









ORIGINAL ARTICLE

Host and Environmental Drivers of Gut Microbiome Variation in Wild *Anolis* Lizards

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ABSTRACT

Animals maintain close associations with diverse microbiota that inhabit their digestive tracts, and these associations can profoundly affect host physiology and fitness. Gut microbiome composition is shaped by both host traits and environmental factors, yet the relative importance of these forces remains unclear in many taxa, including squamate reptiles (lizards and snakes). To address this gap, we analysed the gut microbiomes of seven species of *Anolis* lizards in the lowland tropical rainforest of central Panama. We sought to determine how environmental and host species characteristics shaped gut microbiome composition. Specifically, we examined (1) interspecific variation in the anole gut microbiome, (2) the relative roles of environment and host species in shaping gut microbiomes across two study locations, and (3) patterns of phyllosymbiosis. We found that host-related factors (species identity, body size, and phylogenetic distance) were significant predictors of the composition of *Anolis* gut microbiomes. However, environmental factors, including locality and year of sampling (associated with temperature, humidity, and precipitation), also exerted significant effects. We detected evidence of phyllosymbiosis, but this pattern was moderate, possibly due to the strong effect of environmental variation. Our work contributes to the growing body of literature on lizard gut microbiomes by using comparative observations across habitats and species to identify the factors that shape these communities in the wild.

1 | Introduction

The gut microbiome is a complex and dynamic community of microbes residing in the intestinal tract. Gut microbes can influence aspects of vertebrate physiology including nutrient absorption, immune system development, and metabolism (Biesalski 2016; Cho and Blaser 2012; Kohl and Carey 2016; Levy et al. 2017; McKenney et al. 2018; O'Hara and Shanahan 2006; Robinson et al. 2010; Siddiqui et al. 2022; Sommer and Bäckhed 2013). As such, gut microbial communities can impact

the health, survival, and fitness of their hosts (Shapira 2016). Understanding how and why microbiomes vary across host species, and how they are influenced by the environment, remains a central goal in the fields of vertebrate ecology and evolution.

Gut microbiomes are not random assemblages in most host species. Instead, they are structured communities shaped by a combination of host and environmental factors (Vasconcelos et al. 2023). Host characteristics, including morphology, genotype, and phylogeny, can significantly influence microbiome

composition and function (Maritan et al. 2024). In some cases, host effects result in a pattern where microbial community similarity mirrors host phylogeny, a phenomenon known as phylosymbiosis (Brooks et al. 2016; Kohl 2020). In addition to host effects, environmental variables can also influence microbiome composition and function. These variables include habitat type, temperature, diet, and precipitation, among many others (Williams et al. 2023). However, the relative importance of host characteristics and environmental factors in shaping gut microbiome diversity and composition across host species remains an active area of investigation, particularly in non-mammalian vertebrates such as squamate reptiles (lizards and snakes; Rojo et al. 2017; Sze et al. 2020).

Despite the remarkable ecological and evolutionary diversity of squamate reptiles, our understanding of their gut microbiomes remains limited (Woodhams et al. 2020). Among squamates, lizards are morphologically diverse and occupy a broad range of niches (Pianka and Vitt 2003), making them excellent models for studying host-microbiome relationships. The extent to which host factors and environmental conditions interact to shape microbiome composition and diversity in lizards, including the relative contributions of host phylogeny, morphology, and habitat, remains poorly understood (Colston 2017; Ren et al. 2016; Smith et al. 2025; Vasconcelos et al. 2023). Although diet, locality, and seasonal variation have all been proposed as important drivers of microbiome composition in lizards (Colston 2017), evidence for phylosymbiosis appears weak, with environmental influences often overriding phylogenetic signals (Hernández et al. 2022; Ren et al. 2016; Vasconcelos et al. 2023). Clarifying the extent to which ecological versus phylogenetic factors shape lizard microbiomes remains an open question and is central to understanding host-microbe associations in this group.

To address these knowledge gaps, we used bacterial 16S rRNA sequencing to inventory the gut microbiomes of anole lizards (*Anolis* spp.) in Panama, both in a natural rainforest environment and on an experimentally colonized island where *Anolis apletophallus* represent the fifth-generation descendants of

individuals transplanted from the mainland. *Anolis* lizards are an ideal system for investigating these questions, as they constitute one of the most remarkable examples of adaptive radiation among vertebrates (Losos 2009). They are also one of the most diverse vertebrate genera, with more than 400 species exhibiting exceptional morphological and ecological variation (Losos 2009; Poe et al. 2017; Warheit et al. 1999). All the species we sampled in this study are generalist insectivores and have similar life histories, such as relatively short lifespans, early sexual maturity and one-egg clutches laid primarily during the wet season. The key difference between these species is the microhabitats they occupy (described in Table 1). We sought to evaluate the following: (1) variation in gut microbiome communities of an assemblage of anoles inhabiting Soberanía National Park in Panama, (2) the relative roles of environment and host species in shaping gut microbiomes across two study locations and (3) phylosymbiosis patterns across all species. Specifically, we tested the effects of multiple host and environmental variables, including species identity, body size, locality (mainland vs. island) and sampling year (which correlates with environmental conditions such as temperature, humidity and precipitation) on microbiome structure. We hypothesised that gut microbiome diversity and composition would be shaped by a combination of host and environmental factors, with host species identity and body size exerting the strongest effects, and environmental variation between locality and annual variation in environmental conditions contributing additional structure.

2 | Materials and Methods

2.1 | Site Descriptions and Lizard Collection

Fieldwork was conducted in central Panama at one mainland and one island site (Figure 1A and Figure S1). Mainland sampling was carried out in Soberanía National Park, Panama (9°08.120' N, -79°43.388' W) along a central forest trail Pipeline Road on the first 1000 m after the Juan Grande ravine, as well as in grassy areas adjacent to the forest (near the town of Gamboa,

TABLE 1 | Summary of *Anolis* study species, collection locations, sample size, and microhabitat.

Common name	Species	Collection site	Microhabitat type	Mean SVL ± SD (mm)
Slender anole	<i>A. apletophallus</i>	Mainland (N=14) and Island D (N=9)	Twigs and small perches in tropical forest understory	43.4 ± 1.82
Grass anole	<i>A. auratus</i>	Mainland (N=8)	Tall grass in open habitat	44.8 ± 2.48
Pug-nosed anole	<i>A. capito</i>	Mainland (N=3)	Low perches and forest floor	74 ± 1.73
El Copé anole	<i>A. elcopeensis</i>	Mainland (N=7)	Small and low perches close to the ground in the understory of tropical forests	41.2 ± 1.30
Bridled anole	<i>A. frenatus</i>	Mainland (N=7)	Trunks in the tropical forest upper canopy	98.9 ± 1.64
Dappled anole	<i>A. poecilopus</i>	Mainland (N=2)	Low perches in the tropical forest understory near water	55 ± 2.83
Gaige's anole	<i>A. gaigei</i>	Island D (N=9)	Larger perches, trunks, and forest floor	46.9 ± 1.50

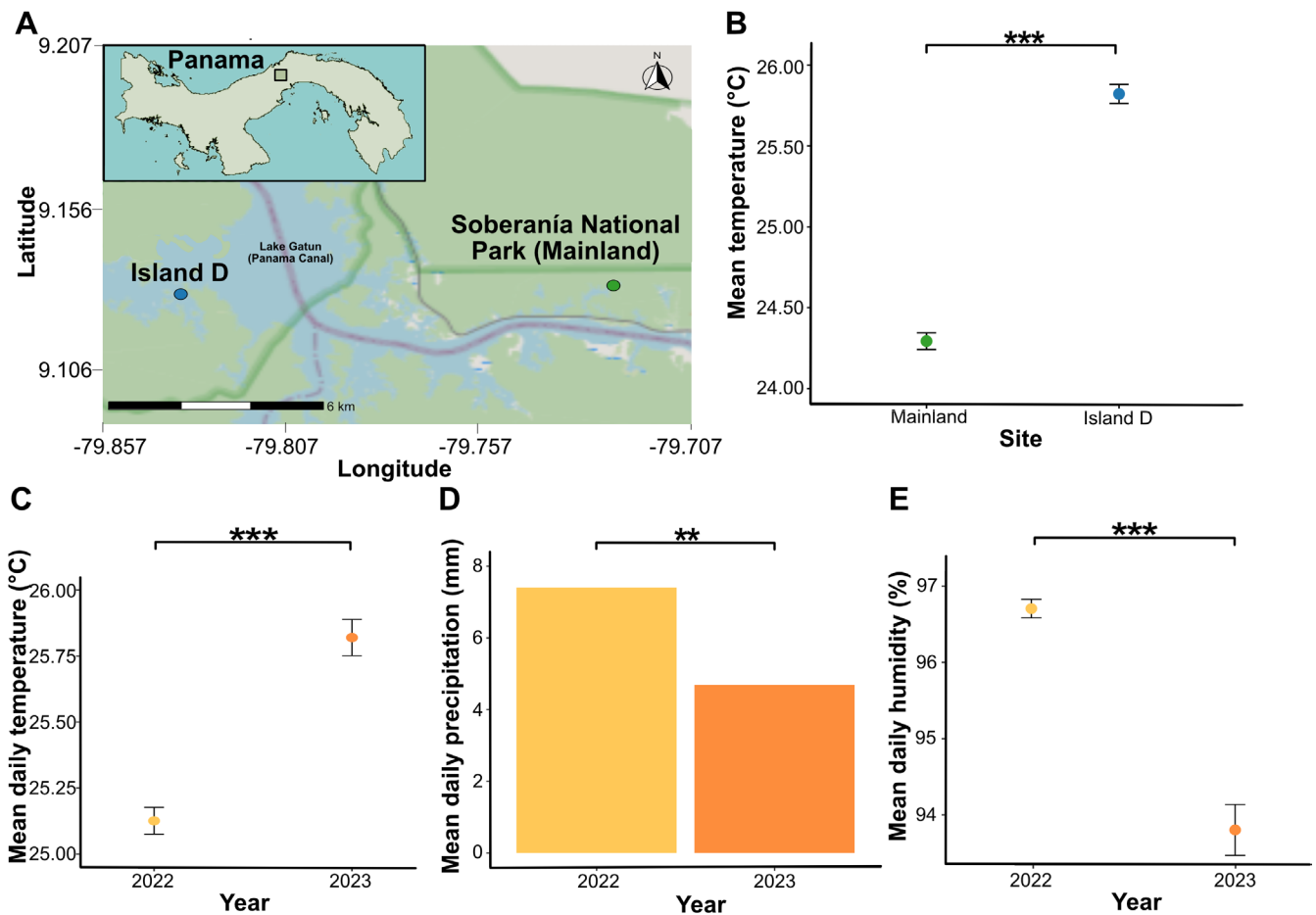


FIGURE 1 | Environmental conditions differed between sites (A, B) and years (C–E). (A) Map of our study sites in the canal zone of Panama. (B) The mainland was cooler than Island D in 2022. (C–E) In the mainland mean daily temperature was higher, while mean daily precipitation and humidity were lower in 2023 compared to 2022. Asterisks indicate significance: *** = $p < 0.001$, ** = $p < 0.01$. Species-specific sample sizes and detailed collection information are provided in Table 1.

9°06.600' N, –79°41.590' W). Island sampling took place on small island in Lake Gatún (Panama Canal) that we call “Island D” (09°07.827' N, –79°50.281' W). This island has a native population of Gaige’s anole (*Anolis gaigei*) and an experimentally translocated population of the Panamanian slender anole (*A. apletophallus*). The small islands in the Panama Canal, including “Island D” were formerly hilltops that became abruptly isolated after central Panama was flooded to connect the north and south entrances to the Panama Canal in 1913 (Leigh et al. 1993). *A. gaigei* is widely distributed throughout mainland Panama, Colombia and Venezuela. As such, it is perhaps most likely that populations of this species were isolated on some of the islands in Lake Gatun as the floodwater rose. However, we cannot rule out the possibility that *A. gagei* colonized Island D through over-water dispersal at some point after the formation of the canal prior to 2017 when we first surveyed this island. We introduced 70 adult *A. apletophallus* (even sex ratio) from Pipeline Road to Island D in 2017 as part of a long-term field experiment (Cox et al. 2020; Nicholson et al. 2022, 2023).

To compare microbiomes, we collected individuals of six *Anolis* species from the mainland community in 2022 and 2023 (41 lizards in total, Table 1). Although they are mostly found in the upper canopy, *A. frenatus* individuals collected in our data set were those that happened to venture into the understory and

were catchable from the ground. Therefore, our dataset may represent a non-random sample of the general population of this species. Although *A. gaigei* is widespread across the mainland in Panama, we did not detect this species in our mainland sampling area, consistent with the difficulty in detecting them consistently in mainland habitats (GBIF 2026; Hofmann et al. 2019; Irschick et al. 1997). On Island D, we collected individuals of both species present on the island in 2022 (18 lizards in total, Table 1). All individuals were captured by hand at both sites.

After capture, lizards were transported to the laboratory at the Smithsonian Tropical Research Institute in Gamboa, Panama and housed individually in plastic Tupperware containers containing a water-saturated paper towel at room temperature (~22°C), with a 12h:12h light/dark cycle for a maximum of 72h. Prior to housing a lizard, the plastic container was sterilized using a 10% bleach solution and a new paper towel was added. Because faecal material represents a high microbial biomass sample type, it is generally less susceptible to background DNA contamination or cross-contamination (Eisenhofer et al. 2019; Fierer et al. 2025). Therefore, potential environmental contamination sources such as container surfaces, water, or paper towels are expected to have minimal impact relative to the high endogenous microbial signal present in faecal material. Due to the short holding period,

no food was provided. This also ensured that we did not introduce novel dietary items that could artificially alter the gut microbiome. Upon arrival at the lab, we measured snout-vent length (SVL; mm) using a digital calliper and measured body mass (g) with a digital balance (nearest 0.01 mm and 0.01 g). Following all measurements and faecal collection, lizards were released either to their initial capture location or euthanized for other studies.

2.2 | Faecal Sample Collection

Faecal samples were collected from all individuals ($N=74$ across all species) while housed in the sterilized containers. Lizard containers were monitored hourly for the presence of faecal material during lizard active hours (6:30 am–6:30 pm). Faecal samples were at most 12 h old. Upon defecation, samples were collected using sterilized tweezers, placed into 2 mL sterile tubes, and immediately placed in a freezer at -20°C .

2.3 | Climatic Data

Climatic variation can impact gut microbiomes, as we have previously shown in *A. apletophallus* (Williams et al. 2022). Here, we compared several climate variables between the 2 years of our study on the mainland (2022 and 2023), as well as between our two study locations (mainland and island D) in 2022 as a potential explanation for why microbiomes varied over space and time. To compare between 2022 and 2023, we analysed data from the Smithsonian Tropical Research Institute's Physical Monitoring Program on Barro Colorado Island (located near our study sites), including precipitation, relative humidity, and temperature at 1 m height. For each variable, we calculated mean daily values and used linear models to assess interannual variation. To quantify thermal differences between the mainland and Island D in the year 2022, we deployed data loggers that measured ambient air temperatures every 100 min. Each temperature logger consisted of an iButton (calibrated at factory: Embedded Data Systems, Lawrenceburg, KY, USA) that was coated in Plasti-Dip (Plasti Dip International, Blaine, MN, USA) for waterproofing and glued to a small piece of wooden trim. We have used these data loggers in the past to reliably estimate the ambient air temperature distributions of understory anoles in Panama (Cox et al. 2020; Logan et al. 2021; Neel et al. 2021). This approach works well in tropical rainforest environments, where heavy canopy cover, stable humidity, and limited direct solar radiation minimize temperature artefacts. Under these conditions, exposed sensors provide accurate estimates of the microclimates that anoles actually encounter, making this method appropriate for our system (Fawcett et al. 2019; Scheffers et al. 2017; Vickers and Schwarzkopf 2016). On the mainland, we deployed temperature loggers in the wet season (May–August) in random locations along a set of transects of 50 m that radiated from Pipeline Road into the forest. Eight perpendicular transects were deployed along a 5.4-km stretch of Pipeline Road (five data loggers per transect). The loggers were fastened to branches using zip ties at random distances along each transect (in 1 m intervals), on a random side of each transect (left or right), at a random height in the vegetation (0.5 to 2 m in 0.5 m intervals), and in a random orientation on the branch (top, side, or bottom).

During the same time period, we deployed 25 temperature data loggers across the experimental island by haphazardly selecting locations that covered the majority of the island, and then randomly assigning a cardinal direction, distance from the branch (0–3 m in 1 m intervals), height (0.5–2 m in 0.5 m intervals), and branch orientation (top, side, or bottom; Williams et al. 2022). We extracted temperature data from iButtons, calculated mean daily values, and used linear models to compare thermal differences between sites in 2022.

2.4 | DNA Extraction, Library Preparation, and Sequencing

Prior to DNA extraction, we homogenized faecal samples from the largest species (*A. capito* and *A. frenatus*) and subsampled to 50 mg, due to these faecal samples exceeding the manufacturer's recommended maximum input material. From all other species, we extracted DNA from the entire sample. We homogenized all samples in Zymo DNA/RNA shield using a sterile pestle, pelleted the solid material, and washed the pellet with TE buffer before lysis with a two-stage method. We first lysed samples enzymatically (using Lysozyme suspended in TE buffer) followed by mechanical lysis with 0.1 mm silica beads suspended in Zymo DNA/RNA Lysis Buffer (Zymo Research, Irvine, CA, USA). We then combined the lysates and extracted genomic DNA using the Zymo quick-DNA HMW Extraction Kit (Zymo Research, Irvine, CA, USA). We quantified DNA concentration for a select number of samples to verify extraction success using a Qubit Fluorometer (Invitrogen, Waltham, MA, USA), and we stored all the samples at -20°C . Finally, we amplified and sequenced the V4 hypervariable region of the 16S rRNA gene for all samples using the Illumina MiSeq v3 platform (Illumina, San Diego, CA, USA) with a 300-bp paired-end sequencing protocol (Williams et al. 2022). To control for contamination during extraction and library preparation, we included and sequenced two extraction blanks and two PCR blanks alongside our samples.

2.5 | Microbiome Data Processing and Analysis

We imported demultiplexed FASTQ files in Casava 1.8 format into QIIME2 (version 2024.2) for processing (Bolyen et al. 2019). We quality-filtered, trimmed paired-end reads to remove primers, and denoised them using the DADA2 plugin to generate amplicon sequence variants (ASVs) (Callahan et al. 2016). We clustered ASVs into 97% operational taxonomic units (OTUs) using the vsearch 'cluster-features-denovo' (Rognes et al. 2016). We constructed a phylogenetic tree by aligning sequences with MAFFT, masking highly variable positions, and building the tree with FastTree, followed by midpoint rooting (Katoh et al. 2002; Price et al. 2010). We assigned taxonomy using the 'feature-classifier-classify-sklearn' plugin with a naive Bayes classifier pretrained on the SILVA 16S rRNA database (release 138, 99% OTUs; Quast et al. 2013). We used the 'decontam' package in R to identify potential contaminant OTUs in our dataset based on extraction kit and PCR control samples (Davis et al. 2018). However, the program did not identify any features as contaminants, and therefore we did not filter any reads out of our dataset. We conducted microbial diversity analyses in QIIME2 and R (version 4.4.1; R

Core Team 2021). For alpha diversity, we calculated OTU richness, Shannon diversity, and Pielou's evenness (Pielou 1966; Shannon 1948) using the 'core-metrics-phylogenetic-97' plugin in QIIME2. For beta diversity, we calculated Jaccard, Bray-Curtis, unweighted UniFrac, and weighted UniFrac distances (Bray and Curtis 1957; Jaccard 1908; Lozupone et al. 2007; Lozupone and Knight 2005).

2.6 | Statistical Analysis

2.6.1 | Interspecific Variation in Gut Microbiomes of Mainland Anoles

To evaluate the differences in alpha diversity across mainland host 'species' and 'year', we used linear models with categorical variables (species and year) followed by pairwise Tukey post hoc tests. To examine the relationship between host body size and alpha diversity, we applied linear mixed-effects models with SVL as a fixed effect and 'species' as a random effect. We tested for significant differences in community composition across 'species', 'SVL', and 'year' using PERMANOVA (adonis2 in R; Li et al. 2022). When the global test was significant, pairwise PERMANOVAs were performed with Benjamini-Hochberg (BH) correction. We used backward stepwise regression to identify the most parsimonious model and visualized patterns of beta diversity using principal coordinates analysis (PCoA) (Anderson et al. 2011). To identify bacterial taxa potentially driving microbiome differences between 'species' and 'years', we used ANCOM differential abundance analyses in QIIME2 at the phylum, family, and OTU levels, after applying an abundance and prevalence filter (minimum frequency=10 reads; minimum prevalence=2 samples) to remove rare features that might not be biologically informative (Mandal et al. 2015).

2.6.2 | Relative Impact of Species Identity and Locality on the Microbiome of *Anolis apletophallus* and *Anolis gaigei*

We compared the relative effects of host species and locality on the gut microbiomes of the two species found on Island D, the slender anole and Gaige's anole. We compared gut microbial communities between three groups: mainland *A. apletophallus*, island *A. apletophallus*, and island *A. gaigei*. Comparisons between mainland *A. apletophallus* and island *A. apletophallus* represent the effect of locality on the microbiome. Comparisons between island *A. apletophallus* and island *A. gaigei* represent the effect of host species (as they occur at the same, highly geographically restricted site). We used linear models to compare alpha diversity across the three populations, all sampled in 2022. Differences in beta diversity between populations were assessed using the same distance metrics and PERMANOVA framework described above. We also compared pairwise distances between populations using Kruskal-Wallis tests, followed by pairwise Wilcoxon tests with BH correction, to determine which comparisons were the most different from one another in microbiome structure. To identify bacterial taxa potentially driving microbiome differences between populations, we used ANCOM differential abundance analyses in QIIME2 at the phylum, family, and OTU levels, after applying a minimum abundance filter

(minimum frequency=10 reads; minimum prevalence=2 samples) to remove rare features that might not be biologically informative (Mandal et al. 2015).

2.6.3 | Phyllosymbiosis Patterns Across Anole Species

To test whether host phylogenetic relatedness was correlated with microbiome similarity, we constructed a phylogenetic distance matrix based on species divergence times obtained from TimeTree (Kumar et al. 2017), which integrates data from thousands of published studies assembled into a searchable tree of life scaled to time. We excluded *A. elcopeensis* due to insufficient phylogenetic data to be accurately placed on the tree. We then calculated average pairwise microbiome distances between species for each of the four beta diversity metrics, using merged species-level samples via the "qiime feature-table group" command in QIIME2 (Bolyen et al. 2019) and computed distance matrices from these aggregated profiles. We used two approaches to test for a phyllosymbiosis signal in beta diversity metrics in R. First, linear models were used to evaluate correlations between species phylogenetic distance and microbiome distance. Second, we applied the Procrustean Approach to Cophylogeny (PACo; Hutchinson et al. 2017) to incorporate tree topology, using the 'paco' package in R to test for congruence between host phylogeny and dendrograms of species' microbiome similarity, with Cailliez-corrected principal coordinates and 999 permutations under the r0 null model. We also tested for a phylogenetic signal in alpha diversity metrics using the phylosignal function in the 'picante' R package (Kembel et al. 2010).

3 | Results

3.1 | Climatic Variation Between Sites and Years

When comparing interannual climatic variation at the regional scale in central Panama, we found that 2023 was a relatively hot and dry year compared to 2022. In 2022, the temperature was 0.45°C lower relative to 2023 (Figure 1C-E; $\beta = -0.45$, $p < 0.001$) and average daily precipitation was 2.7 mm greater (99.3 more cm of rain fell in 2022, Figure 1D; $\beta = 2.7$, $p = 0.008$). Relative humidity was also significantly higher in 2022 (Figure 1E; $\beta = 3.0$, $p < 0.001$). In 2022, at the local scale, Island D was 1.29°C warmer than the mainland (Figure 1B; $\beta = 1.29$, $p < 0.001$).

3.2 | Interspecific Variation in Gut Microbiomes of Mainland Anoles

In the six anole species sampled in mainland Panama in both 2022 and 2023, microbiomes differed significantly by species in alpha diversity (Shannon diversity and the number of OTUs; Figure 2A,C; Shannon: $p = 0.013$, OTUs: $p < 0.001$ for 'species'). Although there was no overall effect of species identity on microbiome evenness ($p = 0.323$ for 'species'), pairwise Tukey test comparisons revealed that *A. elcopeensis* had significantly lower evenness than *A. capito* and *A. frenatus* ($p = 0.030$, $p = 0.018$, Table S1). Host body size (SVL) partly explained species-level alpha-diversity differences: larger-bodied species hosted richer and more diverse microbiomes (Figure 2B,D; OTUs: $p = 0.008$,

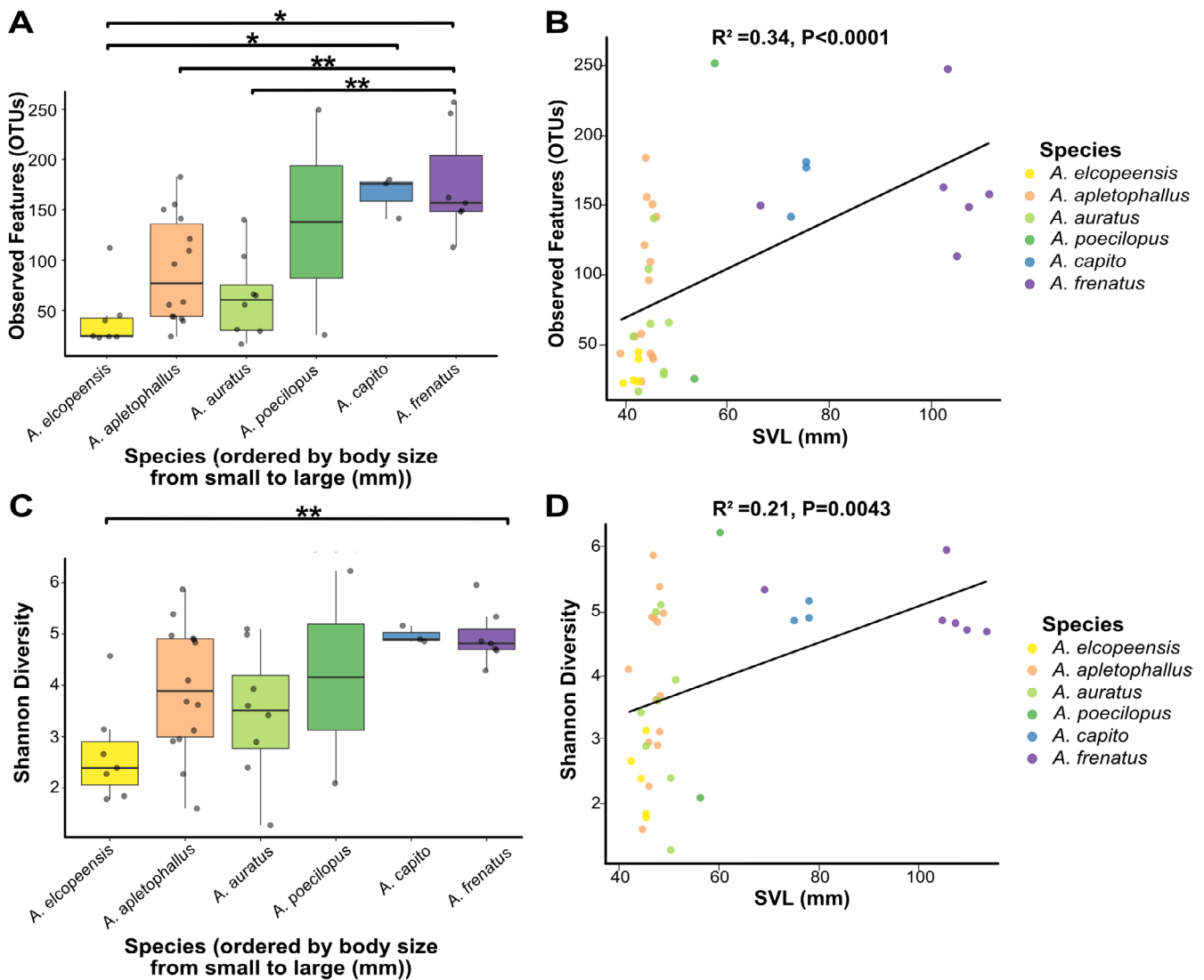


FIGURE 2 | Microbial alpha diversity differed between mainland anole species and was correlated with body size. (A) Comparison of number of OTUs between mainland anole species. (B) Relationship between body size (SVL) and observed OTUs. (C) Comparison of Shannon Diversity in different mainland species. (D) Relationship between body size (SVL) and Shannon Diversity. Brackets represent species pairwise differences. Asterisks indicate significance: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

Shannon diversity: $p = 0.027$). Evenness did not show significant differences across species or body size (Figure S2). Alpha diversity did not differ between the two sampling years for any species (Shannon diversity: $p = 0.235$; OTUs: $p = 0.275$ for ‘year’).

Beta diversity was significantly affected by species identity and sampling year across the six mainland *Anolis* species. Model selection identified species and year, but not body size (SVL), as significant factors explaining beta diversity as measured by Jaccard and Bray Curtis indices (Jaccard: species, $p = 0.001$; year, $p = 0.039$; Bray Curtis: species, $p = 0.001$; year, $p = 0.040$; Figure 3A,B). In metrics that incorporate microbial phylogenetic diversity (unweighted and weighted UniFrac indices), species, but not sampling year or body size, explained differences among individual hosts (unweighted UniFrac: $p = 0.001$; weighted UniFrac: $p = 0.023$; Figure S3A,B). After finding that ‘species’ significantly explained microbiome variation, we ran a pairwise PERMANOVA to identify which species pairs differed for each diversity metric (Table S2).

To determine which microbial taxa underlaid differences in microbial community composition between species, we performed differential abundance testing using ANCOM. At the phylum level, four taxa were found to be significantly different across species (Figure 3C–F): Actinobacteriota ($W = 4$, W statistic reflects the number of pairwise comparisons in which a taxon is detected as differentially abundant, with higher values indicating stronger and more consistent signals of difference across groups), Verrucomicrobiota ($W = 4$), Bacteroidota ($W = 2$), and Campilobacterota ($W = 1$). Actinobacteriota was substantially more abundant in *A. frenatus*, *A. auratus*, and *A. capito*. Verrucomicrobiota was most abundant in *A. capito*. Bacteroidota was most abundant in *A. elcopeensis*, and *A. capito*. In contrast, Campilobacterota remained at very low abundances in most hosts, with only *A. elcopeensis* and *A. frenatus* showing modestly higher abundances relative to the other species. At the family level, two taxa showed significant differences (Figure 3G,H): UCG-010 ($W = 156$; an uncultured bacterial lineage that remains poorly characterized) and Weeksellaceae

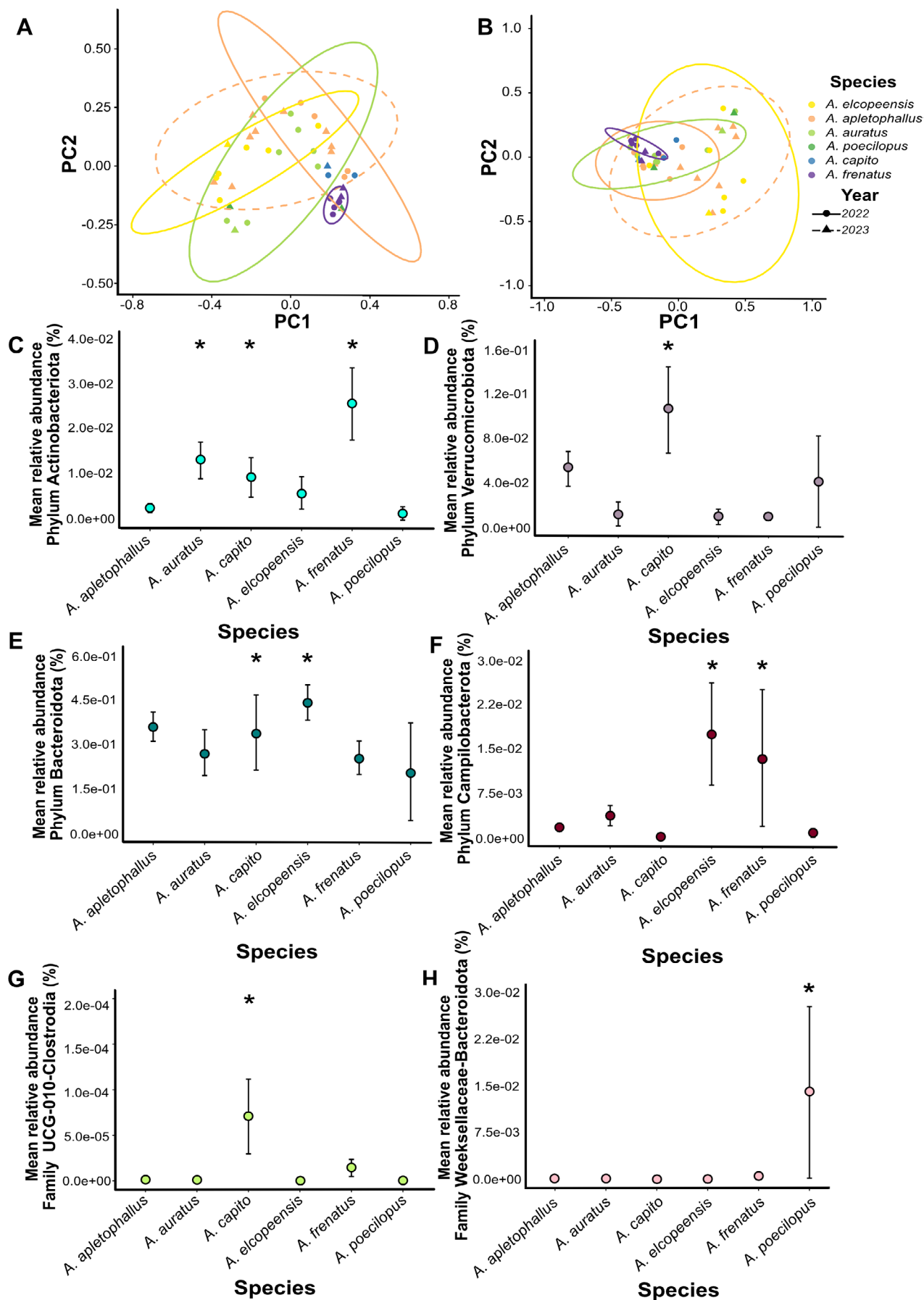


FIGURE 3 | Legend on next page.

FIGURE 3 | Microbial community composition varies across *Anolis* species in the mainland. (A, B) Principal Coordinate Analyses (PCoA) based on Jaccard and Bray–Curtis dissimilarities respectively show distinct species level clustering, indicating that individuals from the same species share more similar gut microbiomes than individuals from different species. Year effects were minimal, with 2022 and 2023 samples clustering closely within species. (C–F) Differentially abundant phyla vary by species: Actinobacteriota and Bacteroidota are elevated in *A. capito* and *A. frenatus*; Verrucomicrobiota is strongly enriched in *A. capito*; and Campilobacterota shows modest increases in *A. elcopeensis* and *A. frenatus*. (G, H) Bacterial families also differ across hosts: UCG 010 (Clostridia) is highest in *A. capito* and *A. frenatus*, while Weeksellaceae is elevated in *A. poecilopus*. Asterisks indicate significance: * = $p < 0.05$.



FIGURE 4 | Gut microbiomes are shaped both by species identity and by site (mainland vs. island D). (A) Principal Component Analysis (PCA) of beta diversity using the Jaccard index for *Anolis apletophallus* and *Anolis gagei* from Island D and the Mainland. Ellipses represent the 95% confidence interval of each of the three populations (*A. apletophallus* mainland (orange circle, solid line), *A. apletophallus* island (orange triangle, dashed line), *A. gagei* island (brown triangle, dashed line)). (B) Pairwise Jaccard distances between individuals from the three groups. Lizard illustrations were created by Yanileth F. López-Tacoaman using Affinity Designer 2.

($W=58$). The first belongs to the phylum Firmicutes and class Clostridia and was most abundant in *A. capito*, and *A. frenatus* compared to the other species. The second belongs to the phylum Bacteroidota and class Bacteroidia and was detected at very low and relatively uniform abundances across most species, except for *A. poecilopus*, which showed markedly higher values (Figure 3H). At the OTU level, just one taxon was identified as differentially abundant. This OTU was classified as belonging to the phylum Firmicutes and class Bacilli ($W=553$). It had significantly higher abundance in *A. frenatus* (3.75%, Figure S3E) compared with the other species. We detected no significant difference in taxon abundance based on year.

3.3 | Relative Impact of Species Identity and Locality on the Microbiome of *Anolis apletophallus* and *Anolis gagei*

Site and species synergistically impacted microbiome community composition. Population (defined as *A. apletophallus* from the mainland, *A. apletophallus* from the island, and *A. gagei* from the island) was a significant (or near significant) predictor of all metrics of microbial community composition (Figure 4A; Jaccard: $p=0.003$; Bray Curtis: $p=0.002$; unweighted UniFrac: $p=0.05$; weighted UniFrac: $p=0.043$). In pairwise comparisons,

species identity and locality had similar degrees of influence over differences in microbial composition (Figure 4B). The largest degree of difference in microbiomes was between *A. apletophallus* inhabiting the mainland and *A. gagei* inhabiting Island D (Figure 4B). The level of microbiome dissimilarity did not differ significantly when comparing the difference between *A. apletophallus* on the mainland vs. *A. apletophallus* on Island D to the difference between *A. apletophallus* and *A. gagei* inhabiting Island D (Figure 4B). Despite differences in microbiome composition by population, alpha diversity was not significantly different across populations (OTUs: $p=0.351$; Shannon diversity: $p=0.340$; Evenness: $p=0.419$).

When comparing populations using differential abundance analysis, we observed bacterial taxa with significantly different relative abundances at both the phylum and family levels. We detected a significant difference in the relative abundance of Campilobacterota ($W=9$). This difference was driven by a significant change in Helicobacteraceae specifically ($W=46$). This family was more abundant in both populations (mainland and island) of *A. apletophallus* and was absent in Island *A. gagei* anoles, indicating that differences in this family are primarily driven by host species identity (Figure S4A,B). Additionally, the family Eubacteriaceae ($W=51$, Figure S4C) was found in higher abundance in island *A. gagei* and less abundant in both island

and mainland *A. apletophallus*. At the OTU level, three taxa in the phylum Bacteroidota showed significant differences: one in the family Marinifilaceae ($W=368$), and two in the family Bacteroidaceae ($W=360$ and $W=327$, respectively). Differential abundance of Marinifilaceae OTUs resulted from their presence in mainland *A. apletophallus*, while it was absent from both island populations (Figure S4D). The other two OTUs were present only in island *A. apletophallus* and island *A. gagei*, and were absent from mainland *A. apletophallus*, suggesting that this taxon is only present in the island environment (Figure S4E,F).

3.4 | Phyllosymbiosis Patterns Across Anole Species

Linear models revealed a significant correlation between microbial Bray-Curtis distance and host phylogenetic distance ($R^2=0.317$, $p=0.029$; Figure 5A), and a marginally significant correlation between microbial weighted UniFrac distance and host phylogenetic distance ($R^2=0.256$, $p=0.054$; Figure 5B). Jaccard ($R^2=0.054$, $p=0.405$) and unweighted UniFrac distance ($R^2=0.006$, $p=0.792$) were not significantly correlated with host phylogenetic distance. PACo analysis revealed significant congruence between host phylogeny and species microbiome similarity for Bray-Curtis ($p=0.022$), Unweighted UniFrac ($p=0.031$), and Weighted UniFrac ($p=0.038$) distance (Figure 5C–E), with a marginally significant result for Jaccard distance ($p=0.051$, Figure 5F). These results collectively suggest that microbiome composition among *Anolis* species is structured by host phylogenetic relatedness, with the strongest signal observed in abundance-weighted metrics. On the other hand, no significant results were found when testing for a phylogenetic signal in alpha diversity metrics (Evenness: $p=0.24$; Observed Features: $p=0.13$; Shannon: $p=0.09$).

4 | Discussion

Gut microbiomes are dynamic communities shaped by both ecological and evolutionary processes. In ectothermic vertebrates such as lizards, where physiology and life history are tightly coupled with environmental conditions, these communities offer a unique opportunity to explore how intrinsic host traits and extrinsic factors interact to structure microbial diversity. We sampled the microbiomes of seven species of *Anolis* lizards across two sites and two years to assess how host characteristics and environmental conditions impact gut microbiomes. We found that traits such as host species identity and body size influenced gut microbiome composition and diversity. Moreover, environmental factors like locality and annual environmental variation may contribute to gut microbiomes variation in anoles. We observed interannual differences in gut microbiome composition across species, which might be explained by the observed differences in temperature, precipitation, and humidity across years. Similarly, gut microbiome composition differed across our study sites which also varied in temperature. Finally, we detected moderate correlations between host phylogeny and microbial community similarity, indicating some degree of phyllosymbiosis in *Anolis* lizards. Together, these findings suggest that while anoles host species-specific microbiomes, environmental effects play a substantial role in modulating this variation.

Species identity emerged as a key factor shaping both the alpha and beta diversity of anole gut microbiomes. Different host species harboured microbial communities of varying richness and diversity, and these differences were partly explained by body size, as larger species hosted more diverse microbiomes. The effect of body size on microbiome diversity has been observed in other species and is hypothesized to be due to the fact that larger guts have more niche space for colonization of diverse taxa, an idea consistent with island biogeography theory (MacArthur and Wilson 2001; Sherrill-Mix et al. 2018). Indeed, the two largest host species we studied had the most distinct microbiomes, and these differences were driven by taxa in the phylum Actinobacteriota, Verrucomicrobiota, Bacteroidota, and Campilobacterota, the family UCG-010 (in the class Clostridia) and an OTU (in the phylum Firmicutes) which have been linked to host diet, bile acid metabolism, xenobiotic processing, and adaptation to changing seasons. Some of these taxa, such as Actinobacteriota, have been recently found in wild individuals of other lizard species (Jiang et al. 2017; Zhu, Chen, et al. 2024; Zhu, Jiang, et al. 2024). This pattern of relatively distinct gut microbiomes between species is consistent with studies in other lizard taxa and vertebrate hosts more broadly (Bunker and Weiss 2022; Eliades et al. 2022; Hernández et al. 2022, 2024; Hong et al. 2011; Kohl et al. 2017; Lazarkevich et al. 2024; Ley et al. 2008; Lim and Bordenstein 2020; Ren et al. 2016; Vasconcelos et al. 2023). Similarly, *A. elcopeensis* has the smallest average body size of species in our dataset and exhibited unique microbiome features such as elevated relative abundances of the phyla Bacteroidota and Campilobacterota. Bacteroidota is a diverse phylum that includes members associated with carbohydrate degradation (Hoffbeck et al. 2023). While Campilobacterota has been detected in other reptile species, their function in these hosts is not well understood (Gilbert et al. 2019). At finer taxonomic scales, additional patterns emerged that highlight the role of species-specific ecology beyond body size alone. The Weeksellaceae family, for example, was detected at uniformly low abundances across most species but showed a pronounced increase in *A. poecilopus*, a semi-aquatic species that is usually found associated with slow moving streams in central Panama (Muñoz et al. 2015). This family has been reported in other aquatic and semi-aquatic reptiles such as turtles and pythons (McKnight et al. 2020). This suggests that this rare and low abundance taxon may be associated with permanent bodies of water. These patterns underscore that while body size helps explain broad-scale differences in microbiome diversity, species-specific ecologies may contribute to the presence of rare or specialized microbial lineages.

Following one of the most severe droughts in Panama's recent history, environmental conditions in 2023 were markedly hotter and drier than in 2022 (Brooks et al. 2023). In the *Anolis* community we studied, gut microbial richness and evenness were resilient to interannual environmental variation, despite significant differences in temperature, precipitation, and humidity between study years. In contrast, microbial community composition differed between years, aligning with previous studies showing that temporal and seasonal variation can influence microbial assemblages in reptiles and other ectotherms (Colston and Jackson 2016; Dubos et al. 2020). In our study system, our group has previously shown that anomalous climatic events (such as drought) can shape the taxonomy and

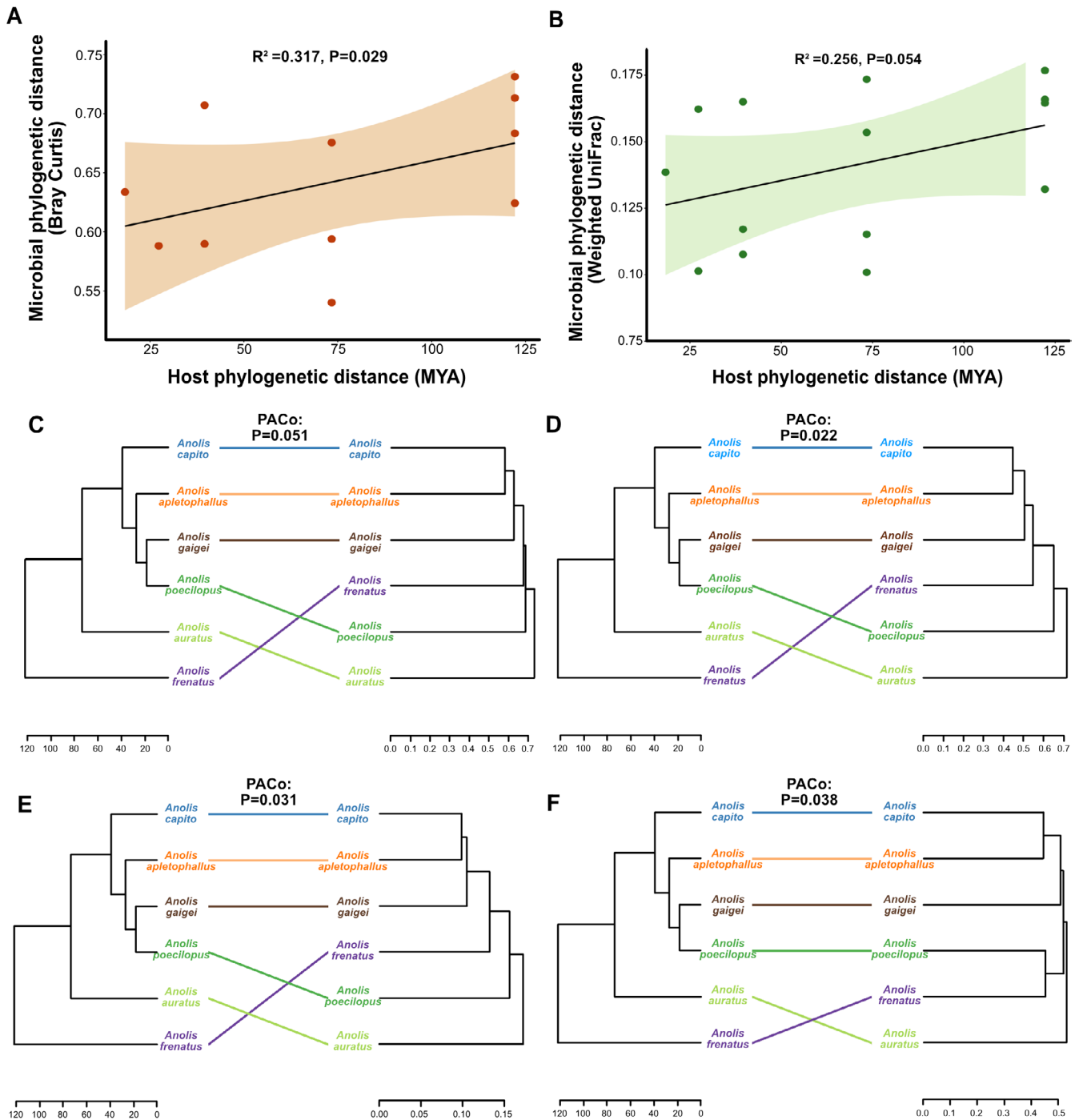


FIGURE 5 | Microbiome distance between *Anolis* species is correlated with host phylogenetic distance by linear model and PACo analysis. Linear relationship between (A) Bray–Curtis and (B) Weighted UniFrac dissimilarity and host phylogenetic distance. Congruence between the *Anolis* species phylogenetic tree (left) and microbiome dissimilarity (right) based on (C) Jaccard, (D) Bray-Curtis, (E) Unweighted UniFrac, and (F) Weighted UniFrac distance between host species.

functional composition of the gut microbiome of *A. apletophallus* (Williams et al. 2022). Given the known sensitivity of microbial communities to temperature, particularly in those of ectothermic hosts like lizards (Moeller et al. 2020), and emerging evidence from mammals that humidity can influence gut microbiomes (Wang et al. 2023), it is plausible that this change in climate between years contributed to shifts in microbiome composition. Whether or not climatic differences directly drive microbiome composition or affect latent

variables that then shape microbiome composition (e.g., prey diversity), remains an open question (Liu et al. 2022; Vicente Liz et al. 2019; Moeller et al. 2020; Zhang et al. 2022; Zhu, Chen, et al. 2024; Zhu, Jiang, et al. 2024). Future studies could directly manipulate these variables in controlled experiments or collect fine-scale climate data that could be correlated with differences in individual microbiomes over time. Regardless, our findings underscore the importance of accounting for temporal variation in understanding variation in gut microbiomes

in wild species, as studies that capture only snapshots of microbial composition in time may be misleading.

When focusing on anoles captured at our mainland site and an experimental island in the Panama Canal (Island D), we found that the gut microbiome was jointly influenced by host species identity and locality. When comparing host species between the mainland and Island D, the greatest difference between microbiomes was between two different species occupying different localities. On the other hand, we observed similar magnitudes of difference when comparing either different species within the same locality or the same species in different localities. This indicates that both host identity and locality combine to influence the microbiome structure of populations. Differential abundance analysis revealed marked compositional differences across host populations at multiple taxonomic levels. While most patterns of differential abundance appear to be associated with host species identity, the detection of one family and two OTUs (from the families Eubacteriaceae and Bacteroidaceae) exclusive to islands points to a potential environmental filtering effect. These taxa may represent microbes adapted to island-specific conditions, or symbionts that provide selective advantages under the distinct ecological pressures of insular environments like adaptation to new diets, season, drought, or warming (Hernández et al. 2023; Hoffbeck et al. 2023; Williams et al. 2022; Yang et al. 2024; Zhu, Chen, et al. 2024; Zhu, Jiang, et al. 2024). Specifically, Eubacteriaceae has been observed in other lizard species after exposure to low temperatures (Zhu, Chen, et al. 2024; Zhu, Jiang, et al. 2024). The *A. apletophallus* individuals on Island D were the fifth-generation descendants of founders that came from the mainland population, while *A. gagei* was present on the island when *A. apletophallus* were introduced. Over this time frame, it appears that the microbiomes of the two species on Island D have converged, underscoring a key role for local environmental conditions in shaping microbial community composition across species, a pattern documented in other ectotherms (Colston et al. 2025; Garcia-Recinos et al. 2019). Candidate drivers of this convergence include the hotter climate of Island D (mean environmental temperature was 1.29°C higher compared to the mainland) and the likelihood that the prey community (arthropods) is more constrained (lower richness) and divergent from that of the mainland. Populations differed the most in microbiome structure when assessed using presence/absence-based metrics (Jaccard, unweighted UniFrac) compared to abundance-based metrics (Bray-Curtis, weighted UniFrac). Thus, locality may primarily influence microbiome structure in anoles by mediating which microbial taxa are present rather than the relative abundance of dominant taxa (Martiny et al. 2011). Other studies have similarly found that sympatric lizard species can share more microbial features than conspecifics living in different geographical locations (Qi et al. 2020).

We found moderate evidence for phylosymbiosis among the anoles we studied. We detected correlations between microbial community composition and host phylogenetic distance when using abundance-sensitive metrics (Bray-Curtis and weighted UniFrac) but not when using presence/absence-based metrics (Jaccard and unweighted UniFrac). This pattern suggests that while related host species may share similar dominant microbial taxa, the overall composition—including the presence/

absence of rare taxa—is less constrained by phylogenetic relatedness (Brooks et al. 2016). When considering topology, our PACo analysis revealed significant congruence between host phylogeny and microbiome community similarity based on all metrics. Although trees were not perfectly aligned, microbiome structure was more congruent with host evolutionary history than expected by chance. While species identity and evolutionary history clearly contribute to microbiome composition, the evidence for phylosymbiosis was moderate relative to patterns observed in other host species. This may reflect both biological and methodological factors. First, phylosymbiosis is often weaker in non-mammalian hosts because opportunities for microbial transmission during live birth, social interactions, and parental care are limited, leading to stronger environmental effects on host-associated microbiota (Kohl 2020; Mallott and Amato 2021). Our results are consistent with these previous studies, as environmental variables strongly influenced microbiome structure in both mainland and island lizard populations. Second, there may be uncertainty in estimated divergence times for *Anolis* species that have diverged relatively recently, or ecological niches may not be sufficiently distinct to have driven co-diversification of gut microbiomes (Ren et al. 2016). Indeed, phylosymbiosis is often more apparent in comparisons across deep evolutionary divergences (i.e., across orders or classes) than among closely related species within a single genus (Trevelline et al. 2020). Nevertheless, we did find that some aspects of gut microbiome structure were predicted by host phylogenetic relatedness, highlighting the fact that host evolutionary history remains an important factor shaping *Anolis* gut microbiomes. Although divergence times from TimeTree provide robust, large-scale estimates of phylogenetic relationships, variation in molecular divergence rates among *Anolis* lineages could obscure fine-scale relationships, potentially reducing the power of our phylosymbiosis tests. Therefore, our findings should be interpreted with caution, as the observed correlation may underestimate true phylogenetic effects among closely related species.

In summary, the gut microbiome of Panamanian anoles is shaped by a variety of factors. Host identity plays a primary role, with a distinct microbial community apparent in each species we examined. The species-level signal may then be modulated by environmental factors like interannual climate variation and geographic location which can restructure communities over time and space. Our work emphasizes the interplay between environmental and evolutionary factors in shaping reptilian gut microbiomes. However, it remains unclear to what extent environmentally driven changes in gut microbiomes impact host ecology and evolution. Exploring the functional impact of shifting microbiomes is a worthy avenue of future study given how rapidly environments are changing across the globe.

Author Contributions

Y.F.L.T., W.O.M., C.L.C., M.L.L., S.S.F. and C.E.W. designed the study. Y.F.L.T., C.E.W., S.S.F., M.A., K.A., L.B., J.D.C., A.G., N.D.G., S.G., R.M.P., N.R., D.R., C.L.C. and M.L.L. collected data and conducted lizard husbandry. Y.F.L.T. analysed data. Y.F.L.T. produced the first draft of the manuscript. All authors revised and approved the final version of the manuscript.

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Ethics Statement

This project was approved under STRI Institute Animal Care and Use protocol number 2017-0308-2020, and permits were issued by MiAmbiente and by the Panama Canal Authority.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Photograph of sample sites. **Figure S2:** Pielou’s evenness did not vary among mainland anole species correlated with body size. **Figure S3:** Microbial community composition differs across *Anolis* species on the mainland. **Figure S4:** Differentially abundant microbial taxa vary across mainland and island populations of anoles: mainland *A. apletophallus*, island *A. apletophallus*, and island *A. gagei*. **Table S1:** Pairwise Tukey post hoc results comparing gut microbiome alpha diversity among host species from the *Anolis* genus on the mainland. **Table S2:** Pairwise PERMANOVA results in comparing gut microbiome beta diversity among host species from the *Anolis* genus on the mainland.