial introgression via hybridization that has been shown to produce signatures of gene tree discordance (Hird and Sullivan 2009; Reid, Demboski and Sullivan 2012; Sullivan *et al.* 2014; Sarver *et al.* 2017). For each locus, models of sequence evolution were evaluated in PAUP*4.0b using decision theory (DT) (Swofford 2002; Minin *et al.* 2003). The best models of sequence evolution for CYTB and ACR were HKY85+I+G and K81uf, respectively. The models were implemented into Garli for ML phylogenetic inference (Zwickl 2006). Garli runs were terminated after a minimum of 30 000 generations without significant topological improvement. We also performed 100 bootstrap replicates to assess nodal support.

Phylosymbiosis

First, we created dendrograms of individual microbiome relatedness for all chipmunk host samples that were successfully sequenced for ACR and CYTB. For subspecies-level analyses, we divided CYTB trees into clades based on tip placement and previous studies (Hird and Sullivan 2009; Reid, Demboski and Sullivan 2012; Sarver *et al.* 2017). Per gene, we created three microbiome dendrograms based on three distance metrics: unweighted and weighted UniFrac (Lozupone and Knight 2005), and Bray–Curtis (Bray and Curtis 1957; calculated using packages 'phyloseq' and 'phangorn'). We created UPGMA trees for the microbiome data using the 'phangorn' package version 2.4.0, following Brooks *et al.* (2016). We transformed trees to binary by randomly bifurcating zero length branches using the multi2di function in the 'ape' package (Paradis, Claude and Strimmer 2004). The microbiome dendrograms were statistically compared to the chipmunk trees using the Robinson–Foulds (RF) distance (Robinson and Foulds 1981), using the 'phangorn' package. We normalized RF distances by the total number of nodes. Normalized RF distances of 0 show complete congruence of tree topology, while a distance of 1 represents no shared nodes. Significance was assessed by randomly shuffling the tip labels of the microbiome dendrogram 1000 times and recalculating the RF distance between the randomized microbiome dendrogram and the chipmunk tree. Microbiome dendrograms and host phylogenies were created at both the individual level and the subspecies level. In addition to the RF index, we calculated correlation between phylogenetic and microbiome matrices using the Mantel test in the ade4 package (Dray and Dufour 2007).

For subspecies level microbiome analyses, ASV counts were averaged over all individuals within a subspecies. A species tree topology was created for the focal subspecies by pruning the species tree from Reid *et al.* (2012), that was estimated using BEAST (Heled and Drummond 2010). Host-microbiome 'tanglegrams' were constructed using the 'tanglegram' function in the 'dendextend' package (v1.8.0; Galili 2015)

Data availability

Sequence data and metadata files are available at Figshare, project number 10.6084/m9.figshare.6905324 (Https://figshare.com/articles/Chipmunk_phylosymbiosis/6905324) and NCBI SRA (Accession: PRJNA574130). DMNS and MSB museum specimen records for chipmunk hosts are available at Arctos: http://arctos.database.museum/SpecimenSearch.cfm. R code is available at https://github.com/KCGrond/chipmunk_R_code.git.

RESULTS

We obtained a total of 4 814 744 raw (microbiome) sequences. We removed chimeras and non-target sequences and continued our analyses with 3 166 367 high quality sequences after QC (68834.1 ± 7677.6SE seqs/sample; range: 15 630–199 753 seqs/sample). Using the 'decontam' package in R, we detected one potential contaminant out of 2354 ASVs, which was removed from our dataset.

Phylosymbiosis

Normalized RF scores ranged from 0.94 to 1, depending on phylogenetic marker used and whether trees were compared on an individual or subspecies level (Table 1). There was no phylosymbiosis detected (Permutational test: $P = 1.00$) on an individual or subspecies level for any of the three distance metrics tested (Table 1, Fig. 2). In addition, we did not detect any significant relationship between fecal microbiome similarity and geographical distance among sampling sites (Mantel test: $r = 0.028$, $P = 0.978$; Figure S1, Supporting Information). Comparison of ACR and CYTB had a normalized RF score of 0.5, which indicates that 50% of nodes matched (Fig. S2, Supporting Information); this is to be expected given the rampant mtDNA introgression documented from these taxa (summarized in Sullivan *et al.* (2014)). Results from the Mantel test were congruent in significance with the RF test for ACR/CYTB–microbiome comparisons (Bray: $P = 0.102$–0.259;

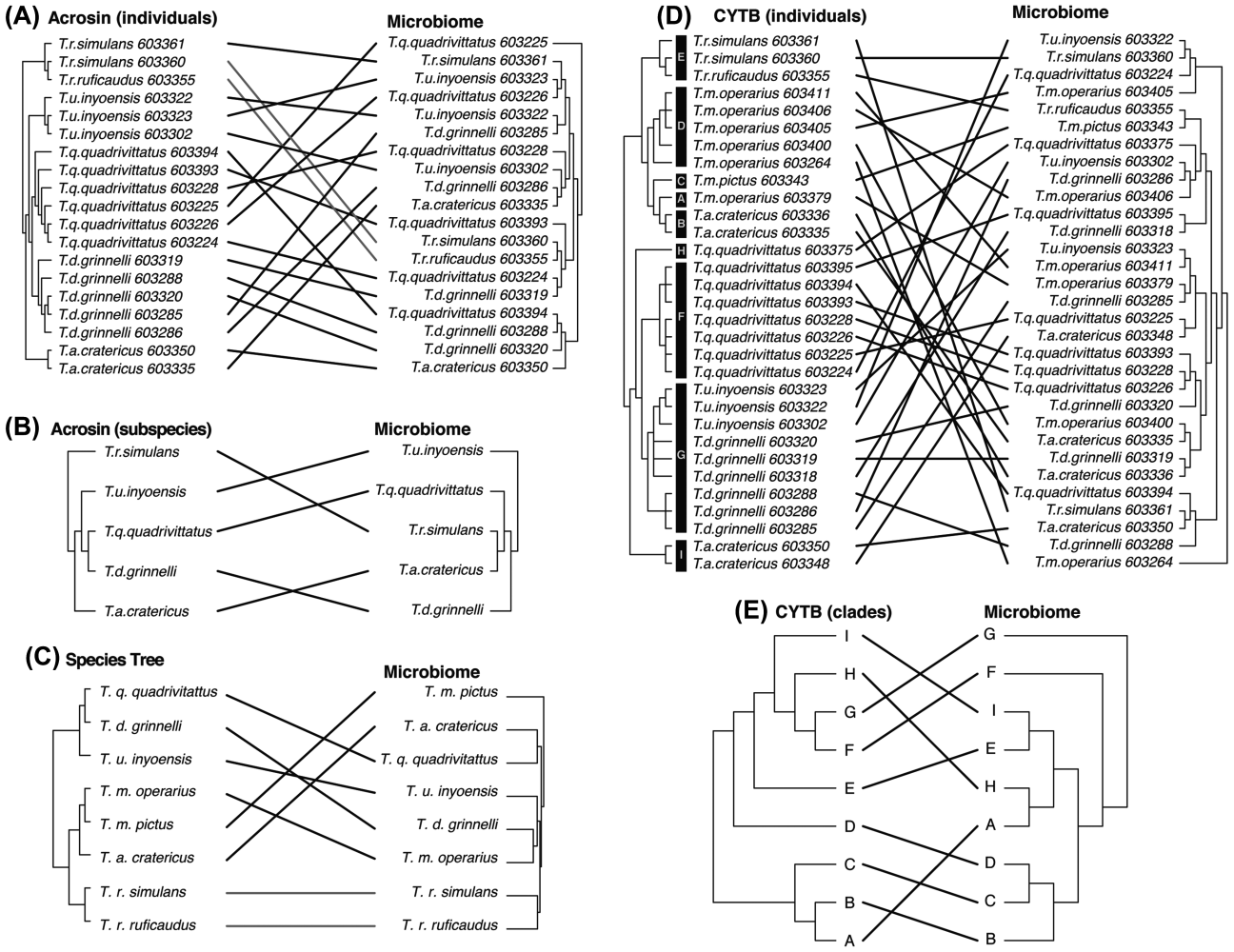

Figure 2. Bray–Curtis distance matrix trees of the chipmunk microbiome plotted against phylogenetic trees of (A) individual level ACR, (B) subspecies level ACR, (C) species tree, (D) individual level CYTB and (E) clade level CYTB. Clades shown in figure E are identified in figure D.

unweighted UniFrac: $P = 0.549–0.998$; weighted UniFrac: $P = 0.832–0.998$).

Alpha diversity

We detected a total of 2354 unique ASVs, resulting in an average of 163.8 ± 9.9SE ASVs per sample (range: 73–313). ASVs were assigned to 156 genera in 15 phyla. We did not detect any statistical differences in richness and alpha diversity among species (richness $F_{7,28} = 0.353$, $P = 0.921$; alpha diversity $F_{7,28} = 0.639$, $P = 0.72$; Figure S3, Supporting Information).

Community composition

A majority of sequences obtained from chipmunk feces belonged to the phyla Bacteroidetes (72.2%), Firmicutes (24.5%) and Proteobacteria (2.4%; Fig. 3A; Figure S4, Supporting Information). Within the Bacteroidetes phylum, 76.5% of sequences belonged to the genera *Prevotellaceae_UCG-001* and *Bacteroides* (Fig. 3B). Both within the class of Bacteroidia, *Prevotellaceae_UCG-001* comprised 46.6% of these sequences, and *Bacteroides* comprised 29.9%. In the Firmicutes, the most abundant genus consisted of the *Lachnospiraceae_NK4A136_group* (28.9%), followed by the *Ruminococcaceae_UCG-014* (8.3%), both within the Clostridia class.

Drivers of microbiome composition

Sampling site, state, species and subspecies were all significantly associated with the variation between microbiomes ($P < 0.002$, Table 2) when using Bray–Curtis distances. Sampling site explained the greatest amount of variation (Bray: $R^2 = 0.424$), followed by subspecies (Bray: $R^2 = 0.149$, $P < 0.001$). Within the two subspecies of *T. ruficaudus* (*T. r. ruficaudus* and *T. r. simulans*), we detected clustering by sampling location for both Bray–Curtis (stress = 0.11) and weighted UniFrac (stress = 0.06) distances (Fig. 4, see Figure S5 (Supporting Information) for full species NMDS), which supports the PerMANOVA results that local environment plays a role in determining microbiome composition. Homogeneity of variances of our ordinations did not differ among chipmunk species for Bray–Curtis (ANOVA $F_{1,11} = 2.43$, $P = 0.147$) and weighted UniFrac distance matrices (ANOVA $F_{1,11} = 2.03$, $P = 0.182$). Host species only explained between 7% of the variance in gut microbiome communities. Following species, the state that chipmunks were sampled in contributed least to variation in chipmunk microbiomes (Bray: $R^2 = 0.130$,

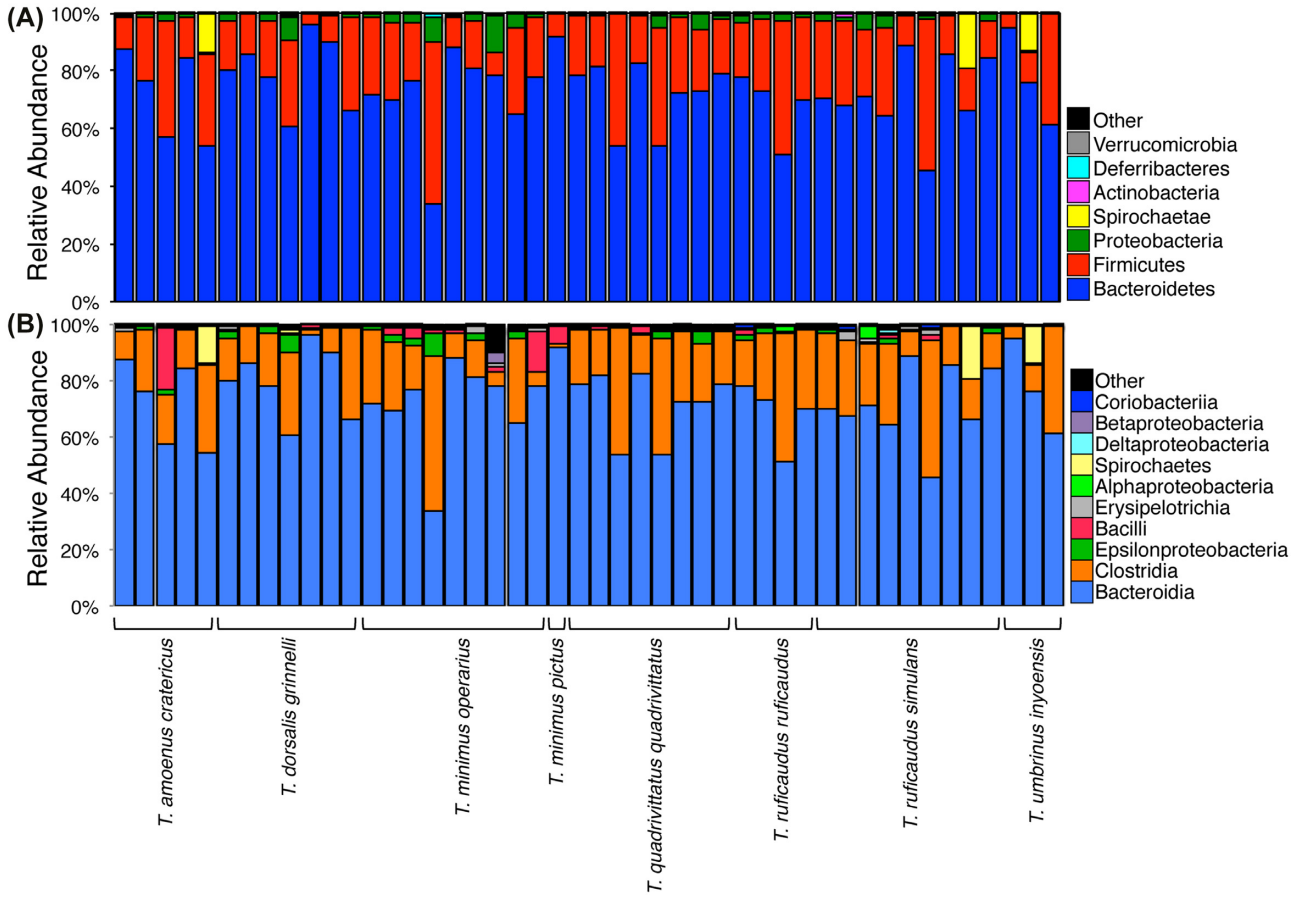

Figure 3. Average relative abundance of bacterial phyla (A) and classes (B) detected in fecal samples of eight chipmunk (sub)species sampled at 15 sites in 4 US states.

Table 2. PerMANOVA (adonis) tests for significance and relative contribution of two environmental and three phylogenetic factors to variation in Bray–Curtis, and weighted and unweighted UniFrac Distance Matrices constructed from chipmunk fecal microbiomes.

	Bray		Unweighted UniFrac		Weighted UniFrac	
	Pseudo-F | R^2	*P*	Pseudo-F | R^2	*P*	Pseudo-F | R^2	*P*
State	2.23 | 0.130	<0.001	1.55 | 0.062	0.008	4.01 | 0.063	0.016
Sampling site	1.68 | 0.424	<0.001	1.34 | 0.214	0.002	1.59 | 0.210	0.248
Species	1.40 | 0.070	0.002	1.4 | 0.141	<0.001	2.19 | 0.151	0.251
Subspecies	1.52 | 0.149	<0.001	1.27 | 0.069	0.064	2.20 | 0.037	0.166
Sex	0.96 | 0.016	0.745	0.98 | 0.017	0.633	0.89 | 0.016	0.993

$P < 0.001$), which is unsurprising since chipmunk (sub)species ranges extend across state lines. Sex of chipmunks did not significantly contribute to variation in their microbiome (Bray: $R^2 = 0.016$, $P = 0.745$).

DISCUSSION

All mammals host complex microbial communities that are involved in myriad aspects of their biology. Understanding the role that the microbiome plays in mammalian evolution requires sampling wild organisms across a spectrum of ecologies and evolutionary histories. Here, we investigated phylosymbiosis of the fecal microbiome in eight Western chipmunk (sub)species. These species belong to a rapidly diverged group of closely related rodents with complex patterns of range and niche overlap. We did not find evidence of phylosymbiosis, which contradicts our predictions that phylosymbiotic relationships would increase with host relatedness, and decrease with geographic distance. Phylosymbiosis is regarded as a common pattern in host-associated microbiomes; our findings contrast with previous mammalian studies, that showed evidence for phylosymbiosis, e.g. in American pika (Kohl *et al.* 2018), humans (Ross *et al.* 2018) and in general across 14 mammalian orders (Nishida and Ochman 2018). The lack of evidence for phylosymbiosis in chipmunks could be attributed to several factors. It seems most likely that niche and/or range overlap of many of our samples, has resulted in homogenization of microbiome communities through horizontal transfer. The horizontal transfer may originate directly from shared environment, from a shared ecosystem, shared dietary elements or physical contact. Patchiness may degrade strong species-specific microbiomes, as different more isolated populations of widespread species could come

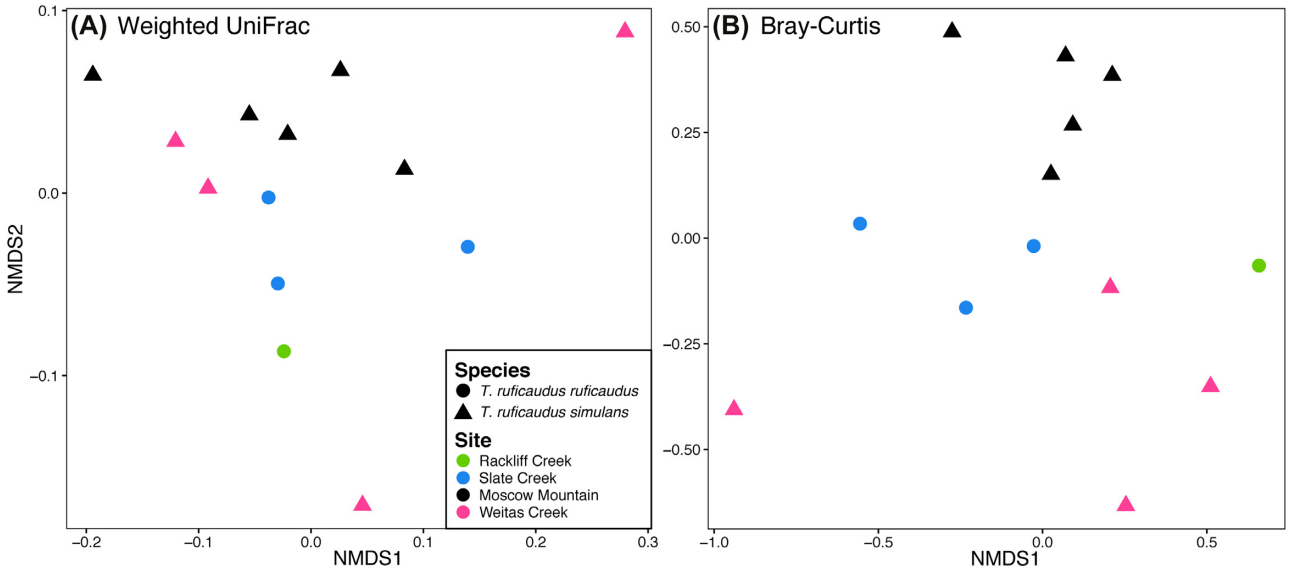

Figure 4. Non-metric Multidimensional Scaling ordination constructed from weighted UniFrac and Bray–Curtis matrices of fecal microbiomes collected from two *T. ruficaudus* subspecies (*T. r. ruficaudus* and *T. r. simulans*) at four sites in Idaho. Shapes represent different subspecies sampled, and colors represent sampling sites

into contact with different sets of neighboring species. Alternatively, the lack of phylosymbiosis could be due to the rapid evolution of the clade or insufficient sample size. These three factors are discussed individually below.

First, the species sampled for our study do not live in geographic isolation. There is significant overlap in species range (Fig. 1), which could facilitate gene and microbiome transfer, (but note there is substantial local patchiness within chipmunk species ranges). Western chipmunks show relatively high levels of introgressive hybridization (Sullivan *et al.* 2014). Acrosin and CYTB tree topologies matched on 50% of nodes, which is not unexpected as mitochondrial introgression is well documented within our focal species and the placement of individuals within clades with introgression events can have a large effect on topology congruence metrics like RF. Mitochondrial haplotype can affect the microbiome (Ma *et al.* 2014; Hirose *et al.* 2017) and the discordance between mitochondrial and nuclear phylogenies in western chipmunks led to the hypothesis that mitochondrial clade may be more strongly related to the microbiome than the nuclear data is.

However, contrary to these expectations, we did not detect any differences in the level of phylosymbiosis between the two phylogenetic markers and the microbiome. Despite the known association between mitochondrial haplotype and microbiome, we found no correlation between microbiome and host for this marker. In addition, the lack of correlation between the nuclear marker, acrosin and the microbiome does not show a phylosymbiotic relationship. Environment and diet can affect microbiome composition. Their range overlap exposes different chipmunk species to similar environments and diets, which could lead to convergence of microbiomes across (sub)species (Muegge *et al.* 2011). We found that sampling site, or the direct environment the chipmunk interacts with, explained almost half of the variation detected in microbiome samples. Chipmunks that are exposed to the same environmental microbiomes could be colonized by the same microorganisms, leading to across-species similarities in gut microbiomes. Our results are incongruent with other rodent studies. A study investigating environmental and host species effects on microbiomes in three rodent taxa, shrews, mice and voles, showed strong host species effects despite similar habitats (Knowles, Eccles and Baltrūnaitė 2019). Similarly, wild house mice (*Mus musculus*) kept in a common garden environment showed continued host-specific gut microbiomes, despite assumed similar microbial environments (Suzuki *et al.* 2019). The strong site effects we detected could potentially be associated with chipmunk-specific behaviors, but we need additional studies to confirm this.

Second, the chipmunk species in our study rapidly and relatively recently diverged, which could result in insufficient time for phylosymbiosis to occur. However, co-divergence of chipmunks and parasitic pinworms was detected (Bell *et al.* 2016; Bell, Demboski and Cook 2018) within this time frame. Although phylosymbiosis does not rely on matching time scales between host and microbiome (Moran, Ochman and Hammer 2019), the co-divergence of higher parasites like pinworms indicates that microbial co-divergence should also be possible over this time frame. From our study species, the two *T. ruficaudus* subspecies, *T. r. ruficaudus* and *T. r. simulans*, split most recently at 326 100 years ago (90% CI: up to 2 854 839 years; Hird and Sullivan (2009)). Kohl *et al.* (2018) found strong phylosymbiosis signal among populations of American pika that split 150 000–900 000 years ago (Galbreath, Hafner and Zamudio 2019), indicating that phylosymbiosis can manifest on timescales similar to the chipmunk divergences in our dataset. However, pika populations have experienced almost complete isolation since they split due to their specialized mountain top habitats (Kohl *et al.* 2018). Nishida and Ochman (2018) found large variation in divergence times of species that showed phylosymbiotic relationships, but a positive relationship between the phylosymbiosis and divergence time was observed across animal clades by Brooks *et al.* (2016). Their mammalian lineage contained six 'Peromyscus' species that diverged 11.7 million years ago and showed complete congruence between phylogenetic and microbiome trees on a species level, although these samples were raised in captivity, which can alter microbiomes (Hird 2017).

Last, larger sample sizes and a more thorough geographic sampling of chipmunk populations may be needed to detect

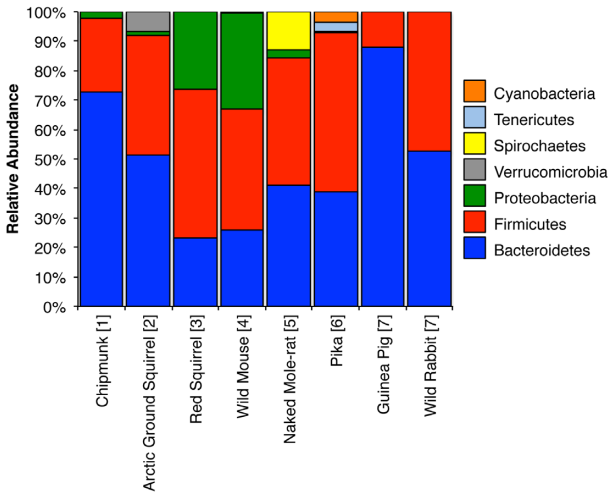

Figure 5. Relative abundance of bacterial phyla in the gut microbiome of chipmunks in our study [1], and 6 additional mammalian taxa. Results were obtained from [2] Hatton *et al.* 2017, [3] Bobbie, Mykytczuk and Schulte-Hostedde, 2017, [4] Rosshart *et al.* 2017, [5] Debebe *et al.* 2017, [6] Kohl *et al.* 2018 and [7] Crowley *et al.* 2017.

phylosymbiosis between such recently diverged hosts. However, the absence of phylosymbiosis in our study indicates that, even with larger sample sizes, the effect is unlikely to be strong. Our limited sample was nevertheless sufficient to demonstrate that chipmunk microbial community similarities were determined mostly by sampling site (42.4%), although subspecies still explained 14.9% of variation. More interspecific comparisons of wild-caught samples will shed light on the frequency of species tracking versus locality tracking.

Characterization of the chipmunk microbiome

The chipmunk microbiome was dominated by Bacteroidetes and Firmicutes. These two phyla frequently make up the majority of mammalian microbiomes (Debebe *et al.* 2017; Rosshart *et al.* 2017), but Firmicutes is usually the more abundant phylum (Fig. 5) (Ley *et al.* 2008; Kohl *et al.* 2018). Bacteroidetes made up over 70% of the chipmunk microbiome and Firmicutes averaged only 24%. As seen in Fig. 5, a high abundance of Bacteroidetes was also observed in another rodent species, the Guinea pig (*Cavia porcellus*), but this trend was not consistent across all rodents (Fig. 5). Bacteroidetes are involved in fermentative degradation of biopolymers (Thomas *et al.* 2011). They have the ability to metabolize more than a dozen plant- and host-derived polysaccharides (Gibiino *et al.* 2018), and the high abundance in the chipmunk gastrointestinal tract may be an adaptation to a predominantly plant-based diet. However, other herbivorous mammals do not show similar high Bacteroidetes abundances, indicating potential other, non-diet related functions.

Moreover, chipmunks are omnivorous, and their diets have been observed to include plant material, seeds, fruits, fungi, insects and bird eggs and nestlings (Ostfeld, Jones and Wolff 1996; Schmidt *et al.* 2001; Kuhn and Vander Wall 2009; Teron and Hutchingson 2013), and further studies into seasonal and local diet preferences of the chipmunk species in our study are warranted to test this hypothesis.

Overall, our findings did not support our prediction that microbiome similarity increased with increasing host phylogenetic relatedness or decreasing geographic distance among hosts. The lack of phylosymbiosis in chipmunks may be due to ecological niche overlap among some (sub)species, but this hypothesis requires further testing given our limited sampling and the propensity for many species to niche partition. Local environment, or sampling site, did explain most variation in microbiome composition, which support this hypothesis.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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