

Gut microbial ecology of Philippine gekkonids: ecoevolutionary effects on microbiome compositions

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Abstract

Given the rapidly changing landscapes of habitats across the globe, a sound understanding of host-associated microbial communities and the ecoevolutionary forces that shape them is needed to assess general organismal adaptability. Knowledge of the symbiotic endogenous microbiomes of most reptilian species worldwide remains limited. We sampled gut microbiomes of geckos spanning nine species and four genera in the Philippines to (i) provide baseline data on gut microbiota in these host species, (ii) test for significant associations between host phylogenetic relationships and observed microbial assemblages, potentially indicative of phylosymbiosis, and (iii) identify correlations between multiple ecoevolutionary factors (e.g. species identity, habitat tendencies, range extents, and maximum body sizes) and gut microbiomes in Philippine gekkonids. We recovered no significant association between interspecific host genetic distances and observed gut microbiomes, providing limited evidence for phylosymbiosis in this group. Philippine gekkonid microbiomes were associated most heavily with host species identity, though marked variation among conspecifics at distinct sampling sites indicates that host locality influences gut microbiomes as well. Interestingly, individuals grouped as widespread and microendemic regardless of host species identity displayed significant differences in alpha and beta diversity metrics examined, likely driven by differences in rare OTU presence between groups. These results provide much needed insight in host-associated microbiomes in wild reptiles and the ecoevolutionary forces that structure such communities.

Keywords: conservation, gecko, microbiome, Philippines, phylogeny, reptile

Introduction

Endogenous microbial communities inhabiting vertebrate and invertebrate hosts are increasingly recognized as essential in maintaining organismal well-being, influencing a variety of traits from host development and behavior to immune response and metabolism (Cho and Blaser 2012, Fraune and Bosch 2010, Lee and Hase 2014). Furthermore, these gut microbiomes likely contribute to host phenotypic plasticity, allowing for rapid adaptation to changing environments (Alberdi et al. 2016, Bahrndorff et al. 2016, Littleford-Colquhoun et al. 2019). Given the dramatic alteration of habitats globally during the Anthropocene, a sound understanding of host-associated microbial communities and the forces that influence them is needed to predict general organismal adaptability to future conditions (Amato 2013, Alberdi et al. 2016, Stumpf et al. 2016, Trevelline et al. 2019, Zhu et al. 2021). Here, we test the impacts of phylogenetic history and contemporary ecology on host species' gut microbiome diversity as a potential correlate of evolutionary plasticity.

At a broad taxonomic scale (generally at the level of family or higher), gut microbial communities often mirror phylogenetic relationships among hosts; a phenomenon known as phylosymbiosis (Amato 2013, Groussin et al. 2017, Ley et al. 2008, Lim and Bordenstein 2020, Sanders et al. 2014, Youngblut et al. 2019). Identifying signs of phylosymbiosis is a requisite first step

toward understanding the ecoevolutionary forces that drive observed assemblages (Lim and Bordenstein 2020). Evidence of this association, however, varies considerably at differing taxonomic levels. Invertebrates and mammals often exhibit observable patterns of phylosymbiosis, while other vertebrate groups show limited evidence for this association (Ley et al. 2008, Youngblut et al. 2019). For example, phylosymbiosis is supported among arthropods such as Hawaiian spiders (Perez-Lamarque et al. 2022) and turtle ants of the genus *Cephalotes* (Sanders et al. 2014), where gut microbiota are strongly correlative with host phylogenetic relationships. Similarly, significant associations between host phylogenetic affinities and microbial communities have been noted among all seven sea turtle species (Scheelings et al. 2020) and among 51 species of passerine birds (Kropackova et al. 2017). Interestingly though, among passerine bird microbiomes examined, most microbial variation in assemblages remains unexplained after accounting for host phylogeny, and factors operating at the within-species level are suspected of contributing to most individual variance (Kropackova et al. 2017). Other studies find less conclusive evidence for the presence of phylosymbiosis. In lizards of the genus *Anolis* and among 31 species of Afrotropical bats, significant, yet weak, associations between host phylogenetic relationships and microbial compositions have been recovered (Lutz et al. 2019, Ren et al. 2016). In both such instances, host microbial

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assemblages in individuals are believed to be influenced more by contemporary ecological features rather than phylogenetic ones (Lutz et al. 2019, Ren et al. 2016). Additional information is needed to better disentangle the influences of various ecoevolutionary factors on observed host-associated microbial communities, particularly within taxonomic groups that have received limited attention from host-associated microbial studies to date.

Reptiles represent one of the most speciose vertebrate groups on the planet, with over 11 000 recognized lineages distributed across all continents except Antarctica (Uetz et al. 2021). These species vary tremendously in body morphologies, habitat preferences, reproductive strategies, and more (Vitt and Caldwell 2013). Despite a striking array of species diversity and a subcosmopolitan distribution, relatively little is known about the symbiotic gut microbiomes of most reptilian species worldwide (Colston and Jackson 2016, Kohl et al. 2017). A critical facet of reptile microbiome research in particular need of further investigation pertains to the ecological and evolutionary traits that structure these gut communities. history influences gut microbial communities

In the few studies that examine multiple reptile species to date, host taxon identity is a prominent indicator of microbial assemblages, with interspecific differences in microbiome compositions generally greater than intraspecific distinctions (Kohl et al. 2017, Lankau et al. 2012, Ren et al. 2016). Analyses of host ecomorphs recover mixed findings. Galapagos land and marine iguanas, which differ significantly in diet, show significantly distinct microbiomes (Lankau et al. 2012) though few features distinguish various Caribbean anole ecomorphs, which all tend to be generalist species (Ren et al. 2016). Within species, individual diet has clear influences on gut microbiota in reptiles (Jiang et al. 2017, Kohl et al. 2017, Lankau et al. 2012, Ren et al. 2016). Host locality shows strong correlations with microbial compositions in Puerto Rican anoles (Ren et al. 2016) and both Galapagos land and marine iguanas (Lankau et al. 2012), but no such significant correlations have been noted in gopher tortoises across the southeastern United States (Gaillard 2014). Host internal microbial community dynamics clearly can be influenced strongly across both ecological and evolutionary scales (Lankau et al. 2012).

In this study, we sought to better understand gut microbial community diversity and structure in reptiles using a unique study system, wild gekkonid lizards in the Philippines. The insular nation of the Philippines in Southeast Asia is home to a remarkable array of reptilian diversity and is considered a global hotspot for reptiles (Mittermeier et al. 1999, Roll et al. 2017). Over 350 species can be found across the ~7500 islands in the Philippines (Uetz et al. 2021). The Philippines archipelago is home to a spectacular assortment of reptile species diversity in part because of its complex geographic history. A total of seven Pleistocene Aggregate Island Complexes (PAICs; Brown et al. 2013a) are generally recognized though many of these PAICs can be divided further still into various endemic biogeographic and even subfaunal regions of native flora and fauna (Heany 1993, Vallejo 2014). In this complex landscape, geckos represent one of the most taxonomically diverse groups of all vertebrates with 49 species described across multiple genera (Uetz et al. 2021). Precise dietary information for all gekkonid species in the Philippines is lacking, though most are thought to be insectivorous (Bauer 2013, Goldberg et al. 2016). Although sharing generalist dietary strategies, Philippine gekkonids display a wide variety of body sizes, distributions, and hypothesized habitat preferences to accompany their phylogenetic distinctiveness (Brown et al. 2008, 2009, 2010, 2011, 2013b, Welton et al. 2010). The marked array of evolutionarily distinct lineages of gekkonids coupled with microendemism and widespread species

across the Philippines provide an exceptional study system to test for phyllosymbiosis among confamilials and to complete ecoevolutionary comparisons of reptile hosts and their microbial assemblages.

We sampled gut microbial communities in 47 individual geckos from nine species and four genera in the Philippines to (i) provide baseline data on endogenous microbiota in these host species, (ii) test for evidence of phyllosymbiosis; microbial community relationships that parallel phylogenetic relationships among gekkonid hosts at the family level, and (iii) test for correlations between broad ecoevolutionary factors and gut microbial community compositions in wild gekkonids, including host species: identity, range, habitat preferences, and maximum body size as well as individual sampling locality and sampling biogeographic region in the Philippines.

Materials and methods

Host species examined

We analyzed gut microbial communities sampled via cloacal swabbing from 47 wild gekkonid lizards. These lizards represent the following nine species and four genera from the Philippines: *Cyrtodactylus philippinicus* ($n = 12$), *Gekko crombota* ($n = 7$), *G. gecko* ($n = 4$), *G. kikuchii* ($n = 1$), *G. mindorensis* ($n = 4$), *G. rossi* ($n = 9$), *Hemidactylus frenatus* ($n = 3$), *H. platyurus* ($n = 3$), and *Luperosaurus macgregori* ($n = 4$).

Sampling localities

To better address the possible influences of locality-specific factors on gut microbiota in wild gekkonids, we sampled hosts opportunistically at seven distinct localities in the central and northern Philippines. These sites were spread across four discrete biogeographic regions: the Babuyan Islands, northern Luzon, the Bicol Peninsula, and Negros Island. We conducted fieldwork on Calayan and Camiguin Norte (Babuyan Island Group), Luzon, and Negros islands in the Philippines during three field expeditions carried out every May and June between 2016 and 2018. All geckos were collected from low- (< 500 ft) to mid-elevation (< 700 ft). Individual sampling localities included Magsidel and Tapao Falls in the Babuyan Island Group, Mariroc and Tulay na Lupa on the Bicol Peninsula of Luzon, Mt. Palali and Nasiping in northern Luzon, and Cagbang on Negros Island (Table 1 and Fig. 1).

Animal and sample collection

Geckos were captured by hand between 1600 and 0200 h and for all locality records we used the WGS-84 datum. We collected cloacal swabs to inventory host-associated gut microbial communities, which have been shown to be effective proxies for endogenous microbiome sampling in reptiles (Colston et al. 2015, Eliades et al. 2021). To collect cloacal microbiome samples, we inserted sterile rayon-tipped swabs approximately 3 cm into the cloacal opening of each animal and rotated them 10 times (Smith et al. 2021). For efficient preservation of DNA, we then placed swabs into individual screw-top 1.5 ml cryovials with 750 μ l Xpedition™ Lysis/Stabilization Solution (Zymo Research Products). Cloacal swabs were stored at ambient temperature while in the field before transportation to the Sam Noble Oklahoma Museum of Natural History for curation and storage in a -20°C freezer until DNA extraction (Smith et al. 2021).

Table 1. Sampling table of 47 gekkonid hosts examined across the Philippines. Specimens included nine species from four genera collected at seven localities in the archipelagic nation. Contemporary factors examined were habitat preferences (forest obligates or human commensals), host species range (widespread vs. microendemic), and host maximum body size (SVL > or < 95 mm).

Genus	Species	Total sampled	Biogeographic region (# sampled)	Localities (# individuals sampled)	Habitat	Range	Body size
<i>Cyrtodactylus</i>	<i>philippinus</i>	12	Luzon (5), Bicol (5), and Negros (2)	Cagbang (2), Mt. Palali (5), Mariroc (2), and Tulay na Lupa (3)	Forest	Wide	Large
<i>Gekko</i>	<i>crombota</i>	7	Babuyan (1)	Tapao Falls (7)	Forest	Micro	Large
<i>Gekko</i>	<i>gecko</i>	4	Negros (1)	Cagbang (4)	Human	Wide	Large
<i>Gekko</i>	<i>kikuchii</i>	1	Luzon (1)	Nasipping (1)	Human	Wide	Small
<i>Gekko</i>	<i>mindorensis</i>	4	Luzon (4)	Mt. Palali (4)	Forest	Wide	Small
<i>Gekko</i>	<i>rossi</i>	9	Babuyan (9)	Magsidel (9)	Forest	Micro	Large
<i>Hemidactylus</i>	<i>frenatus</i>	3	Luzon (2) and Negros (1)	Cagbang (1), Mt. Palali (1), and Nasipping (1)	Human	Wide	Small
<i>Hemidactylus</i>	<i>platyurus</i>	3	Luzon (2) and Negros (1)	Cagbang (1) and Nasipping (2)	Human	Wide	Small
<i>Luperosaurus</i>	<i>macgregori</i>	4	Babuyan (4)	Magsidel (4)	Forest	Micro	Small

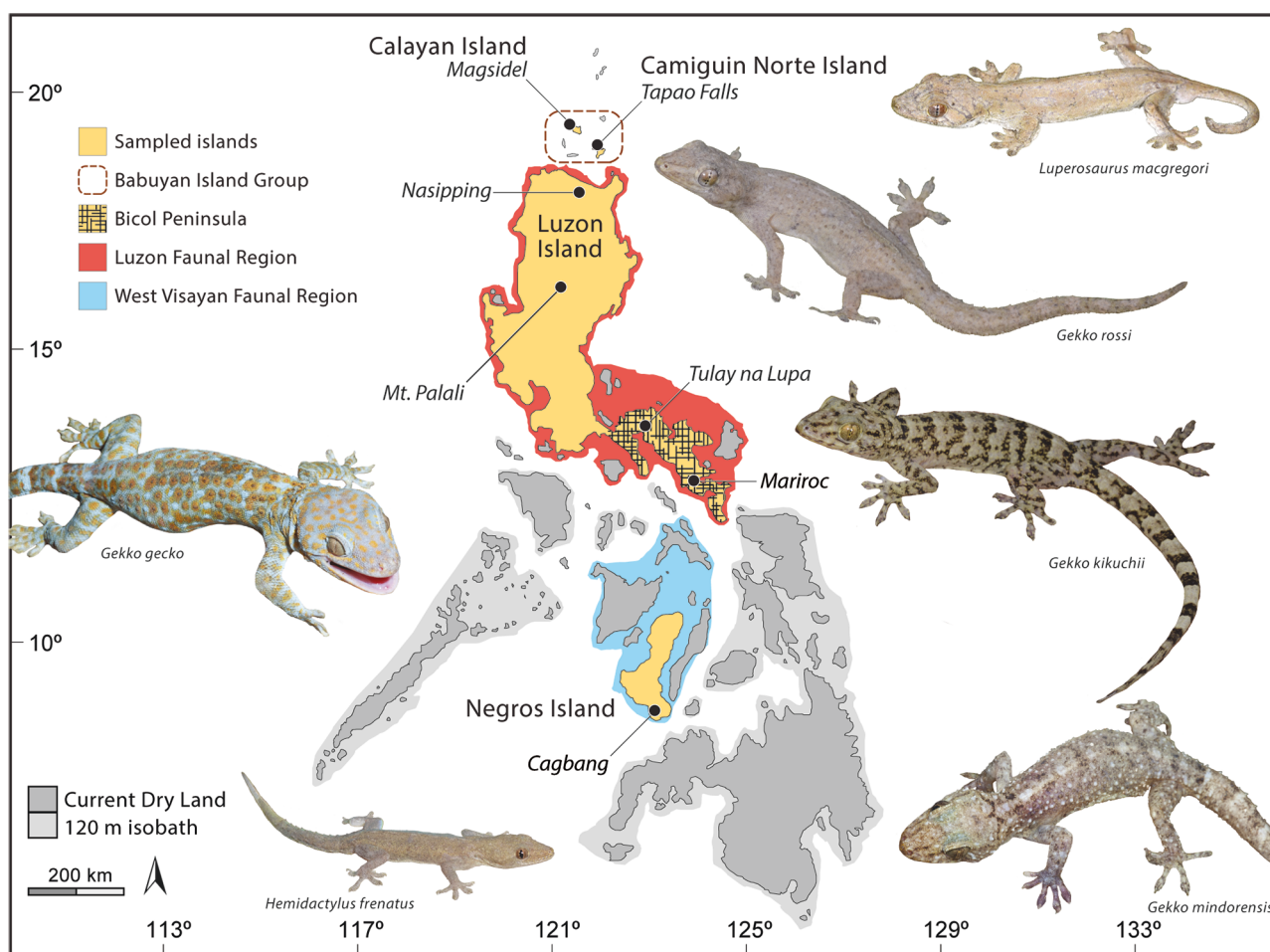


Figure 1. Map of Philippine archipelago with a shaded 120-m isobath around major island groups. Major biogeographic regions of note in this study include the Babuyan Island Group, Luzon Island, the Bicol Peninsula of southern Luzon, and Negros Island. Specific localities sampled in this investigation are included in italics. (Photographs of *G. rossi*, *H. frenatus*, and *L. macgregori* courtesy of Kai Wang, *G. mindorensis*, *G. gecko*, and *G. kikuchii* by C.D.S.).

Microbial inventories

Sample processing, data curation, and analysis closely reflect processes from Eliades et al. (2021). All DNA extraction and library preparation steps were completed at the Sam Noble Museum's

Shared Genetics Laboratories at the University of Oklahoma. We extracted total DNA from 56 gekkonid samples using Zymo Quick-DNA Fecal/Soil Microbe Kits. Cloacal swabs were incubated at 65°C for 15 min on a dry heating block and then vortexed for

15 min on an Eppendorf ThermoMixer® at 23°C and maximum speed (2000 rpm) immediately prior to beginning Zymo's recommended protocol. We amplified the V4 region of the 16S rRNA gene using published protocols index primers and PCR protocols (Kozich et al. 2013). PCR products were cleaned, normalized, and pooled using a Sequel Prep Normalization Plate Kit (Invitrogen). Pooled libraries were purified using Agencourt® AMPure® magnetic bead capture and sent to the University of Oklahoma's Consolidated Core Lab (CCL) for sequencing using 515F and 806R primers targeting 2 × 300 bp reads on an Illumina MiSeq sequencing platform (Caporaso et al. 2012). Libraries were prepared and sequenced in two iterations with 24 samples sequenced in 2018 and 32 samples sequenced in 2019.

Raw sequences from both sequencing iterations were processed concurrently. Reads were first paired and trimmed using AdapterRemoval2 v2.2.2 with default parameters (Lindgreen 2012, Schubert et al. 2016). Cleaned sequences were clustered *de novo* into operational taxonomic units (OTUs) using UPARSE in USEARCH v11.0.667 at a minimum sequence identity of 97% and a minimum abundance of four (Edgar 2013). Remaining sample curation and analysis was carried out in QIIME v1.9.1 (Caporaso et al. 2010). Taxonomies were assigned to OTUs using GreenGenes v13.8 (DeSantis et al. 2006). Archaea, chloroplast, mitochondria, PhiX, and other nonbacterial sequences were removed from processed OTU tables to ensure only bacterial sequences were included in downstream analyses. OTUs found in sample extraction negatives and PCR negatives were filtered and removed from all samples. These samples produced 1063 934 reads with a minimum read depth of 111, maximum of 47 747, and a median of 15 504 reads per sample. All 56 sequences were rarefied to 1000 reads per sample (Good's coverage mean = 0.95 ± 0.03), and samples with insufficient sequencing depth ($n = 9$) were removed from further analyses, resulting in 47 samples examined (Table 1; Appendix S1, Supporting Information). All raw 16S rRNA sequences have been deposited in the Sequence Read Archive (SRA) under accession no. PRJNA879725.

Assessments for phyllosymbiosis

We used Mantel tests in QIIME with default parameters to test whether host phylogeny, as measured in cophenetic genetic distances, is correlated with observed variation in microbial communities of Philippine gekkonid hosts (Caporaso et al. 2010). To generate host genetic distances, we downloaded previously published sequence data available on GenBank for the coding region of the mitochondrial NADH dehydrogenase 2 (ND2) gene for all nine host species included in this study and 10 other extant gekkonid species to improve phylogenetic resolution and to serve as appropriate outgroups (Appendix S2, Supporting Information; Siler et al. 2012). To estimate a time-calibrated phylogeny, we employed an available fossil calibration, *Yantarogekko* (Bauer et al. 2005), in divergence dating analyses (Appendix S2, Supporting Information), which is estimated to date to the Paleogene (33.9–55.8 Ma). Sequence data were aligned in MUSCLE (Edgar 2004) and trimmed to 1041 base pairs of the coding region. We used JModelTest v2.1.10 to identify the substitution model GTR + I + Γ for further use with sequence data (Darriba et al. 2012).

We estimated an ultrametric, time-calibrated topology in BEAST v2.6.3 (Bouckaert et al. 2014), using the Fossilized Birth Death Model following protocols described in Heath et al. (2014), with an initial, minimum date of 33.9 Ma (Bauer et al. 2005) set for the fossil *Yantarogekko*. To calibrate our analyses, we used a uniform prior distribution, U(33.9 and 55.8), branch-specific rates of

substitution were allowed to vary across the tree according to uncorrelated lognormal distributions (Drummond et al. 2006), with exponential prior distributions with a mean of 0.01 for the standard deviation. All remaining priors were left at default values. We ran four independent analyses of 10 million generations, logging parameter values every 1000 generations, and assessed stationarity of the analyses by plotting parameter values and likelihood scores of all four chains over generations to confirm congruence. Conservatively, we discarded the first 20% of samples from each run as burn-in and combined and summarized the remaining 8000 samples across all four independent MCMC chains in TreeAnnotator within BEAST (Figure S1, Supporting Information).

The resulting consensus chronogram was used to generate a cophenetic distance matrix via ape v5.4–1 (Paradis and Schliep 2019) in R v3.6.2 (R Core Team 2013) for use in Mantel tests to assess phyllosymbiosis in gekkonid microbiomes. We used vegan v2.5–6 (Oksanen et al. 2016) to compare cophenetic distances between the nine species sampled in this study and summarized interspecific beta diversity metrics including weighted UniFrac, unweighted UniFrac, and Jaccard distances.

Endogenous microbial community comparisons

We compared a variety of community membership metrics considering multiple ecoevolutionary lenses. For all comparisons, we first calculated alpha diversity measurements including numbers of observed OTUs, the Shannon index (Shannon 1948), and Faith's Phylogenetic Diversity (Faith's PD; Faith 1992). Alpha diversity measurements were compared using analysis of variance (ANOVA) tests in R v3.6.2 (R Core Team 2013) with the Tukey test used for post hoc analyses. ANOVA tests with Bonferroni corrections were used in QIIME to compare relative abundances of bacterial taxa between groups of interest.

Community diversity and structure were compared using principal coordinates analysis (PCoA) on beta diversity metrics including weighted and unweighted UniFrac distances (Lozupone and Knight 2005) and the binary Jaccard index (Jaccard 1901). Beta diversity matrices and PCoA plots were generated from the same rarefied datasets used to measure alpha diversity metrics. The *adonis* function in the vegan v2.3.4 package (Oksanen et al. 2016) of R v3.3.1 (R Core Team 2013) was used on beta diversity distance matrices with 999 permutations to compare community composition between groups statistically. Uncorrected *P*-values of ANOVA and *adonis* tests are presented in-text as corrected *P*-values using the Benjamini and Hochberg procedure in R v3.6.2 did not significantly change findings (Appendix S3, Supporting Information).

We analyzed bacterial composition among all 47 samples to document host-associated microbes in these species and to visualize patterns across microbial communities in Philippine gekkonids. Initial analyses grouped samples first by host species identity and then by a suite of ecoevolutionary categories to identify potential correlations with observed gut microbiomes. These schemes included grouping by host species general habitat tendencies, range extents, and host maximum body sizes. We compared species considered human commensal against those believed to be forest obligates (pers. obs.), then widespread and microendemic species, and finally hosts stemming from larger- (maximum SVL > 95 mm) vs. smaller-bodied (maximum SVL < 95 mm) species (Table 1). After such initial comparisons, we next analyzed samples as grouped by sampling locality and broader biogeographic region.

After analyzing all 47 samples included in this study concurrently, we examined microbial communities from specimens

within the genus *Gekko* ($n = 25$) exclusively to narrow the taxonomic distinctiveness between hosts in analyses and reran eco-evolutionary tests. With this subset, we compared alpha and beta diversity metrics using the same analyses between the following four groups: species identity, habitat tendencies, range extents, and maximum body sizes.

Following these interspecific comparisons within the *Gekko* genus, we next analyzed microbiomes of only *C. philippinicus* specimens ($n = 12$) to focus purely on intraspecific variability among distinct, allopatric populations. We compared both alpha and beta diversity metrics between sampling sites using the methods described above.

Finally, to lessen the influence of locality as a variable separating host species, we compared samples retrieved from three distinct collection sites: Cagbang ($n = 8$), Magsidel ($n = 17$), and Mt. Palali ($n = 11$). Here, multiple, sympatric gekkonid species were sampled. In each subset, we compared our alpha and beta diversity metrics by host species identity to ask whether sympatric species presence and overlapping interspecific ranges may mitigate the host species-specific microbial compositions generally observed in reptiles.

Results

General patterns in gekkonid microbiota

Individual phyla dominating each species' microbial communities varied by host species (Fig. 2), although three phyla, Proteobacteria (54.1%), Firmicutes (20.9%), and Bacteroidetes (16.6%), were most abundant across all rarefied reads. Philippine gekkonid samples averaged 103 OTUs per 1000 rarefied sequences, the Shannon index varied from 1.40 to 6.39 (mean = 4.19 ± 1.39), and Faith's PD varied from 3.62 to 21.78 (mean = 11.25 ± 4.20). A total of six OTUs were found across rarefied sequences from $\geq 70\%$ of all host cloacal samples, including: two *Acinetobacter* spp., *Serratia* sp., *Staphylococcus* sp., *Bacteroides* sp., and an unidentified taxon in the family Enterobacteriaceae.

Across Philippine samples from hosts in the family Gekkonidae, Mantel tests recovered no significant association between host species genetic distances and microbial assemblages as measured by any of the three beta diversity metrics examined (weighted UniFrac $r = 0.207$, $P = .438$; unweighted UniFrac $r = 0.110$, $P = .607$; Jaccard $r = 0.119$, $P = .599$).

In comparing alpha diversity metrics across Philippine gekkonid microbiome samples, we found no significant differences in the number of OTUs or the Shannon index among host species. There was, however, a significant difference between host species in Faith's PD ($F = 2.636$, $P = .021$; Figure S2A, Supporting Information). Tukey *post hoc* analyses identified the *G. mindorensis*–*L. macgregori* and *C. philippinicus*–*L. macgregori* pairwise comparisons as likely driving such differences. Grouping samples by host habitat preferences (human commensal or forest obligate) and maximum body size (larger or smaller) failed to retrieve significant differences in any alpha diversity metrics. In comparing widespread and microendemic samples, significant differences were noted in observed OTUs (mean widespread = 120.11, microendemic = 80.95; $F = 8.225$, $P = .006$), Shannon index ($F = 5.467$, $P = .024$), and Faith's PD ($F = 8.866$, $P = .005$; Figure S3B, Supporting Information). Differentiating by individual host sampling locality and biogeographic region both showed significant distinction in number of observed OTUs (locality $F = 3.672$, $P = .005$; region $F = 5.464$, $P = .003$) and in Faith's PD (locality $F = 4.301$, $P = .020$; region $F = 6.021$, $P = .016$), but not in the Shannon index (Figures S2B and S3A, Supporting Information).

We found strong, significant associations between host species and microbial compositions based on all three beta diversity metrics examined (weighted UniFrac $R^2 = 0.310$, $P = .005$; unweighted UniFrac $R^2 = 0.285$, $P = .001$; Jaccard $R^2 = 0.257$, $P = .001$; Fig. 3). Adonis comparisons of beta diversity metrics showed no differences in human commensal and forest obligate groupings. Significant, yet weak, differences in two beta diversity metrics were found by widespread and microendemic species distribution patterns (unweighted UniFrac $R^2 = 0.060$, $P = .001$; Jaccard $R^2 = 0.051$, $P = .001$) and in the Jaccard index between maximum body size conditions ($R^2 = 0.029$, $P = .043$). Correlations between host locality and microbial communities were found in all three metrics (weighted UniFrac $R^2 = 0.208$, $P = .028$; unweighted UniFrac $R^2 = 0.209$, $P = .001$; Jaccard $R^2 = 0.201$, $P = .001$). Slightly weaker results were recovered in grouping cloacal samples by source host biogeographic region as opposed to specific locality (weighted UniFrac $R^2 = 0.132$, $P = .021$; unweighted UniFrac $R^2 = 0.119$, $P = .001$; Jaccard $R^2 = 0.111$, $P = .001$).

Endogenous microbiota across geckos in the genus *Gekko*

We collected samples from five *Gekko* species at five distinct sites across the Philippines: Cagbang, Magsidel, Mt. Palali, Nasiping, and Tapao Falls (Table 1 and Fig. 1). We only sampled one species of *Gekko* per site and, as such, host species and host locality are confounded in subsequent analyses here and only host species comparisons are included. Microbial compositions within samples from species of *Gekko* varied by host taxon with consistent, high prevalence of Proteobacteria, Firmicutes, and Bacteroidetes (Figs 2 and 4). No OTUs were found to vary in relative abundance across all five host species in the genus *Gekko*. Only nine OTUs were found in $\geq 70\%$ of all rarefied samples from these hosts although within species, a greater number of shared OTUs was common (Appendix S4, Supporting Information). For instance, 24 OTUs were found in all *G. mindorensis* samples while 13 OTUs were identified in all *G. gekko* samples, four in all *G. crombota* samples, and four in most ($\geq 85\%$) *G. rossi* samples.

We found no significant difference in the number of OTUs between host species nor a difference in the Shannon index, though there was significant differentiation in Faith's PD between *Gekko* species ($F = 3.287$, $P = .032$). *Post hoc* analyses indicated this significance was driven by the *G. rossi*–*G. mindorensis* pairwise comparison ($P = .013$; Figure S4A, Supporting Information). Grouping by host range extents and host body size classes, respectively, found significant differences in observed OTUs (widespread mean = 130.22, microendemic = 92.00; $F = 5.198$, $P = .032$; larger body mean = 96.10, smaller = 144.40; $F = 5.909$, $P = .023$; Figure S5A, Supporting Information) and Faith's PD ($F = 6.004$, $P = .022$; $F = 9.846$, $P = .005$; Figure S5B, Supporting Information). No significant differences were found when grouping by forest obligates and human commensals.

Microbial community composition varied significantly by host species in the unweighted UniFrac and Jaccard index metrics ($R^2 = 0.270$, $P = .001$; $R^2 = 0.256$, $P = .001$; Fig. 4), but not in the weighted UniFrac metric. Aside from host species identity, multiple other ecoevolutionary factors showed statistically significant, yet weaker differences between grouping schemes in the unweighted UniFrac and Jaccard distance metrics. These included grouping by species distribution patterns ($R^2 = 0.076$, $P = .014$; Jaccard $R^2 = 0.073$, $P = .003$) and host maximum body size (unweighted UniFrac $R^2 = 0.104$, $P = .001$; Jaccard $R^2 = 0.080$, $P = .001$). Grouping by broad-habitat associations only recovered significant, yet weak, distinctions in the Jaccard metric ($R^2 = 0.064$, $P = .009$).

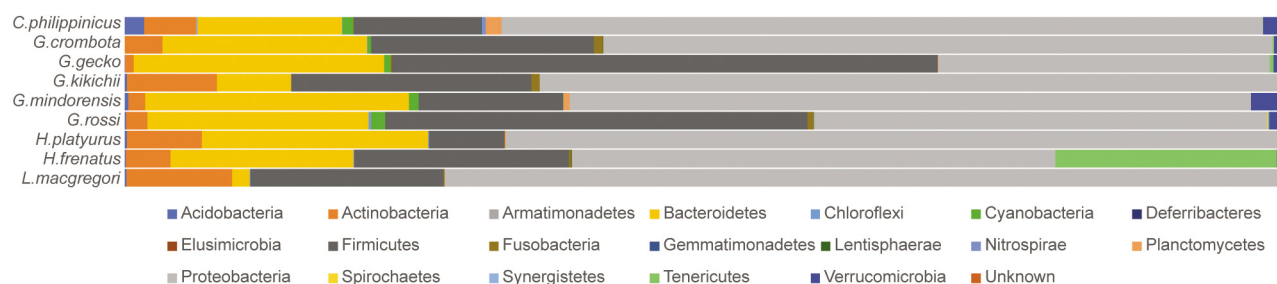


Figure 2. Stacked barplot of average gut microbiome compositions by phyla across Philippine gekkonid hosts.

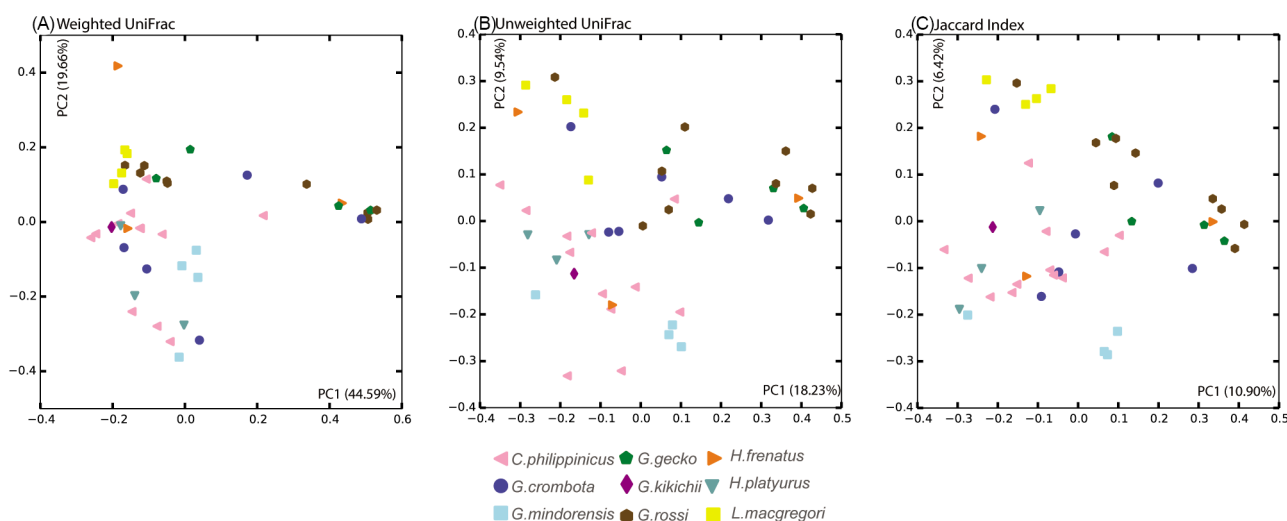


Figure 3. PCoA plots of gut microbiomes from gekkonid hosts as measured by beta diversity metrics including (A) weighted UniFrac, (B) unweighted UniFrac distances, and (C) Jaccard Index.

Endogenous microbiomes at the species level in *C. philippinicus*

We sampled gut microbial communities in 12 *C. philippinicus* specimens at four distinct sites, three on Luzon Island and another on Negros Island, to assess intraspecific variability in host microbiomes at discreet sampling localities (Table 1 and Fig. 1). Phyla dominating microbial compositions in *C. philippinicus* hosts differed by locality, with Proteobacteria always most common (Fig. 5). Firmicutes, Bacteroidetes, and Actinobacteria comprised most of the remaining reads though proportions in individual hosts varied by site (Fig. 5). Just three specific OTUs were shown to differ statistically between localities including *Ochrobactrum* sp., an unidentified taxon in the order Bacillales, and another in the family Bacteriovoraceae (Appendix S5, Supporting Information). A total of three OTUs were found in all *C. philippinicus* specimens sampled, two *Acinetobacter* spp. and a *Serratia* sp., while 13 OTUs were found in $\geq 70\%$ of *C. philippinicus* hosts.

The number of OTUs per 1000 rarefied sequences did not vary significantly between sites, neither did the Shannon index, nor Faith's PD. PCoA revealed a degree of clustering by locality in weighted and unweighted UniFrac measures and pronounced grouping in Jaccard distances. Adonis tests found significant differentiation by locality in all three beta diversity metrics (weighted UniFrac $R^2 = 0.513$, $P = .002$; unweighted UniFrac $R^2 = 0.350$, $P = .035$; Jaccard $R^2 = 0.362$, $P = .001$; Fig. 6), suggesting distinct microbial compositions between sampling sites. Grouping host-associated microbiota by host biogeographic region rather than specific host locality produced similar, though

weaker, results in weighted UniFrac ($R^2 = 0.335$, $P = .014$), unweighted UniFrac ($R^2 = 0.241$, $P = .035$), and Jaccard metrics ($R^2 = 0.245$, $P = .008$).

Locality-specific assessments of microbial inventories in gekkonids

A total of three sites yielded samples from multiple, sympatric gekkonid species: Cagbang, Magsidel, and Mt. Palali. At Cagbang, on Negros Island, we sampled *C. philippinicus* ($n = 2$), *G. gecko* ($n = 4$), *H. frenatus* ($n = 1$), and *H. platyurus* ($n = 1$) hosts (Table 1 and Fig. 1). There was a high degree of intraspecific variation within host microbial compositions at this site (Figure S6, Supporting Information). Most microbiomes were dominated by Proteobacteria, Firmicutes, and Bacteroidetes, except for our single *H. frenatus* sample at this site, with a high proportion of Tenericutes (Figure S6, Supporting Information). We found no significant difference in microbial community structure between host species based on alpha diversity metrics measured. Despite limited sampling sizes, clustering was apparent in PCoA plots with strong, significant distinction in community composition between host species in weighted UniFrac ($R^2 = 0.678$, $P = .0200$), unweighted UniFrac ($R^2 = 0.566$, $P = .008$), and Jaccard distances ($R^2 = 0.534$, $P = .004$; Figure S7, Supporting Information).

At Magsidel, in the Babuyan Island chain, both *G. rossi* ($n = 9$) and *L. macgregori* ($n = 4$) were sampled with marked variability apparent among individual *G. rossi* compositions, where some samples were dominated by Bacteroidetes, others Firmicutes, and others still Proteobacteria (Figure S6, Supporting Information). Cloa-

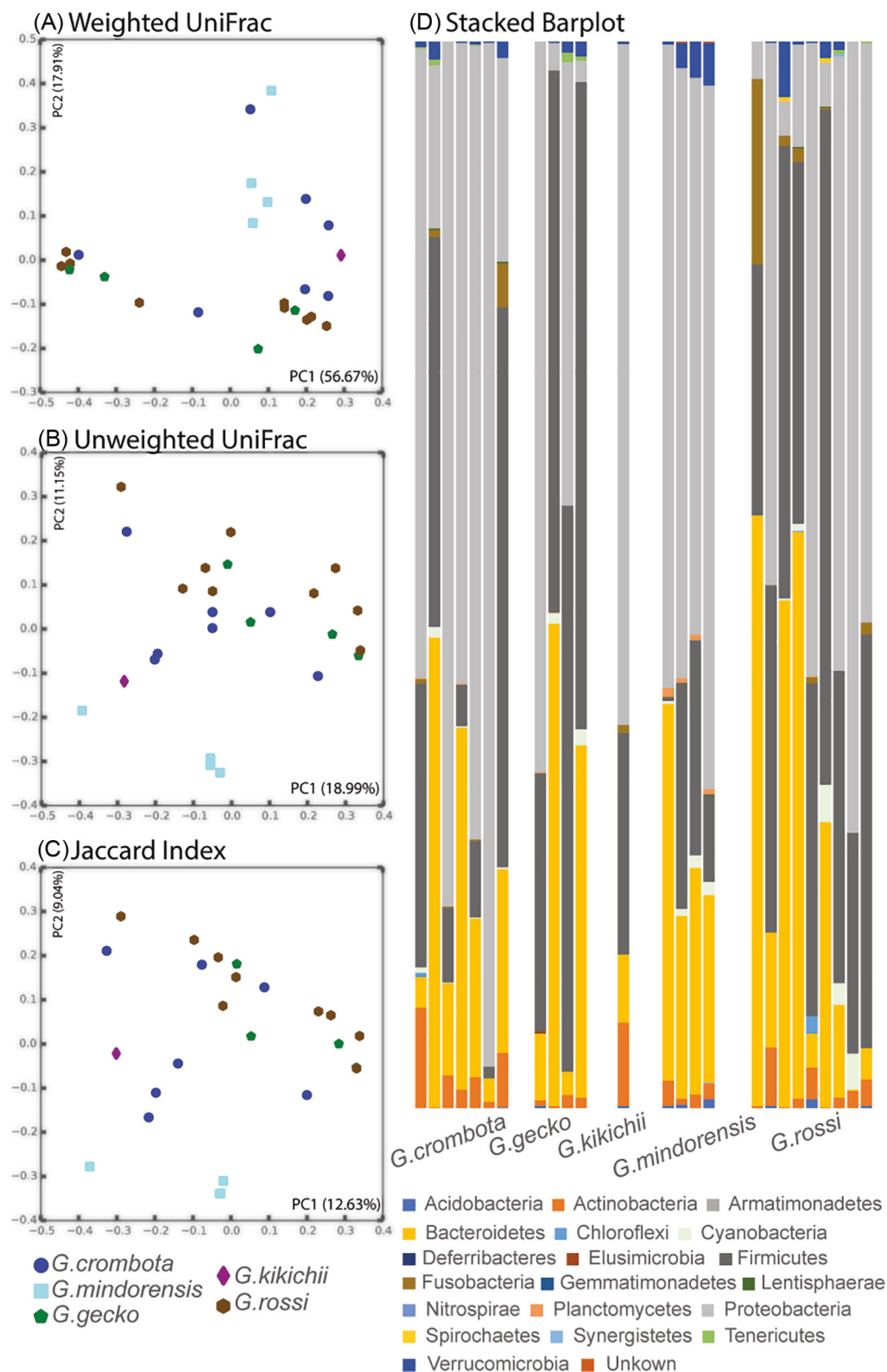


Figure 4. PCoA plots of beta-diversity metrics from geckos in the genus *Gekko* (A)–(C). Stacked barplot (D) of individual microbial compositions of hosts, grouped by taxonomic identity.

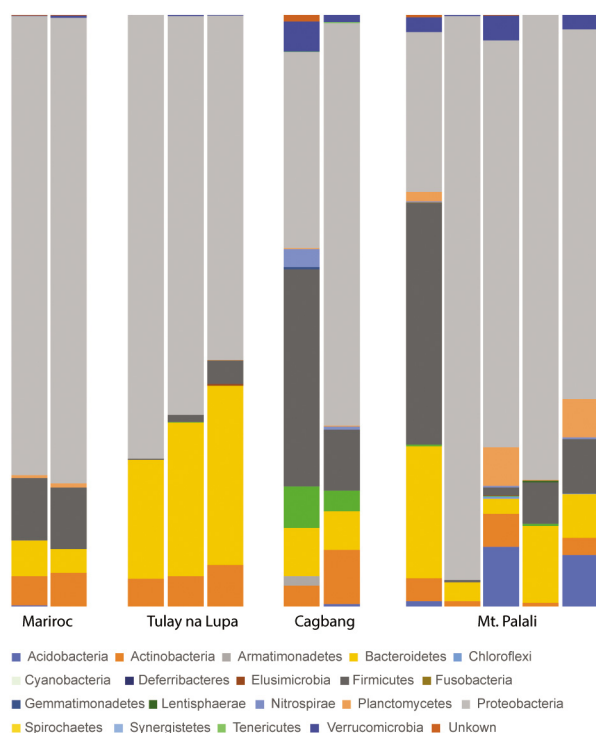


Figure 5. Microbiome compositions at the phylum level from *C. philippinicus* hosts grouped by sampling locality.

cal samples from *L. macgregori* hosts were composed predominantly of Proteobacteria followed by Firmicutes (Figure S6, Supporting Information). Significant differences were found in the number of OTUs observed per 1000 sequences between each host species (*G. rossi* mean = 86.78, *L. macgregori* mean = 36.75; $F = 5.308$, $P = .042$), Shannon index ($F = 6.787$, $P = .025$), and Faith's PD ($F = 5.732$, $P = .036$). Grouping in PCoA plots was unclear, with insignificant differentiation between species in weighted UniFrac measures and significant, yet weak distinctions in unweighted UniFrac ($R^2 = 0.179$, $P = .009$) and Jaccard distances ($R^2 = 0.145$, $P = .008$; Figure S7, Supporting Information).

Finally, we sampled *C. philippinicus* ($n = 5$), *G. mindorensis* ($n = 4$), and a lone *H. frenatus* specimen at Mt. Palali on Luzon Island. Proteobacteria dominated gut microbial communities in geckos sampled on Mt. Palali followed in relative abundance by Firmicutes then Bacteroidetes across all host species (Figure S6, Supporting Information). At this site, no significant differences in alpha diversity metrics were recorded between host species. Statistically significant community clusters between host taxa were most clear in weighted UniFrac composition plots ($R^2 = 0.481$, $P = .015$), unweighted UniFrac ($R^2 = 0.294$, $P = .023$), and Jaccard distance ($R^2 = 0.288$, $P = .007$) clusters were more ambiguous (Figure S7, Supporting Information).

Discussion

This study provides baseline information on symbiotic gut microbes in wild Philippine gekkonids and points toward several eco-evolutionary forces shaping such compositions. At the family level in Philippine gekkonids, we found limited evidence of phyllosymbiosis or host evolutionary history strongly reflecting current microbial compositions. Although previous studies have noted that evolutionary history influences gut microbial communities at various taxonomic levels (Groussin et al. 2017, Ley et al. 2008, Sanders

et al. 2014, Youngblut et al. 2019), such impacts may be unevenly distributed across taxonomic groups. For instance, Youngblut et al. (2019) found that evolutionary history had a stronger effect on intestinal microbiome diversity in mammals than in nonmammalian species. Ren et al. (2016) noted only weak associations between host genetic distances and microbial assemblages in congeneric reptiles of the genus *Anolis*. High degrees of intraspecific variation in host-associated microbiomes may explain the lack of evidence for phylogenetic past reflecting contemporary community compositions here and in other reptile hosts (Brooks et al. 2016, Ren et al. 2016). At the scale of host family, various ecoevolutionary factors outside of phylogenetic histories likely serve pivotal roles in shaping and maintaining gut microbial communities in gecko hosts from the Philippines (Kropackova et al. 2017, Lutz et al. 2019).in host-associated gut microbiomes

Of all ecoevolutionary factors examined, we found that observed host-associated microbial assemblages were most correlative with host species, as seen previously in other reptile groups (Kohl et al. 2017, Lankau et al. 2012, Ren et al. 2016). Sampling locality and broader biogeographic zones irrespective of host species identity were also significantly associated with microbial assemblages, agreeing with previous findings (Lankau et al. 2012, Ren et al. 2016). Within our interspecific comparisons, we note that observed patterns may be at least partially confounded with host species due to uneven opportunistic sampling of wild gekkonid specimens (Table 1 and Fig. 1). Additional sampling efforts that capture spatial variation within and between species would help clarify such conclusions. However, even with limited sampling sizes at select localities, the variation in host-associated assemblages observed in *C. philippinicus* samples across multiple sites (Fig. 6) suggests that individual host locality does influence gut microbiomes significantly within species.

The site specificity seen in compositions from *C. philippinicus* specimens at discrete locations shows that locality can and does influence observed intraspecific variation in microbial compositions (Fig. 6). Site-specific factors that alter these compositions need further investigation in wild hosts (Ren et al. 2016). Preliminary evidence suggests that differences in individual diet at least in captive reptiles may be responsible for marked intraspecific variation in host-associated gut microbiomes (Fong et al. 2020, Jiang et al. 2017). Studies on specific microhabitat tendencies and improved ecological knowledge on host species are needed to better understand drivers of gut microbiome formation and maintenance.

All gecko species included in this study have only broad ecological data available (Brown et al. 2008, 2009, 2010, 2011, 2013b, Welton et al. 2010). The categorizations used in this investigation failed to recover much differentiation based on ecological traits; however, it is possible that more fine-scale ecological partitioning would prove intuitive (Lankau et al. 2012, Ren et al. 2016). Comparative studies of widespread and microendemic species offer promising avenues for more targeted testing, as alpha and beta diversity metrics were significantly distinct between groups (Figure S3B, Supporting Information). Widespread species sampled in this study showed greater OTU diversity, Shannon Index values, and Faith's PD as compared to microendemic counterparts. They also displayed distinct communities in the unweighted UniFrac and Jaccard metrics as compared to microendemic counterparts, suggesting differentiation in rare OTU presence. This significance could be preliminary evidence for a valid ecological phenomenon in which widespread and microendemic hosts exhibit distinct strategies in harboring endogenous microbiome compositions. Internal microbial communities are critical facets of organismal

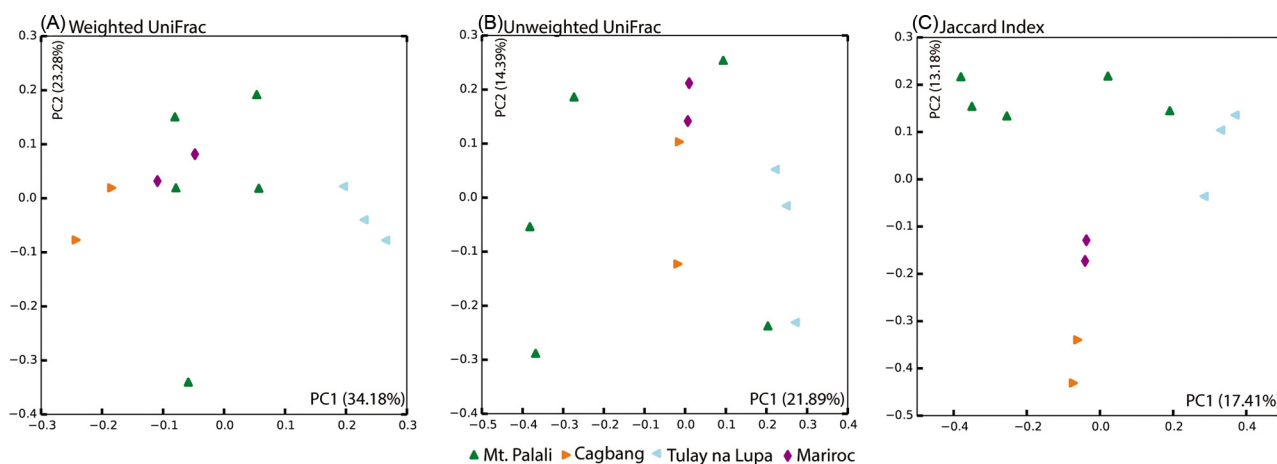


Figure 6. PCoA plots of *C. philippinus* samples designated by sampling locality.

adaptability to novel environments changes in habitat (Amato 2013, Stumpf et al. 2016, Trevelline et al. 2019), and the increased diversity presence or transient microbe acquisition may play a role in widespread species' capacities to persist in novel or changing habitats (Alberdi et al. 2016). Additional insight into the functional capacity of reptile microbiomes and the way endogenous microbiota influence adaptive capacity of hosts is of critical importance for conservation considerations of reptile species in the future (Brooks et al. 2016, Colston and Jackson 2016, Littleford-Colquhoun et al. 2019, Trevelline et al. 2019).

Here, we expand upon what is known on endogenous microbial communities in wild reptiles and identify a suite of contemporary and historical influences that structure such compositions using gekkonids from across the Philippine archipelago. We found no correlations between host genetic distances and observed microbial compositions, suggesting a muted influence of evolutionary history on present variation in Philippine gekkos. Despite this, host species was consistently the greatest determinate in microbial assemblages with marked intraspecific variation observed based on sampling locality. Although these results suggest that contemporary ecological traits may play a more central role than do evolutionary pasts in the maintaining of enteric microbial diversity in gekkonid hosts, future research investigating these factors more precisely in wild specimens remains essential.

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