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HOST MICROBE INTERACTIONS

Bamboo Specialists from Two Mammalian Orders (Primates, Carnivora) Share a High Number of Low-Abundance Gut Microbes

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Abstract

Bamboo specialization is one of the most extreme examples of convergent herbivory, yet it is unclear how this specific high-fiber diet might selectively shape the composition of the gut microbiome compared to host phylogeny. To address these questions, we used deep sequencing to investigate the nature and comparative impact of phylogenetic and dietary selection for specific gut microbial membership in three bamboo specialists—the bamboo lemur (*Hapalemur griseus*, Primates: Lemuridae), giant panda (*Ailuropoda melanoleuca*, Carnivora: Ursidae), and red panda (*Ailurus fulgens*, Carnivora: Musteloideadae), as well as two phylogenetic controls—the ringtail lemur (*Lemur catta*) and the Asian black bear (*Ursus thibetanus*). We detected significantly higher Shannon diversity in the bamboo lemur (10.029) compared to both the giant panda (8.256; $p = 0.0001936$) and the red panda (6.484; $p = 0.0000029$). We also detected significantly enriched bacterial taxa that distinguished each species. Our results complement previous work in finding that phylogeny predominantly governs high-level microbiome community structure. However, we also find that 48 low-abundance OTUs are shared among bamboo specialists, compared to only 8 OTUs shared by the bamboo lemur and its sister species, the ringtail lemur (*Lemur catta*, a generalist). Our results suggest that deep sequencing is necessary to detect low-abundance bacterial OTUs, which may be specifically adapted to a high-fiber diet. These findings provide a more comprehensive framework for understanding the evolution and ecology of the microbiome as well as the host.

Keywords Gut microbiome · Convergent evolution · Feeding strategy · Bamboo specialist · Host-microbiome relationship

Background

Host phylogeny and diet have been established as the two major forces that govern gut microbiome (GM) composition, yet no unifying framework has been proposed to reconcile the effects of phylogeny and diet on GM community assembly. We address this gap by comparing three species of mammal with differing levels of phylogenetic proximity that share a highly derived dietary specialization: bamboo. Two species are distantly related members of the order Carnivora (the giant panda, *Ailuropoda melanoleuca*, and the red panda, *Ailurus fulgens*; diverged 47.5 my [1]) and one is a member of the order Primates (the bamboo lemur, *Hapalemur griseus*, diverged from Carnivora 83 my [2]). By comparing the composition of their GM, we can investigate the similarities and differences in GM composition; some of which will be due to phylogenetic proximity and some of which will be due to diet (i.e., bamboo, in this case). To further examine the effects of diet versus phylogeny, we also compare the bamboo specialists to two phylogenetic controls: the Asian black bear (*Ursus thibetanus*, omnivorous, diverged from the giant panda by 19.5 mya [1]; data from Li et al. [3]) and the bamboo lemur's sister species, the ringtail lemur (*Lemur catta*, a generalist, diverged from the bamboo lemur 11.8 mya [4]; data from McKenney et al. [5]). A recent investigation of GM succession in lemurs detected host species-specific signatures and increased diversity associated with both gastrointestinal

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tract (GIT) complexity and dietary fiber intake [5]. In that study, the folivorous (leaf-eating) lemur, Coquerel's sifaka (*Propithecus coquereli*), exhibited the lowest inter-individual variation, suggesting that the GM is tightly regulated by traits associated with a high-fiber diet (i.e., gut complexity or nutrient availability) in these primates.

Previous studies have revealed remarkable GM convergence that mirrors host diet. For example, ant- and termite-eating mammals show distinct and consistent similarities in their GM, despite vast phylogenetic distance between host species [6]. Diet-induced changes and similarities have been detected in GM communities across mammalian species [7, 8], even driving human GMs to resemble more herbivorous or carnivorous states [9]. Most herbivores have evolved a variety of adaptations to accommodate the challenges associated with consuming high-fiber diets. For example, increased gut complexity and GM diversity are both associated with herbivory and consumption of dietary fiber [10, 11].

The three bamboo specialists targeted by the current study additively span more than 150 my across the phylogenetic spectrum of mammals and represent three independent branches across the phylogeny of mammals: Primates, Ursidae, and Musteloideadae [12] (Fig. 1). These three species also present GIT characteristics that are unique

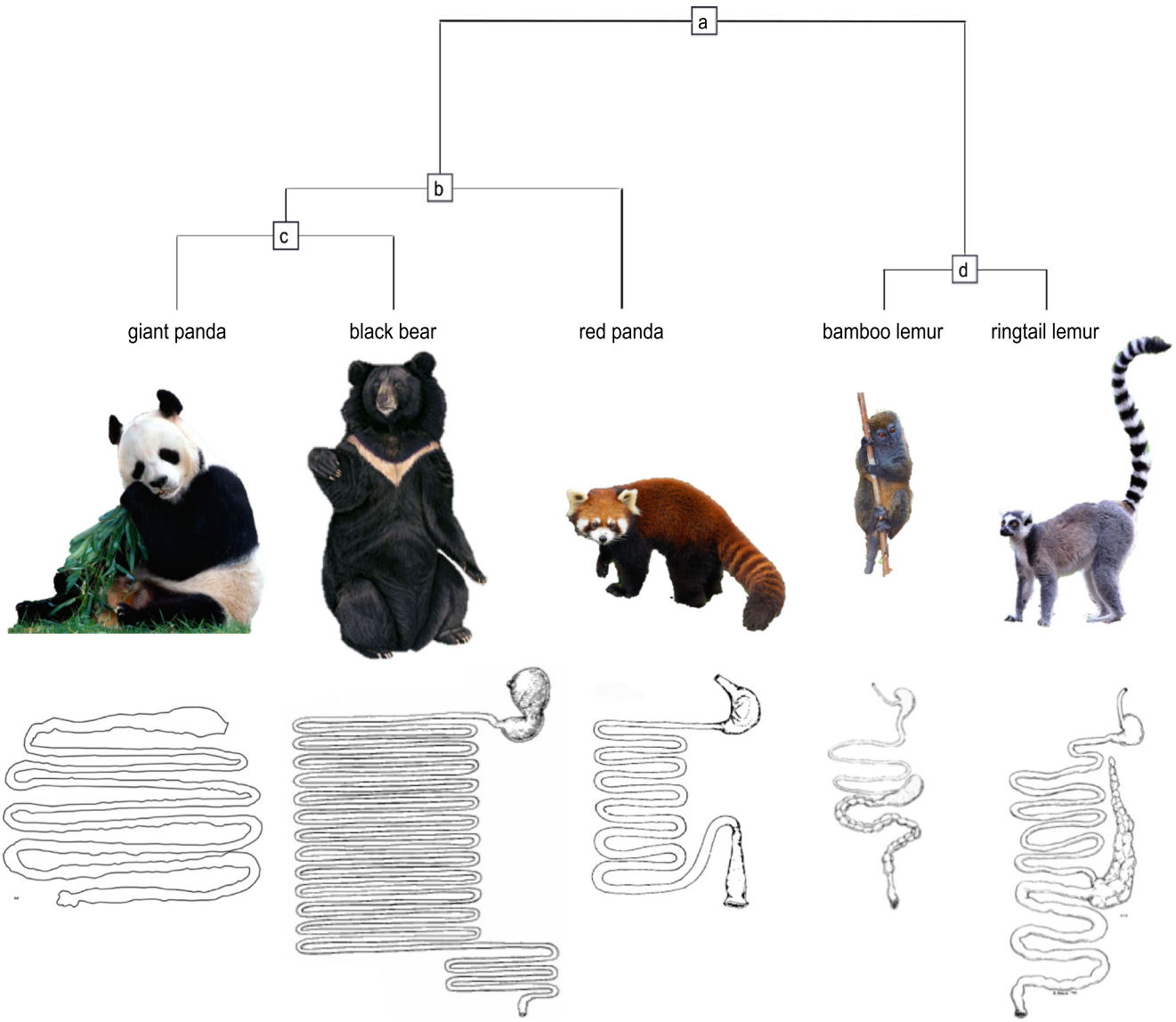


Fig. 1 Bamboo consumption is associated with simple gastrointestinal morphology, and spans the mammalian phylogeny. Divergence times at each node are (a) 83 my [2], (b) 47.5 my [1], (c) 19.5 my [1], and (d) 11.8 my [4]. The giant panda (*Ailuropoda melanoleuca*) gut diagram is adapted from [13]. The Asian black bear (*Ursus thibetans*) and the red panda (*Ailurus fulgens*) guts are represented by the American black bear and the common raccoon [10], respectively. These "surrogate" species are closely related and considered to have a similar gut morphology to the focal species. The bamboo lemur (*Hapalemur griseus*) and ringtail lemur (*Lemur catta*) gut diagrams are adapted from [14]

among herbivores. While herbivores generally exhibit increased gastrointestinal complexity and increased transit time to accommodate high-fiber diets, the bamboo specialists share an overall lack of gastrointestinal complexity and comparatively short total gastrointestinal lengths (Table 1). This apparent lack of morphological adaptation for folivory makes these three hosts an attractive system for comparing the impact of fiber-rich diet versus phylogeny on GM communities. The lack of morphological adaptation to diet might suggest that GM composition will be especially important in these host species for adequately digesting their respective high-fiber diets.

Most studies to date have been unable to detect a strong dietary signature in giant panda GMs [3, 20–22]. In addition to retaining short, simple, carnivore-like gastrointestinal tract, the giant panda harbors extremely low GM diversity [20, 21] dominated by Firmicutes and Proteobacteria [20, 21, 23], with high seasonal variation [21] and limited functional capacity to metabolize cellulose [20]. Moreover, comparative studies have revealed an overwhelming lack of GM convergence between the giant panda and other herbivores, perhaps due to the power of host phylogeny as a driving selective force. In a comparison of GMs isolated from 56 mammalian species, giant pandas clustered with other bears instead of with herbivores [7]. Li et al. also found that the giant panda GM is more similar to that of the Asian black bear than it is to the red panda [3], further suggesting that phylogenetic signal overrides dietary signal in this case.

While Li et al. were the first to compare gut microbes in bamboo specialists using next-generation sequencing [3], relatively low coverage may have precluded detection of low-abundance diet-associated OTUs in previous studies of giant panda and red panda GMs. We predict that deeper sequencing coverage will enable the detection of low-abundance OTUs comprising the "rare biosphere" [24], perhaps revealing a higher GM diversity and more fiber-adapted taxa than have previously been appreciated.

Table 1 Gut length and transit times in bamboo specialists and lemurs.

Species	Feeding strategy	Gut length to body length ratio	Transit time (min)
Giant panda	Bamboo	4[a]	480 ± 90[d]
Red panda	Bamboo	4[b]	225 ± 51[e]
Bamboo lemur	Bamboo	4.1[c]	1836 ± 78[f]
Ringtail lemur	Generalist	5.8[c]	387 ± 76[g]
Coquerel's sifaka	Folivore	15.5[c]	1465.2 ± 102[f]

Previously published measurements from [a] [13], [b,c] [15], [d] [16], [e] [17], [f] [18], and [g] [19]

Indeed, Ochman et al. suggested that sequencing coverage $\geq 10^4$ may be necessary to unmask evolutionary features of GM community structure [25]. To test the *coverage hypothesis*, we analyzed and compared previously published data sequenced on the Roche 454 platform (1280–6879 seqs/sample [3]) with a new data set sequenced on the Ion Torrent platform (66,644–139,554 seqs/sample).

The depth of sequencing coverage produced by our study also offers a novel perspective on the effects of phylogeny versus selection in this highly specialized dietary niche. If convergent evolution shapes the rare biosphere in response to dietary pressures, then bamboo specialists should exhibit shared GM membership despite the phylogenetic distance among the host species. We therefore predicted that diet and phylogeny shape different classes of GM membership. That is, we expect to identify different bacterial taxa, present at different levels of abundance, associated with feeding strategy versus evolutionary history. To test the *differential membership hypothesis*, we compared GM membership across bamboo specialists and their omnivorous phylogenetic controls, to identify four classes of membership: those present in all 12 animals (shared by all), core taxa shared by all nine bamboo specialists, core taxa shared by all seven lemurs, and species-specific OTUs.

Methods

Animals

Bamboo lemurs ($n = 4$) and ringtail lemurs ($n = 3$) were housed at the Duke Lemur Center in Durham, North Carolina. Red pandas ($n = 2$) and giant pandas ($n = 3$) were housed at the Smithsonian Conservation Biology Institute (Front Royal, VA) and the Smithsonian National Zoological Park (Washington, D.C.), respectively. Individual sex and age at time of sampling are listed in Table S1. All animals were born in captivity, and only one individual had a history of antibiotic treatment: a female bamboo lemur, given a 5-day course of thiabendazole for internal parasites, 10 months prior to sampling (Table S1). All animals were healthy, and no individuals were experiencing any kind of gastric disturbance (i.e., mucoidal episodes in giant pandas) at time of sampling. All three species are fed similar diets in captivity (bamboo, supplemented with produce and commercially formulated biscuits; Table 2). Bamboo lemurs were housed in pairs, in indoor/outdoor pens with 146 m^2/animal. Giant pandas have access to 0.5-acre yards (~2023 m^2 per animal), and red pandas are housed in outdoor enclosures (~50 m^2 per animal, and 5 m high). Lemur and giant panda indoor enclosures are hosed daily to remove waste. Red panda waste is collected from a sand-mulch substrate.

Table 2 Representative diets offered to captive bamboo specialists. The weight (kg) and percentage of each item is listed, as fed

	Species								
	Giant panda			Red panda		Bamboo lemur			
Food item	G1	G2	G3	R1	R2	B1	B2	B3	B4
Bamboo[a]	21 kg (96.8%)	30 kg (93.3%)	30 kg (93.5%)	2.7 kg (90.0%)	1.8 kg (88.7%)	0.2 kg (78.4%)	0.2 kg (78.4%)	0.2 kg (70.2%)	0.15 kg (68.2%)
Leaf eater food, gorilla[b]	0.1 kg (0.5%)	0.9 kg (2.8%)	1.2 kg (3.7%)	0.2 kg (6.7%)	0.2 kg (9.9%)				
Leaf eater food, lemur[b]	0.3 kg (1.4%)					0.025 kg (9.8%)	0.025 kg (9.8%)	0.035 kg (12.3%)	0.03 kg (13.6%)
Vegetable mixture[c]	0.3 kg (1.4%)	0.75 kg (2.3%)	0.6 kg (1.9%)			0.015 kg (5.9%)	0.015 kg (5.9%)	0.025 kg (8.8%)	0.02 kg (9.1%)
Fruit rotation[d]		0.5 kg (1.6%)	0.3 kg (0.9%)	0.1 kg (3.3%)	0.03 kg (1.5%)	0.015 kg (5.9%)	0.015 kg (5.9%)	0.025 kg (8.8%)	0.02 kg (9.1%)

[a] Bamboo is primarily *Phyllostachys* spp

[b] Marion Zoological, Plymouth, MN; nutrient content the same

[c] Consists of corn, cucumber, celery, sweet potato, and/or carrot

[d] Consists of apple, melon, grapes, and/or banana

Fecal Samples

A single, fresh stool sample was collected opportunistically from each individual and immediately stored at – 80 °C in an individually labeled Whirlpak bag (Nasco, Fort Atkinson, WI) to prevent microbial reproduction and DNA degradation within feces. Prior to extraction, the exterior of each frozen sample was removed to prevent environmental contamination. DNA was extracted from each sample separately using the QIAamp Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. DNA yields were quantified using a Qubit 3.0 (ThermoFischer Scientific, Massachusetts, USA).

16S rRNA Gene Library Prep and Sequencing

Six hypervariable regions of the 16S rRNA gene were amplified using the Ion 16S Metagenomics Kit large tube (1.5-mL Eppendorf tube) protocol. This kit performs two reactions for each sample, using two primer sets. The first primer set targets the V2, V4, and V8 regions of the 16S rRNA gene; the second primer set targets the V3, V6, and V9 regions. Amplified reads from each region are approximately 250, 288, 295, 215, 260, and 209 bp, respectively. Purified PCR products from each sample were pooled and libraries were prepped using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific, Grand Island, NY). After nick repair, PCRs were purified with Ampure XP (Beckman Coulter, Indianapolis, IN). Unique barcode adapters were ligated, an additional nick repair step was run, and the library was purified a second time using Ampure XP. Ligation is performed on blunt ends and fragments from all variable regions are similar lengths, so this step is not expected to introduce bias. DNA from *E. coli* and distilled water were also prepared and sequenced to provide positive and negative controls, respectively. Library concentration was determined using qPCR. A total of 9 libraries (plus positive and negative controls) were pooled on a 318 chip and sequenced on the Ion PGM platform using the Ion PGM Sequencing 400 Kit (Thermo Fisher Scientific, Grand Island, NY).

Data Analysis

Ion Torrent sequencing data are available in the NCBI Sequence Read Archive under accession numbers SAMN06347171–SAMN06347179. We compared our Ion Torrent data to two previously published 16S rRNA gene data sets. McKenney et al. [5] used the Illumina MiSeq platform to sequence the v4 region in feces collected from three ringtail lemurs (Sequence Read Archive accession numbers SAMN02689938–02689939 and SAMN02689974–02689977). Li et al. [3] compared V1–V3 regions amplified from six captive red pandas, five giant pandas, and six Asian black bears and sequenced using the 454 GS FLX Titanium

platform (Sequence Read Archive accession numbers SRR2584945–2584949, and SRR2584966–2584977). Although comparisons across different sequencing platforms and protocols need to be treated with caution, there is value in comparing the results (i.e., taxonomic classifications of OTUs detected) from these different analytical frameworks for consistency or conflict. Together, these two omnivorous phylogenetic controls provide an opportunity to address the question of whether there is shared membership across bamboo specialists' microbiomes.

We used Quantitative Insights Into Microbial Ecology (QIIME v1.9.1) for all data analysis. All scripts necessary to reproduce the analytical workflow are available in Additional File 1. We demultiplexed Ion Torrent, Illumina, and Roche 454 [3] sequencing data separately to reflect the error models associated with different platforms. We filtered Ion Torrent reads with minimum quality score < 20 and read length < 150 bp to reflect the PGM error model used in the Metagenomics 16S workflow (v4.6) available in the Ion Reporter Software package (v5.0). Illumina sequencing data were demultiplexed by individual and filtered using default parameters, as described previously [5]. Roche 454 data were filtered to remove reads < 200 bp in length and any sequences with ambiguous bases to reproduce previously published filtering methods [3]. QIIME removes barcodes and primers by default during the initial demultiplexing step.

Singletons were removed and genus-level operational taxonomic units (OTUs) were picked based on 97% sequence similarity, using the closed reference UClust [26] option in QIIME. The closed reference method classifies OTUs at the genus level using a pre-defined taxonomy map of reference sequences, thus enabling the comparison of non-overlapping amplicons as described previously [6]. Chloroplasts were filtered from each OTU table, and filtered OTU tables were merged into a single file for downstream analyses.

We plotted rarefaction curves (Fig. S1) and calculated Good's coverage to compare coverage across libraries (Table S2). Beta diversity was quantified using UniFrac distance metrics. We used jackknifed UPGMA clustering to produce sample trees and Principle Coordinate Analysis (PCoA) plots, with biplots to visualize relationships between bacterial membership and host metadata. To test whether discrepancies in sequencing coverage impacted the PCoA, we performed jackknifing twice: first with the full data set (default settings) and second by subsampling each library to a depth of 1280 to match the number of sequences in the smallest library (see Table S2). We calculated two measures of alpha diversity: Shannon diversity, which summarizes community complexity based on the number and frequency of OTUs, and phylogenetic diversity (PD), which incorporates the evolutionary distance between OTUs.

To compare the impact of host diet and phylogeny on OTU membership, we used in-house scripts with Boolean logic statements to identify core taxa shared by all nine bamboo specialists, core taxa shared by all seven lemurs, and species-specific OTUs (present in n members of each species, but $< n$ members of any other species).

Statistical Analysis

We performed an analysis of variance (ANOVA) with post hoc Tukey honestly significant difference to test for differences in diversity data associated with host species and diet. We used a generalized linear model (GLM) approach to test for differences resulting from gut transit time (see Table 1) to accommodate time as a continuous variable. We used linear discriminant analysis effect size (LEfSe; [27]), with default settings and all alpha values set to 0.05, to detect significantly enriched bacterial OTUs in each host species (class = host species; no subclass; sample = SampleID). LEfSe uses a Kruskall-Wallis test to detect differentially distributed OTUs within each class. The enriched OTUs are then ranked by the log of their linear discriminant analysis scores.

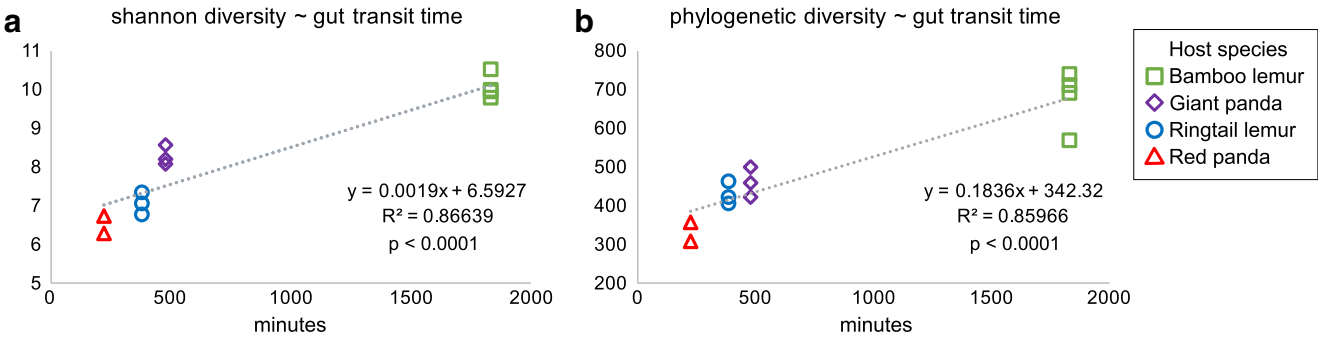
Data Availability Statement The nucleotide sequence data reported are available in the NCBI Sequence Read Archive database under the accession numbers SAMN06347171-SAMN06347179.

Results

While neither Shannon nor ($p = 0.101$) nor PD ($p = 0.325$) was significantly different between bamboo and generalist diets, both measures are significantly and positively correlated with increased gut transit time ($p < 0.0001$; Fig. 2). Shannon diversity measures both bacterial richness (number of taxa) and evenness (distribution of organisms across the taxa), while PD measures the minimum branch length necessary to include all bacterial taxa in each community [28]. We find that bamboo lemurs harbor significantly higher PD than red pandas ($p = 0.00036$) and significantly higher Shannon diversity than any other host species compared in this study (see values in Fig. 2), indicating that not only do bamboo lemurs' gut microbes span more phylogenetic branches than other hosts' GMs, but also that bamboo lemurs' diverse OTUs are more evenly represented.

Support for the Coverage Hypothesis

We find that increased sequencing coverage increases observed diversity and low-abundance taxa. While the rarefaction curves did not completely flatten for either Illumina or Ion Torrent data, the slopes did decrease, indicating that rare bacterial types were sampled (Fig. S1). All Good's coverage values exceeded 0.97 except one (RedPanda, 0.95;



c

Shannon diversity	Giant panda 8.256	Red panda 6.484	Bamboo lemur 10.029	Ringtail lemur 7.030
Giant panda		0.0006771	0.0001936	0.0036344
Red panda	0.0006771		0.0000029	0.2398093
Bamboo lemur	0.0001936	0.0000029		0.0000039
Ringtail lemur	0.0036344	0.2398093	0.0000039	

d

Phylogenetic diversity	Giant panda 456.596	Red panda 331.190	Bamboo lemur 675.278	Ringtail lemur 427.723
Giant panda		0.1252024	0.0031881	0.9096855
Red panda	0.1252024		0.0003586	0.2772677
Bamboo lemur	0.0031881	0.0003586		0.0014323
Ringtail lemur	0.9096855	0.2772677	0.0014323	

Fig. 2 Alpha diversity significantly correlates with gut transit time. We used a generalized linear model to test the strength of the relationship between transit time as a continuous variable and **a** Shannon or **b** phylogenetic diversity. Darker shading indicates higher *p* value significance for pairwise comparisons of **c** Shannon and **d** phylogenetic diversity

Table S2), further indicating that deep sampling captured diversity. A total of 970,948 sequences were retained after quality filtering data sequenced on the Ion Torrent and Illumina platforms, with an average coverage of 107,883 sequences per library (compared to 3889 sequences per library derived from the Roche 454 data; see Table S2). The retained sequences were assigned to 562 genus-level OTUs, compared to 56 total OTUs for the Roche 454 data set.

Discrepancies in sample size and sequencing platforms appear to drive clustering patterns in the PCoA of data sets sequenced on Roche 454, Illumina, and Ion Torrent platforms (Fig. S2). Samples sequenced on the Roche 454 platform cluster separately along PC1, indicating that nearly 40% of the variation in the combined data set is explained by the additional bacterial taxa detected with increased sequencing coverage by Illumina and Ion Torrent sequencing. (By contrast, the difference in bacterial taxa detected by Illumina versus Ion Torrent, along PC2, accounts for only about 4% of the total variation.) This effect was retained when we subsampled each library to an even depth of 1280 sequences, and confirmed when we compared Euclidean distance within and between species, across sequencing platforms (Fig. S3). Both within species (i.e., RP-RP or GP-GP) and between species (i.e., RP-BL, GP-BL, RL-GP, RL-RP, and RP-GP), Euclidean distance was higher for Ion-Roche and Illumina-Roche comparisons than for Ion-Illumina or any within-platform comparisons.

Figure S4 further confirms the prediction that low sequence coverage fails to detect the presence of rare membership in complex GM communities. The OTU heatmap indicates that the relationships underlying the PCoA and UPGMA clustering are driven by the absence of most OTUs from the Roche 454 data. Indeed, only four OTUs were shared by host species across both data sets: *Streptococcus* was detected in all eight giant pandas, *Sarcina* was detected in all eight red pandas and in all three ringtail lemurs, and *Clostridiacium* and an unclassified Clostridiaceae genus were detected in all 29 individuals (across all 4 host species). Additional research, in which all samples are sequenced at similar depth on the same platform, is necessary to thoroughly compare GM membership for evidence of microbial convergence.

Support for the Differential Membership Hypothesis

Diet and phylogeny appear to shape different classes of GM membership. The community structure of the bamboo lemur GM most closely resembles that of the ringtail lemur (Fig. 3), and the distances between carnivores and between lemurs are less than the distances between bamboo specialists (Fig. 4). Jackknifed PCoA (Fig. 5) and UPGMA clustering (Fig. S4) of full and rarefied datasets suggest that community structure appears to be driven by high-abundance OTUs and conserved within host species. Indeed, while the variation in OTU membership and abundance between the red pandas prevented them from clustering together on a UPGMA sample tree (Fig. S4), their samples are still more similar to each other than they are to any other species and only diverge somewhat along the PC3 axis (Fig. 5). Notably, while most mammal GMs are dominated by Bacteroidetes and Firmicutes, our results confirm previous observations [3, 21, 22] that both giant panda and red panda GMs are dominated by Proteobacteria and Firmicutes, but not Bacteroidetes (Fig. 3). The two most

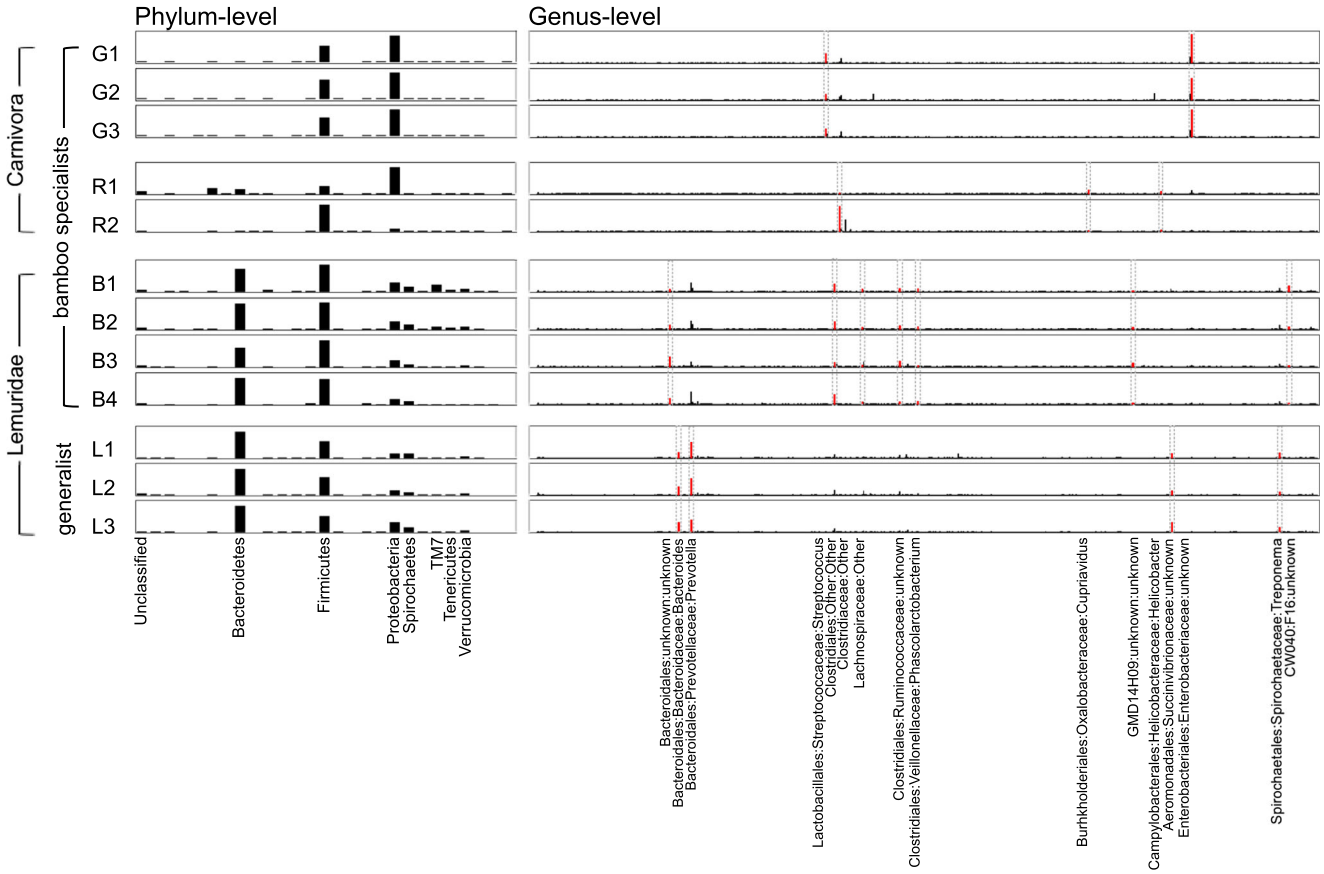


Fig. 3 Bamboo lemurs' gut community structure is more similar to ringtail lemurs than carnivoran bamboo specialists. *G*, giant panda; *R*, red panda; *B*, bamboo lemur; *L*, ringtail lemur. All vertical axes are scaled to a maximum relative abundance of 45%. All labeled genera were also identified as biomarkers (outlined in dashed lines) with significant linear discriminant analysis effect size (LEfSe [27]) scores. We ran LEfSe with default settings and all alpha values set to 0.05, to detect OTUs with significantly different representation between hosts (class = host species; no subclass)

abundant members in giant panda GMs are an unclassified Enterobacteriaceae genus (39.3%; Fig. 5) and *Streptococcus* (12.1%), which also distinguished previously characterized giant panda GMs [3]. Together, these results suggest that majority GM membership is shaped by host phylogeny, in agreement with previous studies [5, 7, 25].

We also detected a greater number of bamboo-associated OTUs compared to lemur- or species-specific bacterial taxa.

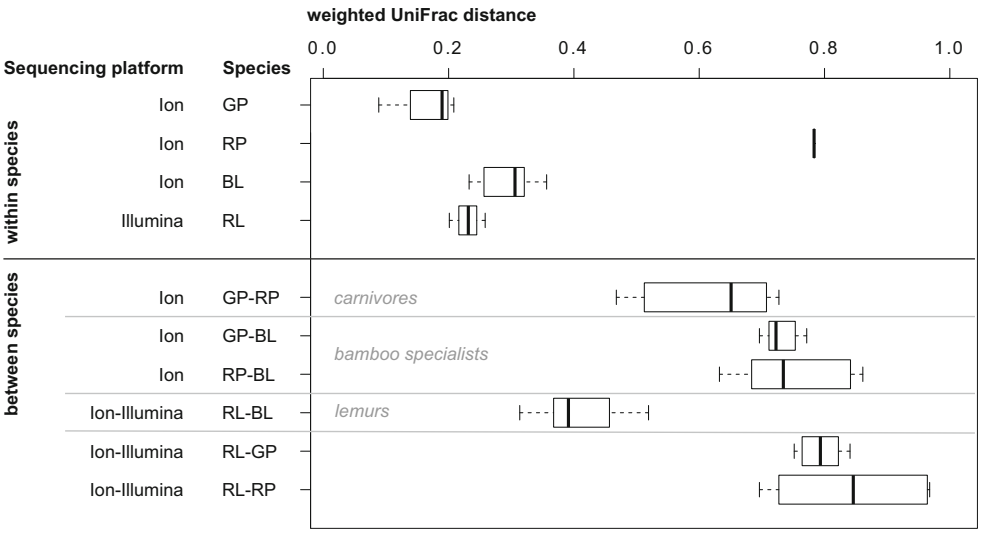


Fig. 4 Weighted UniFrac distance reveals less gut microbial variation between more closely related animals. Weighted UniFrac distance integrates both the phylogenetic differences between different OTUs and their relative abundance in the gut community. *GP* giant panda, *RP* red panda, *BL* bamboo lemur, *RL* ringtail lemur

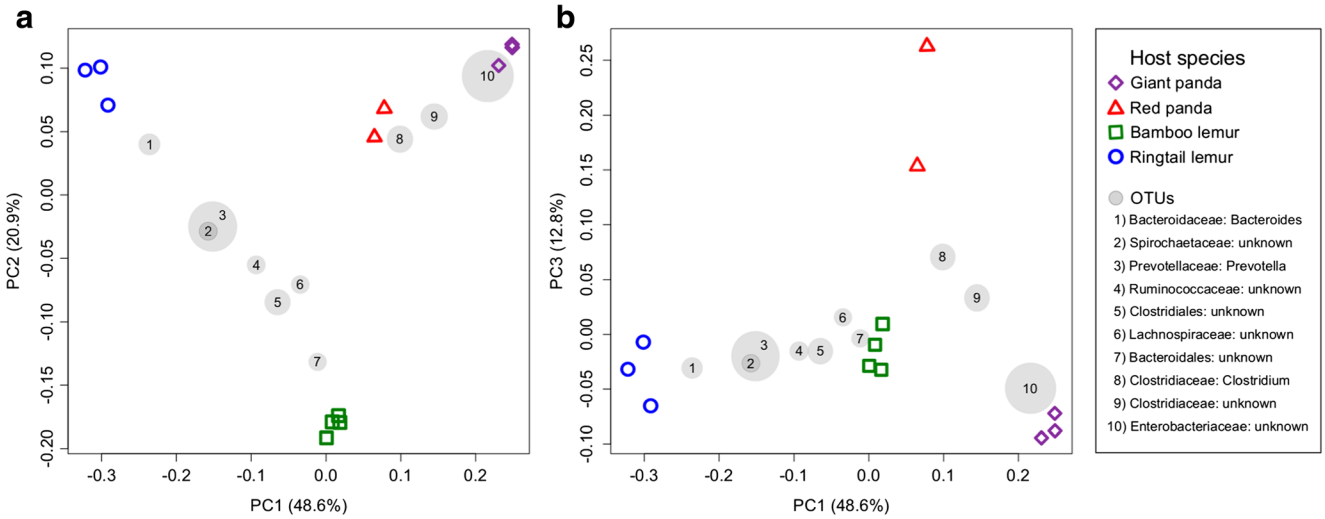


Fig. 5 Principal Coordinate Analysis of jackknifed weighted UniFrac distance reflects OTU-driven variation in the gut microbiome. Each library was subsampled at a depth of 66,000 to match the number of sequences in the smallest library (see Table S2). Weighted UniFrac distance integrates both the phylogenetic differences between different OTUs and their relative abundance in the gut community. The top ten OTUs identified as drivers of PCoA patterns were projected using biplots. These OTUs were also identified as biomarkers with significant linear discriminant analysis effect size (LEfSe [27]) scores and are scaled to reflect their enriched relative abundance

While the bamboo and ringtail lemurs share several high-abundance OTUs (i.e., *Prevotella*, *Treponema*, and an unclassified Clostridiales OTU), bamboo specialists host greater numbers of unique and significantly enriched OTUs (Figs. 3 and 5) and share a higher total number of OTUs (Fig. 6a). Forty-eight OTUs were shared among all bamboo specialists, compared to only eight OTUs shared between the sister lemur species. These core bamboo OTUs were relatively low abundance (Fig. 6 b–e, Table S3), making up only 24.3, 13.0, and 9.6% of the GMs in giant pandas, red pandas, and bamboo lemurs, respectively. These results suggest that diet selects specific OTUs occurring at relatively low abundances, compared with the phylogenetic "class" of GM membership.

Discussion

Coverage

Deep sequencing has only become available at affordable prices relatively recently. As cost and efficiency decreases, limitations have shifted from data production to downstream processing. High-throughput studies now require bioinformatics tools with faster computation times and more extensive databases, lest deep sequencing efforts return ever-increasing proportions of OTUs classified as "unknown" taxa. For example, unclassified bacteria made up 8.5–23.5% of gut communities sequenced from nine wild primates [29].

To ensure detection of low-abundance GM members, we performed deep sequencing of reads amplified from six variable regions of the 16S rRNA gene. Our high coverage data set revealed previously undetected rare membership in the GM structure of both the giant and the red panda, and thus enabled a more thorough investigation of shared membership in their GMs. In this study, we demonstrate that relatively low-abundance taxa comprise the core microbiota shared by bamboo specialists and lemur sister species. Our findings are novel compared to a previous, lower-coverage assessment of the giant panda and red panda [3], suggesting that diet-associated signatures may be masked by more abundant bacterial taxa. We also detected higher Shannon diversity than was previously reported in the giant panda (8.256, compared to 0.342 [3] or 2.4 [21]). This likely results from our detection of low-abundance OTUs; Lemos et al. [30] also found that Shannon values increased with sequencing coverage. Low-abundance taxa, often referred to as the "rare biosphere," are important contributors to both alpha and beta diversity [31].

A previous study detected higher Shannon diversity in Coquerel's sifaka (a leaf-eating lemur) compared to ringtail lemurs [5], suggesting that both dietary fiber and gut complexity increase niche space available to gut microbes. We find that giant pandas harbor similar Shannon diversity to Coquerel's sifaka and that bamboo lemur diversity values are highest as measured by both indices (Fig. 2), despite the bamboo specialists' much shorter gastrointestinal tract lengths (Table 1). It is possible that variation in diet (Table 2) may account for differences in diversity between bamboo specialist species. However, we think that increased consumption of fruit or vegetables is unlikely to account for the greater diversity observed in bamboo lemurs. Sugar and starch (except for resistant starches, such as those found in sweet potato) are easily digested by the host and therefore may not be available as a fermentation substrate to microbes in the hindgut. It is therefore more likely that the greater diversity observed in the

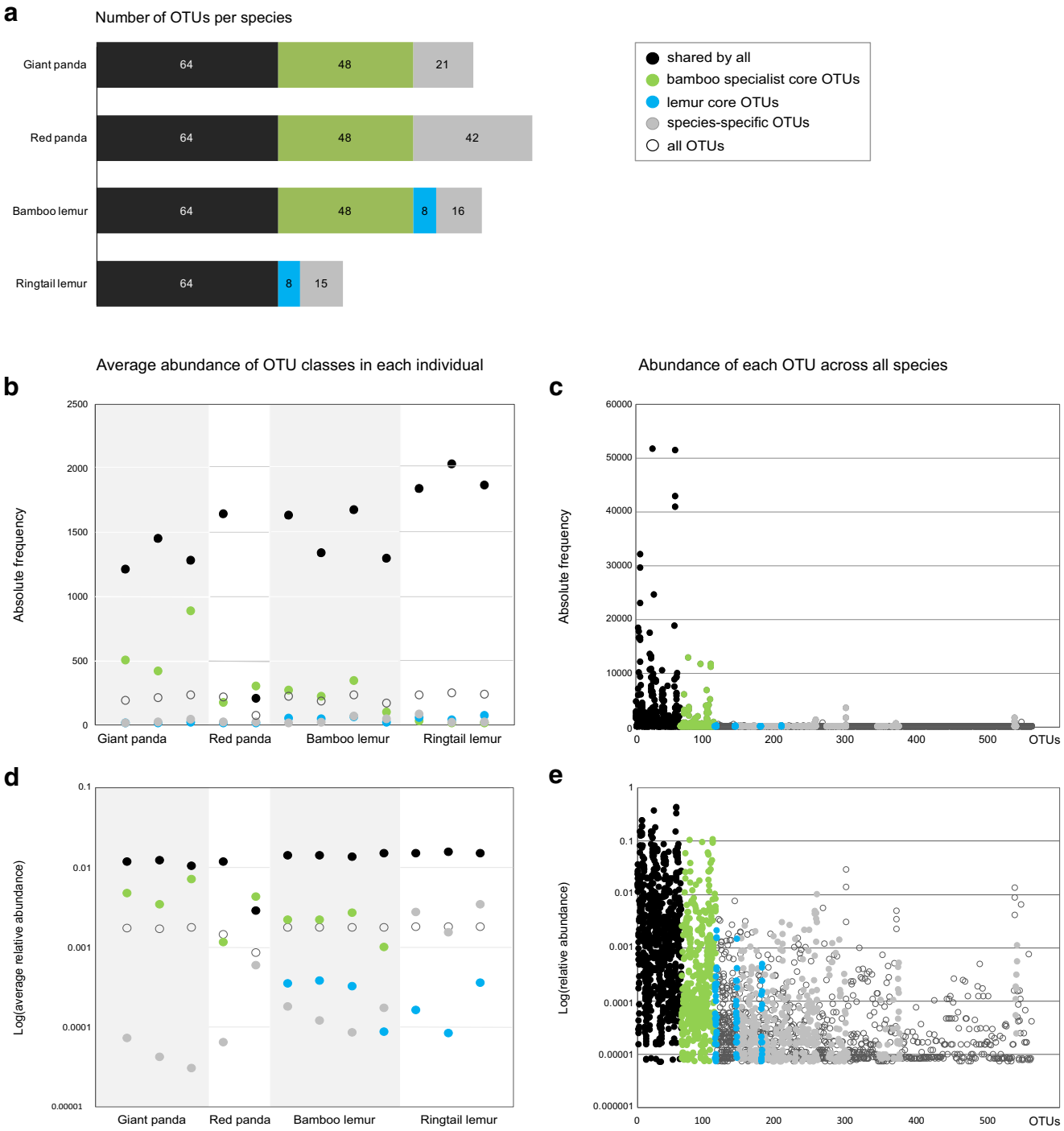


Fig. 6 Phylogeny and diet shape different classes of microbial membership. We defined OTUs as *core* taxa if they were detected in all individuals in a given category. We considered OTUs to be *species-specific* if they were detected in all individuals within that species, but not shared with all members of any other species. Using these definitions, we find that **a** bamboo specialists share more of their microbiome than lemurs and that **b**–**e** species-specific and core OTUs are less abundant (i.e., account for less than 0.12 of community membership)

bamboo lemur is due either to the greater variety of food items in its diet or (most likely) to the increased transit time associated with having a cecum (see Fig. 2).

The bamboo fed to all specialists is richer in fiber compared to the browse fed to folivorous lemurs (Table S4) and may provide additional resources sufficient to increase GM diversity, as previously demonstrated across mammalian species [5, 7–9]. Alternatively, higher Shannon diversity values may indicate that the bamboo specialists' GM communities comprise more transient members compared to Coquerel's sifaka and other lemur species. Rapid transit time may preclude host regulation of GM membership and allow

propagation of additional opportunistic bacterial lineages (i.e., Enterobacteriaceae). Indeed, high diversity may indicate ongoing transition between opportunistic bacteria, augmented by environmental microbes, as seen during GM colonization in other lemur species [5]. Additionally, old age may play a role in diversity differences between bamboo lemurs and other species in this study. Age is associated with decreased diversity and increased inter-individual variation in elderly humans [32]. However, while we do see greater variation within bamboo lemurs compared to ringtail lemurs or giant pandas (Fig. 4), the bamboo lemurs' high microbial diversity values belie their age. Additional, longitudinal studies would be helpful to investigate age-related trends in gut microbial diversity across non-human primates and other species.

We implemented a closed reference OTU-picking approach to compare Ion Torrent sequencing data from bamboo specialists to ringtail lemur data sequenced on the Illumina MiSeq platform [5] and to Asian black bear data sequenced on the Roche 454 platform [3]. While the closed reference approach enables comparisons across 16S rRNA gene regions, batch effects associated with sequencing coverage and platform may bias the distance between samples (Fig. S3 and Fig. 4) and relationships detected with PCoA [33] in Fig. S2 and Fig. 5. We therefore focus our discussion on results derived directly from OTU classification of Ion Torrent and Illumina sequencing data. Specifically, it appears that host phylogeny shapes dominant community structure, while similar dietary pressures select for specific lower-abundance genus-level OTUs. We describe these patterns below.

Different Classes of GM Membership Associated with Diet and Phylogeny

We detected four differentially abundant classes of GM membership associated with phylogeny and diet. OTUs common to all subjects generally occurred at the highest relative abundance, followed by core taxa present in all bamboo specialists, lemur core taxa, and species-specific OTUs (Fig. 6b–e). Evolutionary history appears to shape 64 highly abundant taxa shared by all species, which may possibly indicate a core mammalian microbiome. Community structure is more similar within primates and carnivores (Fig. 3), and Fig. S4 demonstrates topological congruence between gut microbial trees and host relationships (as previously shown in [25, 34]). Diet incurs a more subtle impact: 48 core bacterial taxa are shared across all bamboo specialists, compared to 94 species-specific OTUs (Table S3, Fig. 6a). Core OTUs associated with diet are likely adapted to specific conditions (i.e., high-fiber diet and simple gut morphology) common to bamboo specialists. Yet Table S3 reveals that a unique subset of the shared OTUs appears best adapted to each host species. It is possible that species-specific host characteristics (i.e., immune components or gut transit time) may prevent or favor specific bacterial taxa in each host lineage. Together, these findings support the concept of phylosymbiosis [34].

Phylogenetic Effects

Bacteroidetes and Firmicutes have been identified as the two most dominant phyla in most studies of mammalian GMs. However, we identified Proteobacteria and Firmicutes as the two most dominant phyla in giant pandas and red pandas (Fig. 3), consistent with previous studies [3, 21, 23, 35]. Proteobacteria also dominate the GMs of sloths [36], raising the possibility that Proteobacteria may be more dominant in herbivores with low metabolic rates. The three-toed sloth has a metabolic rate only 75% of the predicted value [37]. The metabolic rate of the red panda is also low (39% of predicted value), presumably to reduce energy requirements [38], and the daily energy expenditure of the giant panda is only 37.7% of the predicted value [39].

The giant pandas' two most abundant bacterial OTUs, an unclassified Enterobacteriaceae genus and *Streptococcus*, were also found to be dominant in previous studies of the giant panda GM [20, 21, 23]. Both taxa have been detected in previous studies of GM succession in newborn humans [40, 41] and lemurs [5], suggesting an ability to colonize simple gastrointestinal tracts. However, Hirayama et al. [35] observed that GM membership in giant panda cubs shifted from *Lactobacillus* and *Bifidobactierium* to Enterobacteriaceae genera with the introduction of solid foods. This transition indicates that, for giant pandas, Enterobacteriaceae may not be invaders so much as a normal part of the healthy adult GM structure in species with relatively simple gut morphology.

Diet May Govern Low-Abundance OTUs

The bamboo lemur shares six times more OTUs with other bamboo specialists than with its sister species (Fig. 6), suggesting that diet shapes a specific class of low-abundance OTU membership (Table S3). While a previous study with lower sequencing coverage (i.e., 10^3 sequences per sample) did not detect dietary signatures in the giant panda or red panda GM [3], our increased coverage approach reveals 48 OTUs comprising a bamboo-associated core GM. Zoo diets do not necessarily capture the diversity of natural diets; however, captive studies do provide relatively more control, where daily dietary intake and other factors that may impact the gut microbiome can be monitored. Furthermore, despite (or, even in light of) the different proportions of bamboo fed to the specialists in this study, bamboo still makes up the bulk of the diet in all three specialists' captive diet. As such, bamboo consumption remains the strongest correlate for the 48 OTUs shared across all three species.

Despite their relatively low abundance, it is possible that OTUs unique to bamboo specialists may play an important functional role for their hosts. Low-abundance bacterial taxa can be disproportionately active [42] and perform key functional roles such as methanogenesis [43] or metabolite production. Therefore, the rare biosphere may inform studies of convergent evolution between long-diverged hosts. Given the small sample size and taxonomic approach in this study, we cannot make conclusive statements about the functions performed by core bamboo taxa. We suggest, however, that the presence of certain low-abundance OTUs across phylogenetically diverse lineages of bamboo specialists is compelling enough to hypothesize their common role in the gut.

Of these shared taxa, *Fibrobacteres* [44] and *Ruminococcus* [45] are known to have fiber-digesting abilities, and other OTUs have been detected in studies of the koala (*Phascolarctobacterium* and *Ruminococcus* [45]) and even the termite gut (*Planctomycetes* [46] and *Synergistes* [47]), demonstrating that diet can convergently shape GM membership across vast host phylogenetic distances. Fourteen of the OTUs detected in bamboo lemurs were previously detected in other lemur species [5] and spanned the bacterial phylogenetic spectrum. Of the OTUs shared among lemurs, *Faecalibacterium*, *Fibrobacter*, *Ruminococcus*, and *Phascolarctobacterium* were enriched in both bamboo lemurs and Coquerel's sifaka (folivorous) compared to other Lemuridae (ringtail lemur and fruit-eating ruffed lemur, *Varecia variegata*) [5]. The increased number of OTUs shared between Coquerel's sifaka and bamboo lemur GMs and among the three bamboo specialists, despite their greater phylogenetic distance, further suggests that these shared OTUs may be adapted to digesting high-fiber diets. Additional research including RNA-based library construction, and measurement of enzymatic rates for cellulose and hemicellulose digestion are needed to confirm which bacterial taxa are active, when, and whether the bamboo lemur's extended transit time enables more effective microbial fiber degradation than is achieved in the giant panda or red panda. Our study also lacks longitudinal leverage. Deep sequencing across additional time points (as performed by McKenney et al. [5]) would verify and extend temporal dynamics (such as the seasonal variation detected by Xue et al. [21]) to the rare biosphere.

Conclusions

With higher sequencing coverage, we were able to detect similarities in rare bacterial membership shared by bamboo eaters. Most likely, these bamboo-associated bacteria yield more benefit in the bamboo lemur (due to its greater transit time) than in the bamboo-eating carnivorans with simpler guts. Despite GI morphological and physiological differences, several OTUs detected across host species suggest that adaptations to bamboo diets may also incur subtle impacts on long-term GM succession and membership. We have integrated these patterns of GM membership across highly diverged bamboo specialists into a governing framework that accounts for and synthesizes the effects of host phylogeny and dietary specialization. Rather than act as directly competing forces, we find that the impacts of phylogeny and diet manifest in different "classes" of GM membership. Specifically, host phylogeny is found to shape the class of highly abundant taxa (which contribute to variation between GMs), while dietary pressures select for a greater number of low-abundance OTUs. The low-abundance OTUs shared among bamboo specialists may be specifically adapted to a nutritional profile rich in fiber. These findings are important for understanding how convergent feeding strategies impact the host-microbiome relationship and, conversely, how gut microbiota may facilitate convergent evolution in phylogenetically diverged species with shared dietary regimes.

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Collected samples: EAM MM
Analyzed and interpreted the data: EAM AR
Contributed reagents/materials/analysis tools: EAM AR ADY
Wrote the manuscript: EAM MM AR ADY

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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