

**Phylogenetically under-dispersed gut microbiomes are not correlated with host genomic heterozygosity in a genetically diverse reptile community**

**Running title:** phylogenetically clustered gut microbiomes

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## Abstract

While key elements of fitness in vertebrate animals are impacted by their microbiomes, the host genetic characteristics that factor into microbiome composition are not fully understood. Here, we correlate host genomic heterozygosity and gut microbiome phylogenetic diversity across a community of reptiles in southwestern New Mexico to test hypotheses about the behavior of host genes that drive microbiome assembly. We find that microbiome communities are phylogenetically under-dispersed relative to random expectations, and that host heterozygosity is not correlated with microbiome diversity. Our analyses reinforce results from functional genomic work that identify conserved host immune and non-immune genes as key players in microbiome assembly, rather than gene families that rely on heterozygosity for their function.

## Introduction

Interactions between animals and their microbiomes, the community of microbes that live on or in host tissues, can exert selective pressure on hosts (Gould et al., 2018; Rosshart et al., 2017), microbes (Garud, Good, Hallatschek, & Pollard, 2019; Guo et al., 2019), or both (Abdul Rahman et al., 2015; Barroso-Batista, Demengeot, & Gordo, 2015; Moeller et al., 2016). The gut microbiome in particular impacts host health, including by protecting hosts from parasites (Duvallet, Gibbons, Gurry, Irizarry, & Alm, 2017; Rosshart et al., 2017; Stough et al., 2016). This function is shared by the microbiomes of other mucosal membranes that interact with the host's external habitat (Jani & Briggs, 2018; Knutie, Wilkinson, Kohl, & Rohr, 2017; Planet et al., 2016; Takeuchi, Fujiwara-Nagata, Katayama, & Suetake, 2021). Despite the clear importance of the gut and other mucosal microbiomes to the survival and fitness of individual hosts, the field lacks a consensus on the expected broad-scale patterns of host-microbiome evolutionary interactions (Hird, 2017; Sharpton, 2018). Empirical studies of host-microbe relationships across the tree of life have findings ranging from tight symbiosis (Jahnes & Sabree, 2020; J. K. Kim et al., 2016) to co-evolutionary arms races (Cable & van Oosterhout, 2007; Eizaguirre, Lenz, Kalbe, & Milinski, 2012; Sarabian, Curtis, & McMullan, 2018). Here, we use the correlation between host genetic diversity and cloacal microbiome phylogenetic diversity across a reptile community to interrogate possible evolutionary processes in host-microbiome interactions. We describe a plausible predicted direction of the correlation between microbiome diversity and host

genetic diversity if higher-diversity microbiomes provide an adaptive advantage to the host and the host genes that structure the microbiome are dependent on heterozygosity for their function. We then compare those predictions to our observed data (Figure 1).

Many studies, particularly in humans and mammalian model organisms, find correlations between host genotypes and gut microbiome structure (Cahana & Iraqi, 2020; Goodrich et al., 2016) and signals of heritability in the bacteria lineages present in gut microbiomes (Grieneisen et al., 2021; Moeller et al., 2017). These studies strongly indicate that adaptive evolution by the host is one of the mechanisms structuring microbiome communities, either through maintaining vertically inherited microbial lineages or by determining how horizontally acquired lineages are filtered by the host. Simultaneously, broad-scale phylogenetic work shows that mammals tend to have more phylogenetic signal in their gut microbiomes than other tetrapods (Song et al., 2020; Youngblut et al., 2019), possibly indicating that different mechanisms structure microbial communities outside of mammals. Determining the degree to which adaptive evolution by the host structures microbial communities across vertebrates and narrowing down the potential mechanisms which do so is a major challenge in fully integrating host-microbe interactions into broader ecological and evolutionary studies. As a further complication to field studies in particular, environmental drivers of gut microbiome assembly are important in all vertebrate systems (Gacesa et al., 2022; P. S. Kim et al., 2021; Wu et al., 2018). Here, we draw on macroecological concepts to identify patterns we would expect to see if hosts were evolving to maximize microbiome diversity (Figure 1A). Although we cannot directly test functional questions, this approach allows us to rule out potential processes that are inconsistent with our observed data. In addition, we use an exploratory science approach to identify patterns within our microbiome communities that could explain our broad-scale findings.

The specific analyses in our study are motivated by a set of broader eco-evolutionary questions, which will require testing using a variety of sources of evidence. These types of questions are key to understanding the relationships between hosts and microbiomes at large ecological and evolutionary scales. For example, we could ask about the expected standing diversity of the genes within a host species that are most likely to be selected on by the cloacal microbiome. This would determine the speed and magnitude of response to selection in the host population. Relatedly, what is the expected rate of adaptive change (if any) of host genetic drivers of microbiome structure over evolutionary time? Finally, what are some characteristics of

host genes that might drive host-microbiome interactions on evolutionary time scales (Hird, 2017; Shapira, 2016; Sharpton, 2018)? Integrating evidence supporting answers to these large-scale questions across empirical datasets and different approaches will allow the field to develop a coherent set of predictors for the eco-evolutionary role of the host-microbiome unit.

### **Microbiome diversity and host fitness**

To generate predictions, we draw on a community ecology hypothesis that higher diversity biological communities can be more stable and resilient than low-diversity communities (de Mazancourt et al., 2013; Lehman & Tilman, 2000; Mougi & Kondoh, 2012). If this argument holds true for microbiomes, higher diversity should provide a fitness advantage to the host (Figure 1A), because diversity maximizes the microbiome's effectiveness at delivering consistent, reliable metabolic services (Gould et al., 2018; Kohl, Weiss, Cox, Dale, & Denise Dearing, 2014; Rosshart et al., 2017). In humans, low diversity in the microbiome is correlated with multiple disease states (Durack & Lynch, 2019; Kriss, Hazleton, Nusbacher, Martin, & Lozupone, 2018; Michail et al., 2012). In some cases, severe disease can occur when low-diversity microbiomes are invaded by opportunistically pathogenic bacteria (Chang et al., 2008), and can be treated by artificially returning the microbiome to a higher-diversity state (Seekatz et al., 2014). In wild vertebrates, low microbiome diversity can correlate with human-induced stressors (Amato et al., 2013; Barelli et al., 2015), indicating that host lineages might experience selection to maximize microbiome diversity. If this is the case, we should be able to observe higher microbiome phylogenetic diversity within hosts than would be expected by chance (shown in Figure 1B column 1). Here, we use observed phylogenetic diversity of our microbial lineages compared to the expected outcome of a random sample of all lineages present. Our observed values should be larger than the expected randomized outcome if our hypothesis is correct (Proches et al 2006). However, there are exceptions to the observation that microbiome diversity correlates with host health, such as animals in disturbed habitats exhibiting higher-diversity microbiomes than those in more intact habitat (Huang, Chang, Huang, Gao, & Liao, 2018; Littleford-Colquhoun, Weyrich, Kent, & Frere, 2019; J. N. Phillips, Berlow, & Derryberry, 2018), and dietary specialists optimizing fitness with a low-diversity, consistent microbiota (Dill-McFarland, Weimer, Pauli, Peery, & Suen, 2016; Greene et al., 2021). These

exceptions underscore the need for systematic tests of the pattern in host communities outside the laboratory.

### **Relationship of host genetic diversity to microbiome diversity**

The hypothesis that higher host heterozygosity could be related to higher microbiome diversity is similarly biologically plausible. Vertebrate hosts interact with their gut microbiome through a variety of heterozygosity-dependent systems, including the innate and adaptive immune systems (Milligan-Myhre et al., 2016; Postler & Ghosh, 2017; Rudman et al., 2019; Small, Milligan-Myhre, Bassham, Guillemin, & Cresko, 2017), the olfactory and taste receptors (R. M. Carey & Lee, 2019; Harmon, Deng, & Breslin, 2021; I. Khan et al., 2015; Leung & Covasa, 2021; Pluznick, 2014), and the nuclear receptor genes (Little et al., 2022; Ning, Lou, Zhang, & Xu, 2019; Sivaprakasam, Bhutia, Ramachandran, & Ganapathy, 2017). These gene categories often occur in large families of similar but non-identical copies derived from gene duplication events, as greater diversity in the proteins they encode allows the host to recognize a larger number of substrates. In addition, this type of gene can undergo rapid pseudogenization, leading to both functional and non-functional alleles for each gene copy circulating in a population. As a result, individuals with lower heterozygosity are more likely to have two pseudogenized alleles at a given locus, thereby reducing their overall sensory repertoire (Hasin et al., 2008; Hasin-Brumshtein, Lancet, & Olender, 2009).

Because a variety of host gene families interact with the gut microbiome, using a genome-wide estimate of heterozygosity provides an approach that is agnostic to the function of any particular family. In addition, field-based studies indicate that genome-wide heterozygosity can be predictive of selective outcomes. Previous studies have shown correlations between genome-wide heterozygosity and fitness or disease prevalence in reptiles (Shaner, Chen, Lin, Kolbe, & Lin, 2013). Other work has shown correlations between neutral-allele heterozygosity and MHC heterozygosity (Elbers, Clostio, & Taylor, 2017; Santonastaso et al., 2017), although the pattern is not found in every population investigated (Hacking, Stuart-Fox, Godfrey, & Gardner, 2018). Both the variation in outcomes of neutral allele-MHC diversity correlations and the variety of gene families that depend on underlying diversity to drive function demonstrate that the microbiome-host genomic heterozygosity relationship requires further empirical testing.

## Study system

We collected samples of host genetic material and cloacal microbiomes from lizards and snakes in the Peloncillo Mountains in southwestern New Mexico during June and July of 2015. This community has high species diversity, increasing our chances of capturing a range of host heterozygosity levels (Grundler, Singhal, Cowan, & Rabosky, 2019). In addition, squamate reptiles lack the shifts to herbivory that drive microbiome assembly across the mammalian phylogeny, thereby reducing potential confounding factors in our work (Harrison et al., 2021; Ley et al., 2008; Youngblut et al., 2019). We sampled a host community of 18 lizard and snake species with a large range of variation of host genetic diversity both within and between species (Table 1), including three lineages of clonal teiid lizards (Reeder, Cole, & Dessauer, 2002). The clonal lizards arise from hybridization events between two diploid parent species, leading the triploid lineages to have high within-individual heterozygosity but little to no diversity between conspecifics. In addition, we have a range of dietary specializations represented. Horned lizards in the genus *Phrynosoma* are relatively specialized on ants (Lemos-Espinal, Smith, & Ballinger, 2004; Meyers, Herrel, & Nishikawa, 2006; Montanucci, 1989; Pianka & Parker, 1975), while other members of the family Phrynosomatidae are generalist insectivores. Dietary specialization is correlated with reduced gut microbiome diversity in some vertebrate taxa (Dill-McFarland et al., 2016; Greene et al., 2021), although the pattern is not universal (Bolnick, Snowberg, Hirsch, et al., 2014). Additionally, our single location, cross-community sampling allows for testing host-microbiome diversity hypotheses at both inter- and intraspecific levels without the potential confounding effects of geographical variation (Amato et al., 2013; Barelli et al., 2015; Ingala, Becker, Bak Holm, Kristiansen, & Simmons, 2019).

Reptiles, like most other vertebrates but unlike mammals, tend to have gut microbiomes dominated by bacteria from the phylum Proteobacteria, with a lower proportion of the community comprised of Firmicutes, Bacteroidetes, and Actinobacteria (Colston & Jackson, 2016). We sampled the reptiles using a minimally invasive cloacal swab, a common approach in reptile cloacal microbiome studies (Colston, Noonan, & Jackson, 2015). Among tetrapods, all orders except mammals have a cloaca, an opening that houses the terminus of the digestive, urinary, and reproductive tracts. The cloacal microbiome is seeded in part by the lower intestinal microbiome but includes aerobic lineages that can survive in its semi-oxygenated environment (Grond, Sandercock, Jumpponen, & Zeglin, 2018). The cloacal microbiome is distinct from the

lower gut and fecal microbiome, with some studies finding little correlation between the communities (Kers, Fischer, Stegeman, Smidt, & Velkers, 2019; Lee et al., 2020; Williams & Athrey, 2020), while others do find similarities (Andreani, Donaldson, & Goddard, 2020; Berlow, Kohl, & Derryberry, 2020; Bodawatta, Puzejova, Sam, Poulsen, & Jønsson, 2020; Zhou et al., 2020). The cloacal mucosa is integral to health in reptiles, with a variety of pathogens causing symptomatic infection in the area (Curtiss et al., 2015; Johne et al., 2002; Styles, Tomaszewski, Jaeger, & Phalen, 2004; Tillis et al., 2021). In addition, the cloacal microbiome in lizards seeds eggshells with anti-fungal microbial lineages, a key fitness benefit (Bunker, Martin, & Weiss, 2021). For these reasons, we predict host-level selection enabling some degree of host control over the assembly and function of cloacal microbiomes.

### **Study aims and predictions**

In our sampled community, we hypothesize that individual microbiomes will show inflated phylogenetic diversity relative to random expectations (Figure 1B, left column). We further hypothesize that host heterozygosity should be correlated with microbiome diversity (Figure 1B, lower row). Identifying this relationship will provide evidence about the standing host genetic diversity available for selection due to host-microbiome interaction and the expected rate of change of microbiome structure over evolutionary time. If microbiome assembly is driven by heterozygosity-dependent, quickly evolving gene families, we would expect host-driven rapid adaptive evolutionary change to the microbiome structure. If assembly instead is more driven by conserved host genes, host genetic controls on microbiome structure should evolve more slowly. We further investigate possible mechanisms behind our observed results using descriptive approaches to identify instances in which our empirical outcomes differ from random community assembly expectations.

### **Materials and Methods**

#### **Sample collection**

During May and June of 2015, we conducted surveys for squamate reptiles (snakes and lizards) on Antelope Pass in the Peloncillo mountains in New Mexico (Table 1, Table S1). We surveyed for approximately eight hours a day between sunrise (between 6:00 and 6:30 am) and

sunset (between 8:00 and 8:30 pm). We avoided sampling during the heat of the day to reduce stress on the animals. For each captured individual, we recorded species, snout-vent length (SVL), mass, date and time of capture, and GPS coordinates (Table S1). We took a tissue sample (tail tip for lizards, ventral scute clip for snakes) and a cloacal swab from each animal. We inserted a sterile rayon urethral swab (MW113 by Medical Wire & Equipment) fully into the cloaca but not into the lower digestive tract and left it in for a count of five. If the animal was large enough, we moved the swab to cover the entire cloaca. By gently applying pressure to the lower abdomen of the animals, we were able to slightly evert the cloacal mucosa and insert the swabs without touching skin.

Tissues and swabs were stored in at ambient temperature in RNALater in the field and carried with the researchers in a backpack to avoid direct sunlight. At the end of each day, samples were deposited in a shaded but not climate-controlled storage location for the duration of the field season. Samples were stored at  $-20^{\circ}\text{C}$  upon returning to the lab. We captured at least one individual from 18 snake and lizard species (Table S1). They fell into six major taxonomic groups, the lizard families Teiidae, Phrynosomatidae, Eublepharidae, and Crotaphytidae, and the snake families Vipiridae and Colubridae. We collected under New Mexico Game and Fish permit 3606 issued to Michael Grundler, and University of Michigan UCUCA protocol #PRO00006234. UCUCA is the equivalent of the more commonly used IACUC acronym.

We sequenced host DNA for 104 individuals and then sequenced microbiome DNA for 94 of those individuals, for a total of 94 individuals with attempted sequencing for both data types. We eliminated some individuals due to either host or microbiome sequencing failing our quality control checks, retaining 63 individuals with high-quality sequences for both microbiome and host DNA (Table 1, Table S1). We used a RADseq approach to quantify host heterozygosity. RADseq heterozygosity values correlate with values derived from other marker types such as microsatellites, other sources of SNP data, and whole genome sequences (Bradbury et al., 2015; Cariou, Duret, & Charlat, 2016; Lemopoulos et al., 2019). RADseq heterozygosity values also correlate with metrics of census population size in lizard species that are ecologically similar to some of our focal taxa (Singhal et al., 2017). For microbiome diversity, we used a metabarcoding approach with the widely used 16S rRNA V4 barcode region, which provides phylogenetic resolution across broad taxonomic scales (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013).



## Laboratory methods

We extracted total DNA from tissue samples and cloacal swabs using DNeasy Blood and Tissue spin column kits from Qiagen. We prepared the tissue DNA according to a RADseq protocol from Peterson, Weber, Kay, Fisher, & Hoekstra, 2012, using the restriction enzymes EcoR1 and Msp1. DNA fragments up to 400 bp in length were sequenced on an Illumina HiSeq platform using version four reagents over two paired end runs at the University of Michigan Sequencing Core Facility. For bacteria metabarcoding, we used an Illumina MiSeq platform at the University of Michigan Microbiome Core Facility to barcode a 252 bp sequence from the V4 region of the 16S rRNA gene (Kozich et al., 2013). The library was prepared by the core and sequenced in a single lane with a negative (water) and positive (extracted DNA from a mock community) control.

## Bioinformatic pipelines

For our RADseq data, we used a modification of the pipeline presented in Singhal et al., 2017. This pipeline was tested against pyRAD (Eaton, 2014) and determined to provide more reliable estimates of heterozygosity (Singhal et al., 2017, electronic supplementary material). We first removed low-quality sequences and adapter sequences using Trimmomatic v0.33 (Bolger, Lohse, & Usadel, 2014) and assembled the reads using Rainbow v2.0.4 (Chong, Ruan, & Wu, 2012). We then generated a separate pseudogenome for each host individual by clustering the assembled reads using vsearch (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Finally, we mapped the raw reads back to the pseudogenome using bwa v.0.7.12 (Li & Durbin, 2009) and called SNPs and indels using the GATK function haplotypeCaller (McKenna et al., 2010; Poplin et al., 2017). Assembling per-individual rather than per-species pseudogenomes allowed us to avoid biases introduced by variation in the number of fragments per individual and differences in sample size between species (Cariou et al., 2016). Using the GATK haplotypeCaller function allowed us to specify the ploidy of the hosts as an input variable for the program. Using published values, we specified that our unisexual species were triploid (Reeder et al., 2002), while the other hosts were diploid. We retained all sequenced DNA fragments with a read depth greater than 20 and called heterozygote bases when the rarer of the two alleles was represented in more than 40% of the reads for diploids or when the rarest of the three alleles was represented in

more than 20% of the reads for triploids. We retained host individuals that had more than 100 DNA fragments of a length greater than 200 bp that passed our filters (Table S1).

For the bacterial 16S rRNA metabarcode data from cloacal swabs, we used the program *mothur* v.1.48 (Schloss et al., 2009). For our 94 microbiome samples and the negative sequencing control, we made contigs from our paired-end sequences, selected fragments within 8 bp of our target 252 bp length, and removed reads with homopolymers over eight bp long or with ambiguous base calls. We then aligned the sequences against the reference bacteria in the SILVA 16S rRNA database release 138 (Quast et al., 2012; Yilmaz et al., 2014), and removed the sequences that did not overlap the target alignment region on the reference dataset or aligned with less than 80% similarity. We removed chimeric sequences then obtained the taxonomic classification of our remaining sequences by aligning them against the June 2020 RDP data release (Cole et al., 2014; Wang, Garrity, Tiedje, & Cole, 2007). We retained only those sequences that aligned to bacteria references. We clustered sequences at a 97% similarity threshold to obtain OTUs using the OptiClust algorithm (Westcott & Schloss, 2017), then assigned a final taxonomic classification to our OTUs using the RDP dataset. We used this set of OTUs in all further analyses. Finally, we generated a phylogenetic tree using the ‘clearcut’ command (Sheneman, Evans, & Foster, 2006).

Using a custom script in R, we removed the 12 OTUs that occurred in the negative control from our dataset. Following visual inspection of the distribution of sample sizes, we retained the data from all hosts with more than 2,000 total sequences. The threshold of 2,000 reads occurred at a natural breakpoint in our histogram of sequences recovered per host (Figure S1 A). We also plotted rarefaction curves (Figure S1 B) using the ‘rarecurve’ function in the R package ‘vegan’ (Oksanen et al., 2018). Because some of our downstream analyses are vulnerable to unbalanced sample sizes (Gotelli & Colwell, 2001), we rarefied our host-by-OTU matrix to 2,000 sequences per host using the ‘rrarefy’ command from ‘vegan.’

The largest sequencing depth for any OTU that occurred in the negative control was two. We therefore set all OTU calls in our dataset with sequencing depths less than four to zero. This threshold conservatively accounted for potential rates of index hopping that could have caused the sequences to appear in our negative control. We also modified *mothur* output files to generate a taxonomy file for the OTUs, a fasta file with one representative sequence per OTU taken from

the first listed sequence name in the OptiClust output, and a phylogenetic tree of the OTUs. Custom scripts and input files are available on Dryad ([doi.org/10.5061/dryad.f7m0cfxzb](https://doi.org/10.5061/dryad.f7m0cfxzb)).

### **Quantifying host heterozygosity**

We quantified host heterozygosity by finding the mean proportion of heterozygous sites in each RADseq DNA fragment for each host (Table S1), a method shown to reduce bias relative to measuring heterozygosity at polymorphic sites only (Schmidt, Jasper, Weeks, & Hoffmann, 2021). To determine whether we were using an adequate number of fragments to retrieve a reliable heterozygosity measure, we calculated a running average heterozygosity by sequentially adding fragments from one to the total number recovered for each individual (Figure S1 C). We determined the number of fragments necessary for the running average heterozygosity value to be within 0.001 of the final value recovered for that host individual (grey bar in Figure S1 C). We produced these values with a custom script using the ‘vcfR’ package (Knaus & Grünwald, 2017). For each host, we recorded the total number of fragments recovered and the number of fragments necessary to achieve a stable heterozygosity estimate (Table S1).

### **Microbiome alpha and beta diversity**

To calculate alpha (within-host) diversity, we used the phylogenetically informed Faith’s diversity index implemented in ‘picante’ (Kembel et al., 2010). To test for sub-kilometer landscape level processes that might lead to correlation between low host genetic diversity and lower microbiome diversity within our sampled area, we used a Procrustes analysis implemented in the R package ‘vegan.’ We tested a geographic distance matrix of sampling locations against a pairwise Bray-Curtis community distance matrix and used the function ‘protest’ to identify whether a significant correlation existed. To assess the degree to which microbiomes cluster by host taxon, we performed a PERMANOVA using the adonis2 function from the R package ‘vegan.’ We tested host family and host heterozygosity as explanatory variables. Understanding the degree of host phylogenetic clustering in microbiomes allowed us to construct appropriate null distributions for microbiome richness comparisons. Since samples were stored at ambient temperature in the field for a variable length of time, we included storage length as an additional explanatory variable. In addition to the PERMANOVA, we regressed microbiome OTU species richness and phylogenetic diversity against the time the samples were stored at ambient

temperature. If there was a correlation, we would be concerned that our analyses would be impacted by sample degradation.

### **Relationship between microbiome diversity and host heterozygosity**

We used the R package ‘nlme’ to perform a phylogenetic generalized least squares model to test whether host heterozygosity or diet significantly predicted cloacal microbiome phylogenetic diversity (Pinheiro & Bates, 2000; Pinheiro, Bates, & R Core Team, 2022). We retained tips from a squamate wide phylogenetic tree that either matched our host species or were the nearest available proxy (Tonini, Beard, Ferreira, Jetz, & Pyron, 2016). Since there were no instances in which two sexually reproducing sampled host species were more related to each other than to a species included in the tree, no branch lengths were altered by this approach. Only one of our three clonal whiptail species, *A. uniparens*, was represented on the tree. We used *A. uniparens* to represent both *A. sonorae* and *A. flagellicaudus*, as all three lineages share the same parent species (Reeder et al., 2002). We used the host phylogeny as a correlation structure in the PGLS. To visualize host phylogenetic signal in cloacal microbiome diversity, we plotted the average microbiome diversity for each species on a phylogenetic tree.

We used a null model approach to determine whether observed gut microbiome community phylogenetic diversity was higher or lower than a random expectation. We used the ‘ses.pd’ function in the R package ‘vegan’ to perform 10,000 randomization runs and 9,999 iterations of the taxon labels of our host-by-OTU community matrix. The function reports observed phylogenetic diversity, the mean phylogenetic diversity of the randomized communities, and a p-value reflecting the quantile of the observed compared to the randomized samples. Since our PGLS and PERMANOVA found phylogenetic signal in cloacal microbiome diversity and composition, we repeated the randomization on subsampled matrices representing the OTUs found in each of our three most abundant host families: Teiidae, Phrynosomatidae, and Colubridae. For each family, we removed any OTUs that did not occur in any of our samples from that family, so existing OTUs could not be randomized into those taxa. We examined the within-family subsets because we were concerned that randomizing across the full community could produce signatures of lower than expected phylogenetic diversity purely due to host phylogenetic signal in gut microbial communities, and sampling from within single host families

would be less prone to this bias as it would account for some of the evolutionary history driving such an effect.

For visualization purposes, we generated a community-wide null expectation for microbiome phylogenetic diversity. To test for the relationship between Faith's diversity and species richness in our samples, we performed a linear model in base R. Since our species richness and Faith's phylogenetic diversity values were significantly correlated, we improved visual comparability between samples by dividing our phylogenetic diversity values by species richness. We randomly permuted values in the rows of our host-by-OTU matrix using the R function 'sample.' We calculated individual phylogenetic diversity values from the resampled matrix. For our 1,000 random draws, we recorded the number of times the true phylogenetic diversity value for each host was greater than the values calculated from a random permutation of that host's read depth values on the full OTU matrix. We then found the mean of the corrected diversity values for each run, repeated the procedure 1,000 times, and found the mean of those 1,000 means. For each of our three focal host families, we subsampled 1,000 subsets of seven individuals each to generate a family-specific null expectation for diversity. We chose the cutoff to match the size of the smallest of the three sample sizes. For each subsample, we then followed the procedure outlined for the full dataset.

### **Within-host microbiome community structure**

In our previous analyses, we found that gut microbiomes tended to be phylogenetically under-dispersed relative to random expectations. To further explore patterns in the community structure that might drive this observation, we calculated pairwise Euclidean sequence distances between all OTU sequences in each host. We used the `dist.alignment` command the R package 'seqinr' (Charif & Lobry, 2007), which reports the square root of the measured pairwise distance. We squared the output to regain the true distance values. For comparison, we drew equal numbers of OTUs from our full community and found the pairwise distances between the OTUs. Visual inspection of the true-community histograms relative to the null community histograms showed a shift toward lower values across the distribution (Figure 4A, 4B). To test the strength and generality of this pattern, we compared the mean value of each host's pairwise distances to 100 richness-matched random draws and determined how many of the random

means were lower than the true mean. We excluded hosts with fewer than 10 OTUs for this approach.

To better understand the drivers of this pattern as a potential explanation for our observation of lower than expected phylogenetic diversity of cloacal microbiome communities, we tested whether host-specificity by the OTUs could explain our observed differences. To do so, we took all OTUs that occurred in more than one host and found the mean pairwise distance of those OTUs from all other OTUs in each community. Then we took the mean of those means. We plotted these values against the total patristic distance, or total length of all branches connecting a set of tips on a tree, of all hosts the OTU occupied. Since the total number of hosts could drive total patristic distance, we also plotted the mean values against the total number of occurrences of each OTU in our dataset. For both comparisons, we performed a linear model in base R.

## **Results**

### **Host heterozygosity**

We calculated the proportion of heterozygous sites per nucleotide in each host as the independent variable in our analyses. To determine whether the number of fragments available was adequate to reach a reliable value, we identified the number required to achieve a value within 0.001 of the final heterozygosity value in each individual. Host heterozygosity ranged from 0.004-0.019 sites per bp (Figure 2A, Table S1). All individuals had more fragments than necessary to reach a measure within 0.001 of the full-sample heterozygosity value (Table S1).

### **Microbiome diversity**

As a dependent variable, we calculated a phylogenetically informed alpha diversity for our cloacal microbiome communities. The values of the Faith's diversity index of the cloacal microbiome communities ranged from 0.137 to 7.706 (Figure 2B, Table S1). Our Procrustes analysis found no significant spatial structuring of microbiome community distances ( $p=0.399$ , correlation=0.123). Our PERMANOVA found significant clustering according to host family ( $p=0.001$ ,  $R^2 = 0.190$ ), but not host heterozygosity ( $p=0.632$ ,  $R^2 = 0.011$ ) or time kept at field ambient temperature ( $p=0.414$ ,  $R^2 = 0.014$ ). Neither Faith's diversity ( $p=0.979$ ,  $R^2 = -0.016$ ) or

species richness ( $p=0.816$ ,  $R^2 = -0.015$ ) in our retrieved communities showed a correlation with time stored at field ambient temperature.

### **Relationship between microbiome diversity and host heterozygosity**

Our PGLS found no significant correlation between microbiome phylogenetic diversity and host heterozygosity ( $p=0.760$ ) or diet ( $p=0.500$ ). Microbiome diversity did show host phylogenetic signal (Pagel's  $\lambda = 0.772$ ). To test whether microbiomes were under- or over-dispersed relative to null expectations, we used a randomization test implemented with the 'ses.pd' function in the R package 'vegan.' For the full host-by-OTU matrix, we found that our observed Faith's diversity values were smaller than the mean value of the randomizations for all but one individual. Forty-nine of 63 microbiomes had p-values less than 0.05. When we applied a Bonferroni correction to the 0.05 p-value for corrected significance level of 0.0008, 27 microbiomes were still below the threshold. For the within-Colubridae comparisons, all eight observed microbiome diversity values were smaller than the mean of the randomizations. Seven of eight had p-values below 0.05 and six of eight had p-values below the Bonferroni corrected value of 0.007. For the Phrynosomatidae, all 20 observed microbiome diversity values were below the mean for the randomizations, with 15 reaching significance at  $p=0.05$  and 10 at the Bonferroni-corrected  $p=0.0025$ . For the Teiidae, 24 of 25 microbiomes diversities were lower than the mean of the randomizations, with 19 of 25 having a significant p-value at 0.05 and 16 of 25 reaching significance at the corrected p-value of 0.002.

For visualization (Figure 3), we permuted the rows of our host-by-OTU matrix and found the mean Faith's phylogenetic diversity values for the individuals in the permuted matrix, then repeated the permutations 1,000 times. For all values in the figure, we divided the Faith's diversity value by the species richness of the microbiome to increase visual comparability. The relationship between species richness and phylogenetic diversity was highly significant in a linear model ( $p < 2.2 \times 10^{-16}$ , adjusted  $R^2 = 0.964$ ). The mean of the 1,000 permuted values was 0.119. All but six individual values were lower than the mean (Figure 3A). Due to the host phylogenetic signal in our dataset, we repeated the randomization on the OTUs present in sets seven confamilial hosts drawn from our three most abundant host families, the Teiidae, Phrynosomatidae, and Colubridae. The values of the mean randomized phylogenetic diversity within families were lower than the overall mean value, with Teiidae being 0.097 (two

individuals from the family above), Phrynosomatidae 0.117 (three individuals above), and Colubridae 0.103 (two individuals above).

### **Within-host microbiome community structure**

We found that 29 of the 49 hosts for which we compared mean pairwise sequence distance between OTUs had a smaller mean distance than 90% of the matched null communities (Table S1). This outcome indicates that low microbiome phylogenetic diversity in some hosts could be driven by groups of microbes with closer-than-expected genetic distances that tend to cooccur in microbiomes. This pattern could be due to microbes that specialize on specific host lineages. To test this possibility, we found the mean distance between each OTU and the OTUs with which they co-occur. This value should be lower in OTUs that tend to co-occur in related groups. We compared these values to the mean patristic distances between hosts in which the OTUs occurred. Patristic distances should be smaller for host-specific microbes. We found that Proteobacteria and Firmicutes tended to have lower mean pairwise distances than Bacteroidetes, and that small average pairwise distances are not restricted to OTUs with low patristic distances (Figure 4C). The linear model for this comparison has a p-value of 0.0036, but an  $R^2$  of only 0.0294, showing a very weak relationship. In particular, some Proteobacteria OTUs with low pairwise distances are widespread across hosts in our sample (Figure 4D), indicating that these OTUs may drive a portion of our observed skew in mean pairwise distances relative to the null distributions. The linear model for this comparison was not significant ( $p=0.209$ ), and also had a very low correlation coefficient ( $R^2 = 0.0023$ ).

### **Discussion**

We tested whether a biologically plausible hypothesized correlation between the heterozygosity of reptile genomes and the diversity of their cloacal microbiomes could be detected in a natural host community. Using a single community with a range of host heterozygosity values allowed us to focus on patterns of host and microbe diversity without the confounding factor of landscape-scale processes that could impact both host genetics and microbiome diversity (Amato et al., 2013; Barelli et al., 2015; Ingala et al., 2019). We found no evidence of a correlation between host heterozygosity and microbiome phylogenetic diversity. In addition, we showed that microbiome communities were phylogenetically under-dispersed



relative to random expectations, specifically including more groups of closely related OTUs than would be expected by chance (Figure 1, Figure 3). We had hypothesized that a phylogenetically over-dispersed microbiome would reflect a history of selection on the host lineage toward recruiting diverse cloacal microbes and that a positive correlation between host heterozygosity and cloacal microbiome diversity would indicate that host-microbiome interaction was driven by specific categories of host genes that rely on diversity for their function (Figure 1) (Bolnick, Snowberg, Caporaso, et al., 2014; A. A. Khan et al., 2019; I. Khan et al., 2015; Sumiyama, Saitou, & Ueda, 2002).

### **Potential sources of error and bias**

We sampled the semi-oxygenated habitat of the reptile cloaca (Grond et al., 2018), which has a distinct microbiota relative to the gut (Kers et al., 2019; Lee et al., 2020; Williams & Athrey, 2020). As such, our results should be interpreted with caution for microbial communities in the gut or other environments within the host. In addition, sampling error due to variation in swabbing, extraction efficacy, or PCR error could reduce our ability to detect correlations in our data (Bonk, Popp, Harms, & Centler, 2018; Pollock, Glendinning, Wisedchanwet, & Watson, 2018). Finally, we used a reduced-representation approach to sequence host DNA (Peterson et al., 2012). Since restriction enzyme cut sites vary across species and even at the individual level, the DNA fragments we recovered from this process are not homologous across hosts. Further study of the microbiome-host heterozygosity relationship is necessary, incorporating microbiome communities from a range of tissues and different host DNA markers.

In addition to sources of bias, we may have failed to detect a correlation due to decoupling of heterozygosity between our hypothesized target genes and the background heterozygosity of the host genome. In some instances, local selection can maintain high heterozygosity at immune genes even when neutral loci lose diversity (Knafler, Grueber, Sutton, & Jamieson, 2017; Oliver & Piertney, 2012; Strand et al., 2012). In addition, cloacal microbiome diversity maintenance might be more critical to host survival at particularly physiologically stressful times. Examples of stressors known to impact gut microbiomes in vertebrates include heat stress (Chen et al., 2018; Sepulveda & Moeller, 2020) and the changes in physiology associated with hibernation (H. V. Carey, Walters, & Knight, 2013; Tang, Wang, Wan, & Fang, 2019; Tong, Hu, Du, Bie, & Wang, 2020). Since we sampled only during spring and early

summer, when food was relatively abundant and conditions were less stressful, we may have failed to confirm relationships that are detectable under different circumstances. Further work will be necessary to fully understand the vertebrate microbiome's response to external stressors and the fitness consequences of the interaction between host and microbiome during stressful conditions.

In addition, codiversification and adaptation by some microbial lineages to specific host taxa could drive our observed lower than expected phylogenetic diversity (Figure 3), particularly when coupled with possible phylogenetically conserved dietary ecology in the hosts (Youngblut et al., 2019), although this effect may be weaker outside of mammalian hosts (Harrison et al., 2021). If specialization to host taxa was the major driver of our observed signal of significantly low phylogenetic diversity in our full host-by-OTU matrix, we would expect that our family-specific randomizations would show a greater proportion of microbiomes within the expected distribution of random samples, as they would account for a portion of the host evolutionary signal driving the pattern in the full dataset. We did not find this to be the case, indicating that our observed patterns were not driven by codiversification alone (Figure 3B).

### **Selection on the host genome by the microbiome**

Several mechanisms could explain our finding that cloacal microbiome phylogenetic diversity is uncorrelated with host heterozygosity, including both environmental and host-driven processes. Focusing on host-driven mechanisms, positive correlations between microbiome diversity and host genetic diversity seem to be observed in the literature when host heterozygosity is low, either due to natural processes (Couch et al., 2020), or because the study uses genetically identical lab animals (A. A. Khan et al., 2019; Kubinak et al., 2015). More-heterozygous host populations tend to show absent or even negative correlations between gut microbiome diversity and host genetic diversity (Bolnick, Snowberg, Caporaso, et al., 2014; Steury, Currey, Cresko, & Bohannan, 2019). Given this context, it is possible that hosts with even lower genetic diversity than those that appear in our samples would experience reduced fitness due to low microbiome diversity. As an alternate but not mutually exclusive explanation, transcriptomic studies between populations of hosts displaying distinct gut microbiome communities often identify expression levels of more-conserved host genes as key correlates of gut microbiome composition (Milligan-Myhre et al., 2016; Rudman et al., 2019; Small et al.,

2017). Our results may provide an additional line of evidence that this category of more-conserved genes are key in managing the host-microbiome relationship, as host heterozygosity is not directly related to their function.

If further studies confirm the generality of the finding that more-conserved host genes tend to impact host-associated microbiome community structure, it may explain a common finding that vertebrate gut microbiome community structure tends to be consistently differentiated between hosts from different families, but less so between hosts within a family, particularly when dietary differences are accounted for (Amato et al., 2019; Gaulke et al., 2018; Groussin et al., 2017; Kropáčková et al., 2017; Moeller et al., 2012; Nishida & Ochman, 2018; C. D. Phillips et al., 2012). Vertebrate-wide comparative analyses indicate that the host-family level differentiation pattern is frequent among non-mammalian vertebrates, while mammals may more often show finer-scale phylogenetic signal in host-microbe interactions (Song et al., 2020; Youngblut et al., 2019). The hypothesis that more-conserved, slower-changing genes determine gut microbiome structure is consistent with family-level microbiome distinctions, as conserved genes are less likely to vary between members of the same genus than they are between members of different families (Jiggins & Hurst, 2003; Milligan-Myhre et al., 2016; Mukherjee, Sarkar-Roy, Wagener, & Majumder, 2009).

### **Microbiome phylogenetic community structure**

To identify possible drivers of our observed pattern of lower-than-expected phylogenetic diversity in microbiome communities, we examined the phylogenetic structure of cloacal microbiomes relative to null expectations. Within individual cloacal microbiomes, the signal of low phylogenetic diversity may have been driven by clusters of related bacteria, particularly some widespread OTUs from the phylum Proteobacteria (Figure 4C, 4D). Given the large host breadth of these OTUs and the lack of signal with host patristic distance, host specificity by microbes is unlikely to completely explain our observed low phylogenetic diversity. We explore two non-mutually exclusive hypotheses that could fit our observation, although other mechanisms such as habitat filtration could also apply. First, some bacteria might alter the host environment for their own establishment, thus facilitating colonization by related bacteria (Bragonzi et al., 2012; Burmølle, Ren, Bjarnsholt, & Sørensen, 2014; Rathi, Singh, Osbone, & Babu, 2015; Shiri et al., 2013). Second, the host immune response might favor a less-diverse

cloacal microbiome. Most vertebrates go through a juvenile period of high lineage turnover in their microbiomes (Hornef & Torow, 2020). As hosts become adults, their microbiome settles to a more consistent community, with the host immune system ‘learning’ a set of permitted OTUs and mounting immune defenses against other bacteria (Erturk-Hasdemir et al., 2019), perhaps favoring genetically or ecologically similar groups of bacteria.

Given our finding that the most widespread bacterial lineages in our samples also tended to have low mean pairwise distances (Figure 4D), we propose a re-examination of assumptions behind the ecology and host benefits of widespread OTUs. The ubiquity of a bacterial OTU within a host species has been used as a proxy for that OTU’s importance to host health or fitness (Hernandez-Agreda, Gates, & Ainsworth, 2017; A. A. Khan et al., 2019; Shade & Handelsman, 2012; Zhu, Wang, & Li, 2010). Differences in ecological generalism are another reason to apply caution to assumptions about the health impacts of frequently detected bacteria OTUs (Vieira-Silva et al., 2016). Bacterial ecology, including dormancy capabilities (Locey et al., 2020; Xu et al., 2022) and dispersal ability (Moeller et al., 2017; Stothart et al., 2021), can heavily influence habitat breadth in a taxon, and should be considered when interpreting OTU host breadth in ecological or evolutionary terms.

## Conclusions

Our observed outcomes of no correlation between host heterozygosity and gut microbiome diversity and significantly lower than expected phylogenetic diversity in microbiomes are consistent with and provide additional context to frequently encountered patterns across vertebrate host-microbiome evolutionary studies. First, microbiome differences are often best predicted by the allelic states or expression levels of more-conserved (immune and non-immune) host genes (Jiggins & Hurst, 2003; Milligan-Myhre et al., 2016; Mukherjee et al., 2009; Rudman et al., 2019; Small et al., 2017). Second, vertebrate gut microbiomes, particularly outside of mammals, often differ predictably at the host family level, but not below when dietary differences are accounted for (Amato et al., 2019; Gaulke et al., 2018; Groussin et al., 2017; Kropáčková et al., 2017; Moeller et al., 2012; Nishida & Ochman, 2018; C. D. Phillips et al., 2012). We argue that these empirical outcomes could have implications for the expected standing diversity for the genes involved in host-microbiome interaction, and hence the evolvability of the host-microbiome unit.

More fully incorporating cloacal and other host-associated microbiomes into ecology and evolutionary biology can shed light on a wide variety of natural processes. Through metabolic services to their hosts, gut microbes can impact ecosystem-scale processes, for example by altering trophic webs (Amato et al., 2015; Kohl et al., 2014) or curtailing community-wide impacts of epidemic disease (Smith-McKenna et al., 2014). On smaller scales, gut microbiomes facilitate host-lineage adaptation to new resources, opening up larger geographic ranges (Kohl et al., 2014) and novel ecological niches (Alberdi, Aizpurua, Bohmann, Zepeda-Mendoza, & Gilbert, 2016; Moeller et al., 2014). Reptile microbiomes contain many lineages that are acquired through environmental interactions or horizontal transfer (Kohl et al., 2017). Clarifying the host genetic or other mechanisms that govern potentially adaptive community assembly in the horizontally acquired portions of the microbiome is a key challenge for the field.

Together, our finding suggests testable principles for future work on host-microbiome evolution. First, predictable differentiation of cloacal microbiome communities between vertebrate host lineages may occur on longer timescales than speciation processes. If evidence supports the first claim, then community-wide host-associated microbial diversity might be better predicted by host family diversity than host species diversity. These observations could suggest that losing host families, but not host species, from a community is likely to reduce the regional diversity of cloacal microbiome lineages. Second, the literature includes examples of quick adaptations to novel environments or resources via host-associated microbiomes (Alberdi et al., 2016; Kohl et al., 2014). If our hypotheses above are upheld by empirical work, such rapid adaptations may be maintained by mechanisms other than direct interaction between host genotype and microbial lineages. Understanding potential mechanisms may be key for conservation planning and establishing a broader understanding of the biology of host-microbe interactions in vertebrates.

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### Data Accessibility Statement

Both 16S rRNA microbiome data and host RADseq data are available on NCBI's Short Read Archive, as BioProject PRJNA746253 and PRJNA744273 respectively. We provide processed versions of several files available on DataDryad (doi:10.5061/dryad.f7m0cfxzb). These include vcf files of the RADseq sequences for each host, the 16S rRNA OTU reference sequences, the host-by-OTU table, and the 16S rRNA-derived phylogenetic tree used in our analyses.

### Figure and Table legends

#### Figure 1: Possible relationships between host genetic diversity and microbiome

**phylogenetic diversity to test evolutionary hypotheses.** A) We identify the evolutionary process of interest, describe the pattern that process should generate, and describe how we will measure the pattern. While other assembly processes could account for the patterns we discuss, we focus on process that could be driven by host genetics. B) Four possible outcomes that show how selective processes might drive the host-microbiome relationship. Higher than expected phylogenetic diversity of microbiome lineages (blue lines, first column) point toward a fitness benefit of cloacal microbiome diversity, in contrast to lower than expected phylogenetic diversity (red lines, second column). Correlation of microbiome diversity and host heterozygosity would

be consistent with positively selected, high-diversity gene (bottom row) maintaining the host-microbiome relationship. We predict that the microbiome will be over-dispersed and show microbiome diversity correlated with host heterozygosity (black box).

**Figure 2: Both genetic diversity and gut microbiome diversity varies within and between host species.** A) Host diversity varies within and between species, with heterozygosity values for each host family distributed through the total observed range. B) Microbiome phylogenetic diversity shows variation within host species as well as phylogenetic signal across the host tree.

**Figure 3: Gut microbiome diversity is not correlated with host genetic diversity and is under-dispersed relative to random expectations.** A) Our observed outcome does not show evidence of a positive correlation of gut microbiome and host genetic diversity, as would be expected if gut microbiome diversity increased host fitness and heterozygosity-dependent genes drove gut microbiome diversity. B) Gut microbiomes are phylogenetically under-dispersed relative to random samples from the total regional pool of bacteria (dashed grey line), or size-matched samples of the three most abundant host families (dashed colored lines) and gut microbiome phylogenetic diversity is not correlated with host genetic diversity.

**Figure 4: Low phylogenetic diversity within microbiome communities may be driven by a few widespread OTUs.** The distribution of pairwise distances in a real gut microbiome community (A) and an equal number of randomly sampled OTUs from the regional pool (B), shows a larger number of shorter distances in the real community. C) Mean distance from other OTUs is not predicted by the mean patristic (branch length) distance between hosts in which the OTU occurs, as would be predicted if host specialization by groups of OTUs explained the signal of low phylogenetic diversity in the microbiome. D) A few widespread Proteobacteria lineages have low mean distance from other OTUs in their communities, indicating that these lineages may be one driver for our observed pattern of full microbiome communities.

**Table 1: Host-species level characteristics.** Provides taxonomic and ecological information and sample size per host species.

## Supplementary Figure and Table legends

**Table S1: Individual-level data.** Columns 1-13 provide capture history and basic statistics for each host. Columns 14-20 provide data on individual-level genomic or microbiome parameters.

**Figure S1: Defining cutoffs for retaining individuals in analysis.** A) Example of a heterozygosity accumulation curve for one individual. Dark line indicates heterozygosity measure at  $n$  fragments, grey bar indicates a range within 0.001 of the full-dataset heterozygosity value. B) Histogram of the number of reads in the 16S rRNA cloacal microbiome dataset for each individual. C) Species accumulation curves for the 16S rRNA cloacal microbiome data, with vertical line indicating the cutoff for retaining a host in the dataset.

**Figure S2: OTU and sequence-level phylum assignment of gut microbiomes across species.**

**Figure S3: host family-level PERMANOVA based on microbiome Bray-Curtis distances**

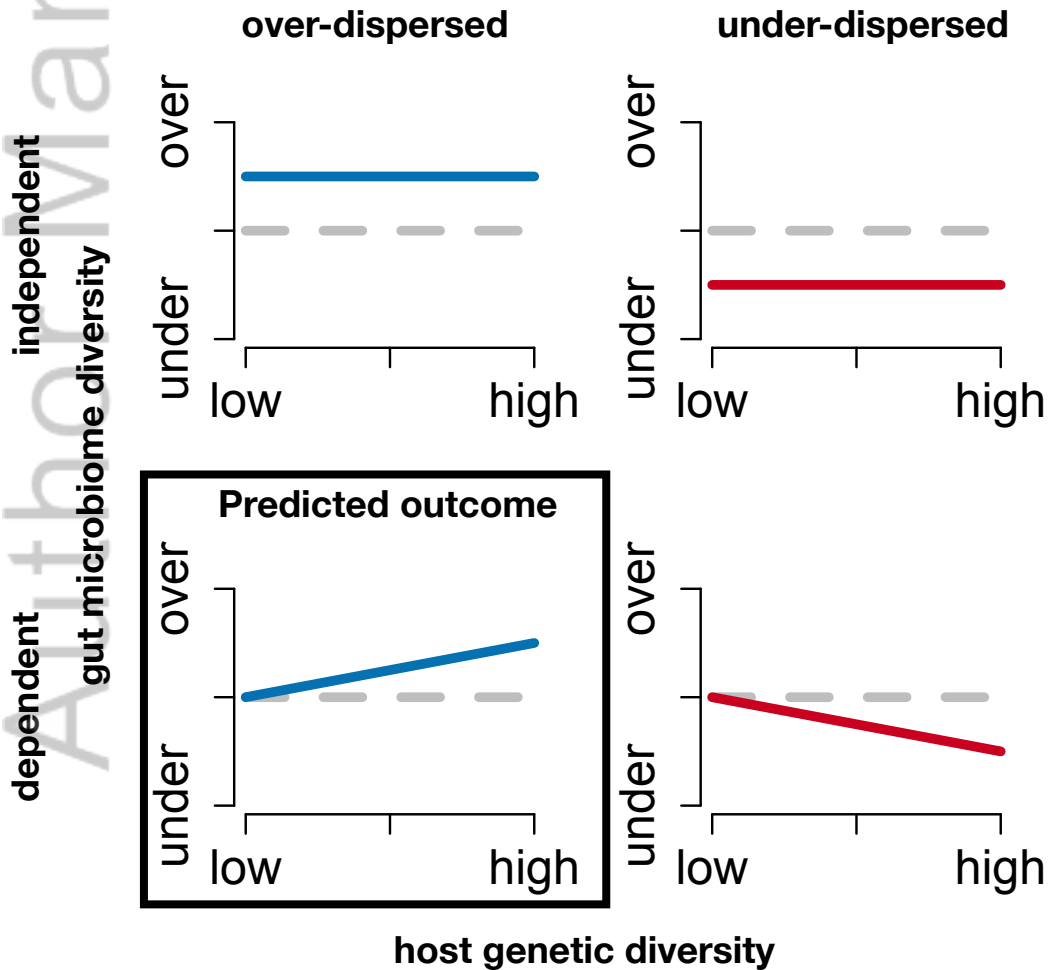
Family	Genus	Species	Reproductive strategy	Sample size	Diet
Teiidae	Aspidocelis	flagellicaudum	clonal	2	arthropods
Teiidae	Aspidocelis	sonorae	clonal	9	arthropods
Teiidae	Aspidocelis	uniparens	clonal	6	arthropods
Teiidae	Aspidocelis	tigris	sexual	8	arthropods
Vipiridae	Crotalus	atrox	sexual	1	vertebrates
Colubridae	Coluber	bilineatus	sexual	1	vertebrates
Crotaphytidae	Crotaphytus	collaris	sexual	5	both
Phrynosomatidae	Cophasaurus	texanus	sexual	2	arthropods
Eublepharidae	Coleonyx	variegatus	sexual	3	arthropods
Crotaphytidae	Gambelia	wislizenii	sexual	1	both
Colubridae	Pituophis	catenifer	sexual	2	vertebrates
Phrynosomatidae	Phrynosoma	cornutum	sexual	4	arthropods
Phrynosomatidae	Phrynosoma	modestum	sexual	5	arthropods
Colubridae	Rhinochilus	lecontei	sexual	1	arthropods
Phrynosomatidae	Sceloporus	clarkii	sexual	4	both
Colubridae	Salvadora	hexalepis	sexual	4	vertebrates
Phrynosomatidae	Urosaurus	ornatus	sexual	3	arthropods
Phrynosomatidae	Uta	stansburiana	sexual	2	arthropods



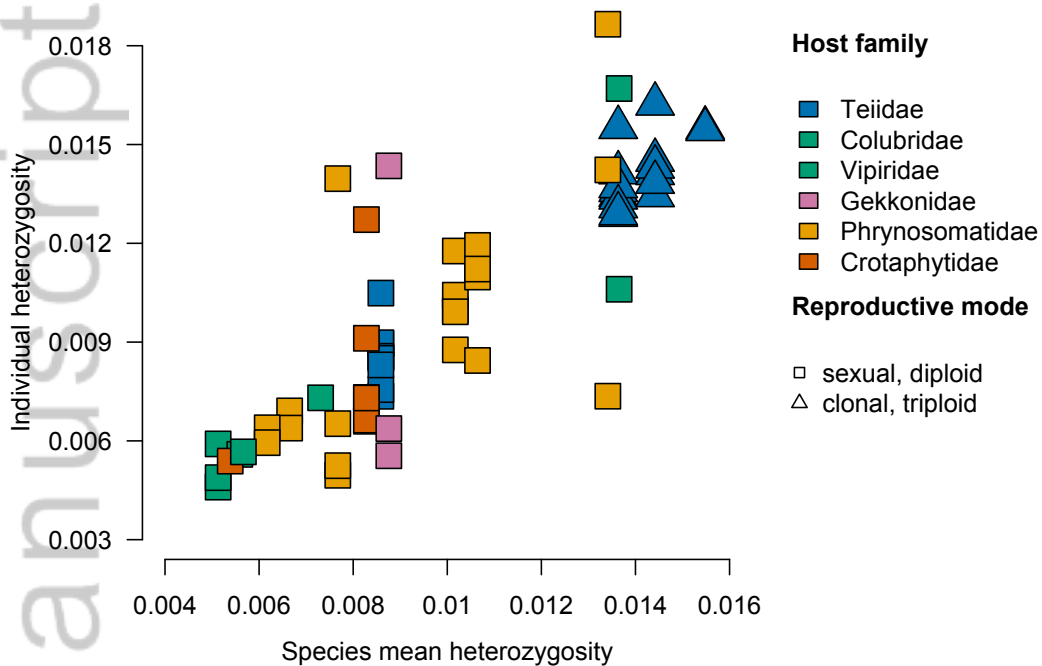
## A) hypothesis testing with observable patterns

	evolutionary process	current state of system	measurable outcome
microbiome	higher microbiome diversity benefits hosts	hosts preferentially retain microbes that increase the phylogenetic diversity of their microbiome	cloacal microbiome phylogenetic diversity is inflated relative to random expectation
host genetics	key host-microbiome interactions occur through positively selected genes	hosts with higher heterozygosity in immune genes have more diverse microbiome	host genome heterozygosity correlates with cloacal microbiome diversity

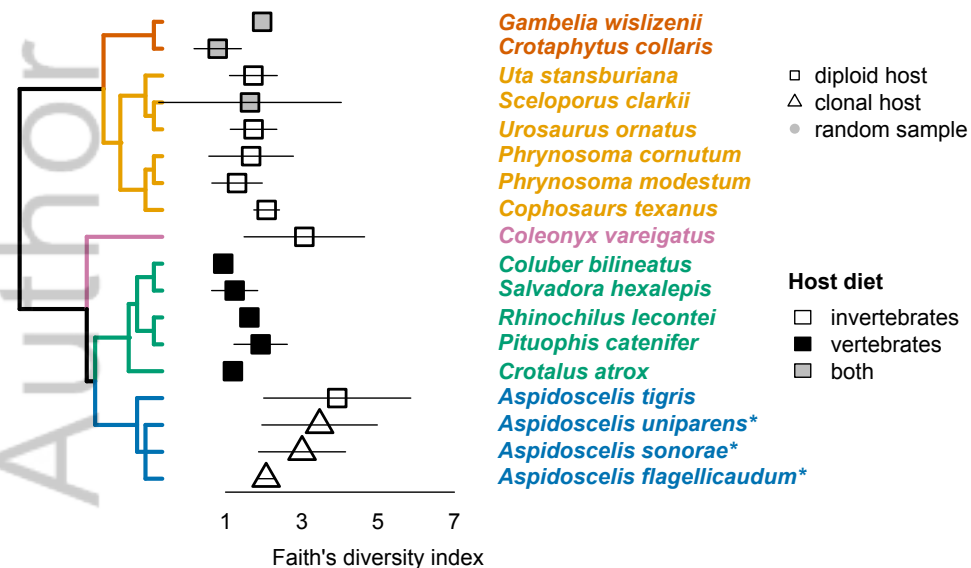
## B) possible host/microbiome diversity relationships

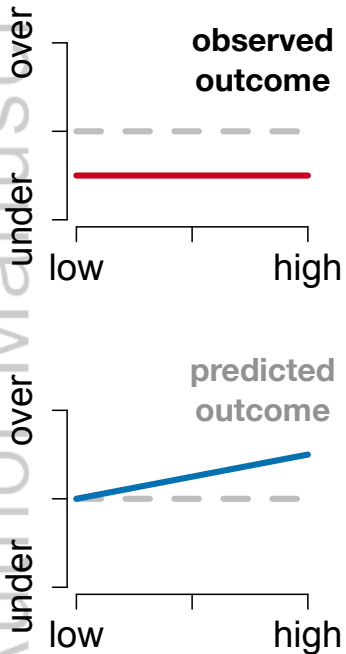
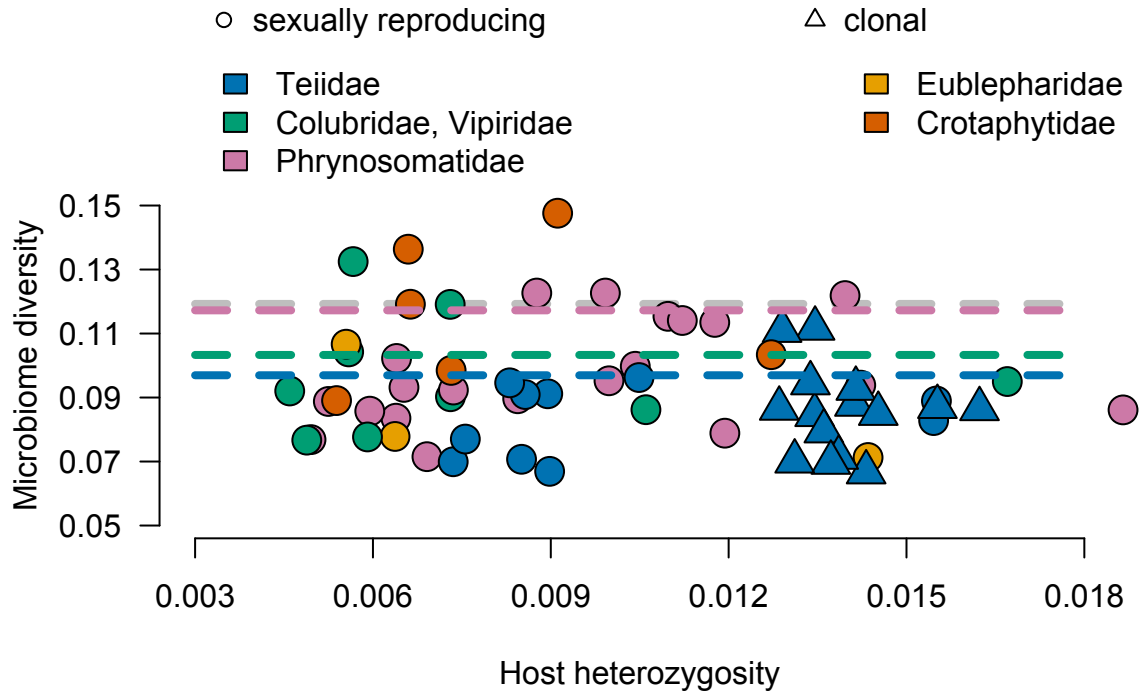


## A) genome diversity varies within and between host species

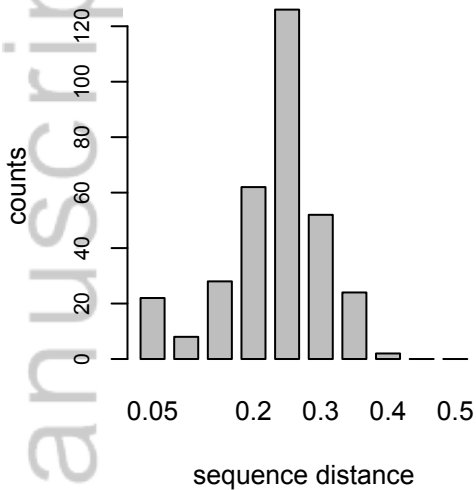


## B) microbiome diversity varies across host phylogeny



**A) hypothesis not supported****B) no correlation between host and microbiome diversity**

**Real data**



**Random sample**

