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Species-specific but not phylosymbiotic gut microbiomes of New Guinean passerine birds are shaped by diet and flight-associated gut modifications

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Animal hosts have evolved intricate associations with microbial symbionts, where both depend on each other for particular functions. In many cases, these associations lead to phylosymbiosis, where phylogenetically related species harbour compositionally more similar microbiomes than distantly related species. However, evidence for phylosymbiosis is either weak or lacking in gut microbiomes of flying vertebrates, particularly in birds. To shed more light on this phenomenon, we compared cloacal microbiomes of 37 tropical passerine bird species from New Guinea using 16S rRNA bacterial gene sequencing. We show a lack of phylosymbiosis and document highly variable microbiomes. Furthermore, we find that gut bacterial community compositions are species-specific and tend to be shaped by host diet but not sampling locality, potentially driven by the similarities in habitats used by individual species. We further show that flight-associated gut modifications, coupled with individual dietary differences, shape gut microbiome structure and variation, contributing to the lack of phylosymbiosis. These patterns indicate that the stability of symbiosis may depend on microbial functional diversity rather than taxonomic composition. Furthermore, the more variable and fluid host–microbe associations suggest probable disparities in the potential for coevolution between bird host species and microbial symbionts.

1. Introduction

Gut microbial symbionts play a multitude of dietary, defensive and developmental functions facilitating their host's life-history strategies [1,2]. Consequently, we often observe tight associations between symbiotic microbial communities and their hosts. In some instances, these associations are strong and lead to phylosymbiosis, in which phylogenetically related host species harbour compositionally more similar microbial communities than distantly related species [3]. Evidence for phylosymbiosis is found in many animal clades, including corals [4], many clades of non-flying mammals [5,6], spiders [7] and some insects [8,9]. However, phylosymbiosis is not apparent in multiple animal groups such as bats [10], chipmunks [11], teleost fish [12] and birds [10,13]. In birds, host phylogeny has a weak, or at least only a secondary influence on gut community structures [10,14–17], but rather follows a random model of evolution [13].

Physiological gut adaptations associated with flight have been proposed to explain this weak association [10]. The evolution of flight has led to

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modifications in the digestive tracts of birds; specifically, shorter digestive tracts and faster gut retention times compared to similar-sized non-flying mammals [18]. These modifications could potentially affect the stability of avian gut microbial communities [10], because shorter gut retention leads to more fluctuating gut environments and potentially higher microbial turnover. Bird microbiomes are also highly variable between individuals of the same bird species [19–21] and tend to be malleable to dietary and environmental changes [19,21–26]. Consequently, diet tends to affect gut microbiomes more than host phylogeny [17]. Since diet, and potentially gut length, influence avian gut microbiome structure, phylosymbiotic signals may only be apparent when examining closely related bird species with similar body size and diet, while controlling for sex and environment [14]. With the exception of one recent study [16], no studies have explored a diverse group of wild passerine birds (order Passeriformes) with multiple individuals per species to test the relative importance of phylogeny, diet and body size (i.e. gut retention time) on gut microbial compositions and the potential absence of phylosymbiosis.

Here, we compared microbiomes from 356 cloacal samples across 37 passerine species (14 families) from multiple highland and lowland localities on the tropical island of New Guinea. First, we investigated host taxon effects on microbial community composition, a pre-requisite for the presence of phylosymbiosis. Despite the strong effect of host taxon, our data suggest the absence of phylosymbiosis. To determine what factors may be causal to this, we first tested if gut community composition reflected dietary guilds of hosts [17,19]. Second, we tested the prediction that locality could cause gut microbial compositions to be more similar in individuals of the same bird species [21]. Finally, we evaluated whether gut retention time affected passerine microbiome composition and variability, using body mass, which is strongly associated with retention time, as a proxy [27–29]. Larger birds with slower gut retention times [27,28] would have less fluctuating (more stable) gut environments compared to smaller birds, potentially influencing microbial diversity and stability. If gut length and retention time influence bird gut microbiome compositions, we expect host body mass to be negatively correlated with microbial diversity and heterogeneity.

2. Methods

(a) Sample collection

Cloacal swabs were collected from passerine birds (order Passeriformes) captured through mist netting at four high elevation localities (2200–3700 m.a.s.l), two mid-elevational localities (1700 m.a.s.l.) and three lowland localities (200 m.a.s.l) in Papua New Guinea in 2017, 2018 and 2019 (electronic supplementary material, figure S1). All samples were collected during the dry season, except for 28 samples collected at Baitabag in the rainy season in 2019. The forest types and climatic variables were similar in different localities at similar elevations [30–32]. Swabs were collected using Copan mini FLOQ swabs (Brescia, Italy) and stored in either 70% EtOH (2017) or RNAlater (2018 and 2019) at approximately 20°C for up to two weeks until they were transferred to the field station and stored at −20°C. Storing samples in RNAlater at ambient temperature for less than two weeks is not expected to have a strong impact on the microbiomes [33]. To determine if the storage buffer (EtOH or RNAlater) affected microbial profiles, we also collected samples from up to six individuals per buffer type for two species in 2019 from the same locality (see Results). Birds were weighed in the field and assigned to feeding guilds (frugivore, insectivore, frugivore + insectivore, insectivore + nectarivore, or granivore), based on extensive sampling of stomach contents by Sam *et al.* [32].

(b) DNA extractions and MiSeq amplicon sequencing

A total of 555 cloacal samples were collected from 85 passerine species (electronic supplementary material, table S1). The full swab, along with 100 µl of RNAlater (for the samples that were stored in EtOH we only used the swab without EtOH) were used for DNA extractions, and all samples were extracted at the same time. DNA extractions and MiSeq amplicon sequencing of the v4 region of the bacterial 16S rRNA gene was performed as described in Bodawatta *et al*. [20].

(c) Miseq sequence analysis, alfa diversities

MiSeq sequences were analysed using the DADA2 pipeline [34] within QIIME2 [35]. Sequences were assigned to amplicon sequence variants (ASVs) with 100% similarity and then to taxonomy using the SILVA 132 database [36]. Archaeal, mitochondrial and chloroplast sequences were removed using QIIME2. We generated the rooted bacterial phylogeny using the 'align-to-tree-mafft-fasttree' function in QIIME2 [35]. For the final analyses, we only included bird species with three or more individuals (electronic supplementary material, table S1). ASVs with <10 total sequences across all samples were excluded, and the dataset was rarefied based on the sample with the lowest number of sequences (2480 sequences) using the 'rarefy_even_depth' function in phyloseq [37] (electronic supplementary material, figure S2). Final analyses were conducted on the rarefied dataset in R 4.0.2 [38] (detailed information in electronic supplementary material, file S1).

Observed ASV richness and Shannon's diversity were calculated using the phyloseq [37] and microbiome [39] packages. Faith's phylogenetic diversity (PD) of microbiomes was calculated using the picante package [40]. High PD values indicate high phylogenetic diversity within a microbiome. We calculated the standardized effect size (SES) of PD using species-level mean abundance of each ASV and conducting 1000 random iteration (shuffling taxa across the bacterial phylogeny), using the 'SES.pd' function in picante [40].

(d) Effects of host species, feeding guilds and locality on gut microbiomes

Bacterial community differences were calculated using Bray–Curtis, Jaccard, weighted and unweighted UniFrac, and β mean nearest taxon distances (βMNTD). Except for βMNTD, all distances were calculated using phyloseq [37]. βMNTD distances were calculated using the 'cophenetic' function in the ape package [41] and the 'bmntd' function in iCAMP [42]. Overall, statistical differences between bacterial communities of different host species, feeding guilds, localities and buffer types (EtOH and RNAlater) were evaluated using permutational multivariate analysis of variance (PERMANOVA) using the adonis2 function in vegan [43] with 10 000 permutations. We tested all variables in one model, with the 'by' parameter set to 'margin' in adonis2 to estimate the marginal effects of different variables and to control for covariation between them.

Centroids (±s.e.) of bacterial communities of different species were visualized in nonmetric multidimensional scaling (NMDS) plots. We investigated the heterogeneity (individual variation) of microbiomes through calculating the microbial divergence

(distance to group median) using the 'divergence' function in the microbiome package [39]. This was only calculated using Bray–Curtis dissimilarity as the divergence function is unable to use UniFrac distances. Larger divergence values indicate higher bacterial community variation. As divergence is influenced by sample size, we also calculated species-level microbial heterogeneity using three randomly picked individuals per species and repeated this five times. We evaluated the core microbiome of each bird species using the 'core' function in the microbiome package [39], assigning ASVs with a relative abundance of at least 0.0001% in at least 50% of the samples to cores.

For feeding guilds, we used the wrapper package pairwise.adonis [44], to investigate the bacterial community-level pairwise differences. We investigated the 30 most relatively abundant bacterial genera between five feeding guilds using the 'amp_heatmap' function within ampvis2 [45]. We further evaluated the species-level effect of habitat on gut microbiomes in species captured at two or more localities with a minimum of three individuals per locality. Statistical differences were determined using PERMANOVAs and the data were visualized using principal coordinate analyses (PCoA).

(e) Host species phylogeny and testing for phylosymbiosis

We created a host species phylogeny using a concatenated alignment of three mitochondrial and three nuclear genes sourced from GenBank (electronic supplementary material, table S2) using BEAST v. 1.8.4 [46]. We applied the general time reversible nucleotide substitution model to the concatenated data and ran the analysis for 100 million generations using a relaxed uncorrelated lognormal distribution for the molecular clock model, and assuming a birth-death speciation process as a tree prior. Convergence diagnostics were assessed in Tracer v. 1.6 [47], by determining the effective sample sizes and mean distribution values. The final output tree was summarized in TreeAnnotator v. 1.8.3 [46] as a maximum clade credibility (MCC) tree after discarding the first 10 million generations as burn-in.

To test for phylosymbiosis, we used both correlation (Mantel test) and cluster-matching (Robinson–Foulds distances—RF) analyses between the host phylogeny and the microbial communities. These analyses require the number of tips (samples) in the phylogeny to match the number of samples in the microbial distance matrix. Thus, to acquire one microbial community to represent each bird species, we both (i) calculated the average abundance per ASV within a bird species [14] and (ii) randomly picked one individual per bird species [10]. Random picking was repeated 10 times. Microbial community distances were then calculated using all the aforementioned distance matrices.

Mantel correlations were calculated using vegan [43] through Pearson's correlation tests between host phylogenetic distances (measured with the adephylo package [48]) and microbial community similarities. The significance of correlations was evaluated using 10 000 random permutations. For the cluster-matching method, we used the RF.dist function in the phangorn package [49] to investigate the normalized RF distances (normalized by the total number of nodes) between host phylogeny and the microbial dendrogram. A normalized RF distance of 0 represents perfect congruence of the topology of tested trees while a value of 1 represents no matching nodes. The significance of observed values was determined through comparing mean RF distances acquired from 10 000 randomly generated microbiome dendrograms using the rNNI function in phangorn [49].

(f) Association between microbiome structure and body mass

To investigate the relationship of species-level average ASV richness, PD, microbial heterogeneity with body mass (g), we used phylogenetic generalized least-squares (PGLS) tests in caper [50], accounting for phylogenetic non-independence between host species. We used each of the microbiome characteristics as dependent variables and body mass (g) as the independent variable. Categorical comparisons were conducted using Kruskal–Wallis rank-based tests (KW) and Dunn's *post hoc* tests using the FSA package [51]. All results were visualized using the ggplot2 [52] and viridis [53] packages in R 4.0.2 [38].

3. Results

For the final analyses, we used 356 cloacal microbiomes from 37 bird species (average number of individuals ± s.e. = 10 ± 1.5) representing 14 families with a total of 11 457 557 sequences (average ± s.d.: 32 184 ± 19 483). After removing ASVs with less than 10 sequences, but prior to rarefying, sequences were assigned to 24 110 ASVs from 41 phyla (electronic supplementary material, table S3). Rarefaction of the dataset led to 21 686 ASVs, dominated by Proteobacteria (36.7%), Firmicutes (17.7%), Epsilonbacteraeota (12.3%), Actinobacteria (9.7%), Bacteroidetes (8.4%) and Tenericutes (5.5%). Only 0.4% of the sequences could not be assigned to phylum. The average relative abundances of bacterial phyla differed markedly between bird species (electronic supplementary material, figure S3 and tables S4 and S5). Both in the full dataset (table 1) and in the two selected species we did not find an effect of storing buffer (EtOH and RNAlater) on microbiome composition (electronic supplementary material, figure S4 and table S6). Thus, we analysed all rarefied data from the three years together.

(a) Passerine microbiomes are species-specific with a small core microbiome

Alpha diversity indexes differed significantly between bird species (ASV richness, KW: $\chi^2 = 74.29$, d.f. = 36, $p = 0.0002$; Shannon's diversity, KW: $\chi^2 = 89.16$, d.f. = 36, $p < 0.0001$; PD, KW: $\chi^2 = 66.52$, d.f. = 36, $p = 0.0015$; electronic supplementary material, figure S5). *Post hoc* tests revealed that these patterns were mainly driven by a few species-level differences (electronic supplementary material, table S7). Standardized effect sizes of PD indicated that PD was significantly lower than expected by chance in all but three bird species (electronic supplementary material, figure S6 and table S8), suggesting non-random phylogenetic composition of communities.

Host taxon (species) significantly affected microbial beta diversity and explained a large proportion of the variance, irrespective of the distance matrix used (table 1 and figure 1), suggesting species specificity in microbiome composition. Heterogeneity (divergence) differed between bird species (KW: $\chi^2 = 170.1$, d.f. = 36, $p < 0.0001$; electronic supplementary material, figure S6b), suggesting intraspecific variation. Despite this strong effect, core microbiomes included on average only 1.7% (s.d.: ± 1.6) of the total number of ASVs. Core richness was marginally significantly negatively associated with sample size (electronic supplementary material, figure S7a). In contrast, total species-level ASV richness was significantly positively correlated with sample size (electronic supplementary material, figure S7b). However, the average ASV richness of a species was not associated with sample size (electronic supplementary material, figure S7c), suggesting that it is more robust to sampling depth.

Table 1. Effects of host species and feeding guild on microbial community structures measured with five different matrices using PERMANOVAs with 10 000 permutations. Significant results are highlighted in italics.

distance matrix	variable	*F*	d.f.	R^2	*p*
Bray–Curtis	*host species*	*2.345*	*33*	*0.1777*	*<0.0001*
	feeding guild	*1.474*	*1*	*0.0034*	*<0.0001*
	locality	*1.519*	*8*	*0.0279*	*<0.0001*
	buffer type	1.182	1	0.0027	0.1058
Jaccard (unweighted)	*host species*	*1.733*	*33*	*0.1436*	*<0.0001*
	feeding guild	*1.241*	*1*	*0.0031*	*0.0003*
	locality	*1.244*	*8*	*0.0249*	*0.0002*
	buffer type	1.079	1	0.0027	0.1582
UniFrac (weighted)	*host species*	*1.489*	*33*	*0.1283*	*<0.0001*
	feeding guild	0.0014	1	0.0000	0.9911
	locality	0.8557	8	0.0141	0.5072
	buffer type	0.1598	1	0.0003	0.5758
UniFrac (unweighted)	*host species*	*1.489*	*33*	*0.1283*	*<0.0001*
	feeding guild	1.145	1	0.0029	0.2441
	locality	1.059	8	0.0221	0.2564
	buffer type	0.7364	1	0.0019	0.8572
βMNTD	*host species*	*3.476*	*33*	*0.2133*	*0.0002*
	feeding guild	−1.212	1	0.0023	0.9739
	locality	*3.706*	*8*	*0.0551*	*0.0126*
	buffer type	3.421	1	0.0064	0.1194

Table 2. Results of testing for phylosymbiosis between host phylogeny and species-level average gut microbiome dissimilarity using both correlation-based Mantel tests and cluster matching with Robinson–Foulds distances.

	correlation-based analyses		cluster matching with Robinson–Foulds distances		
distance matrix	Mantel *r*	*p*	RF (observed)	null mean (10 000 trees)	*p*
Bray–Curtis	0.0493	0.0908	0.9143	0.9481	0.9952
Jaccard	0.0491	0.0994	0.9143	0.9483	0.9934
UniFrac (weighted)	0.0109	0.3415	0.9429	0.9657	0.9992
UniFrac (unweighted)	−0.0042	0.5229	1	0.9947	0.8194
βMNTD	−0.0344	0.8381	0.9714	0.9819	0.9829

The proportion of core ASVs was not associated with the feeding guild (electronic supplementary material, figure S7d). Overall, this underlines the importance of sample sizes for estimating microbial diversity and core microbiomes of passerine birds.

(b) No evidence of phylosymbiosis between host phylogeny and microbial communities

Despite species specificity, we did not find evidence for phylosymbiosis irrespective of the method used for species-level average microbiomes (table 2 and figure 2). Overall, randomly selected microbiomes (one individual per species) also did not consistently show signs of phylosymbiosis. Only four out of 10 randomly selected communities showed significant Mantel correlations with host phylogeny for Bray–Curtis and Jaccard dissimilarities, while only one of the 10 communities showed a significant association for UniFrac and βMNTD (electronic supplementary material, table S9). Furthermore, there was no evidence for phylosymbiosis in any of the 10 sets when using the RF matching cluster method.

(c) Host dietary guild secondarily influence the structure of microbiomes

Observed ASV richness (KW: $\chi^2 = 11.45$, d.f. = 4, $p = 0.0219$) and Shannon's diversity (KW: $\chi^2 = 19.41$, d.f. = 4, $p = 0.0004$) differed between dietary guilds, while PD did not (electronic supplementary material, figure S8). Pairwise feeding

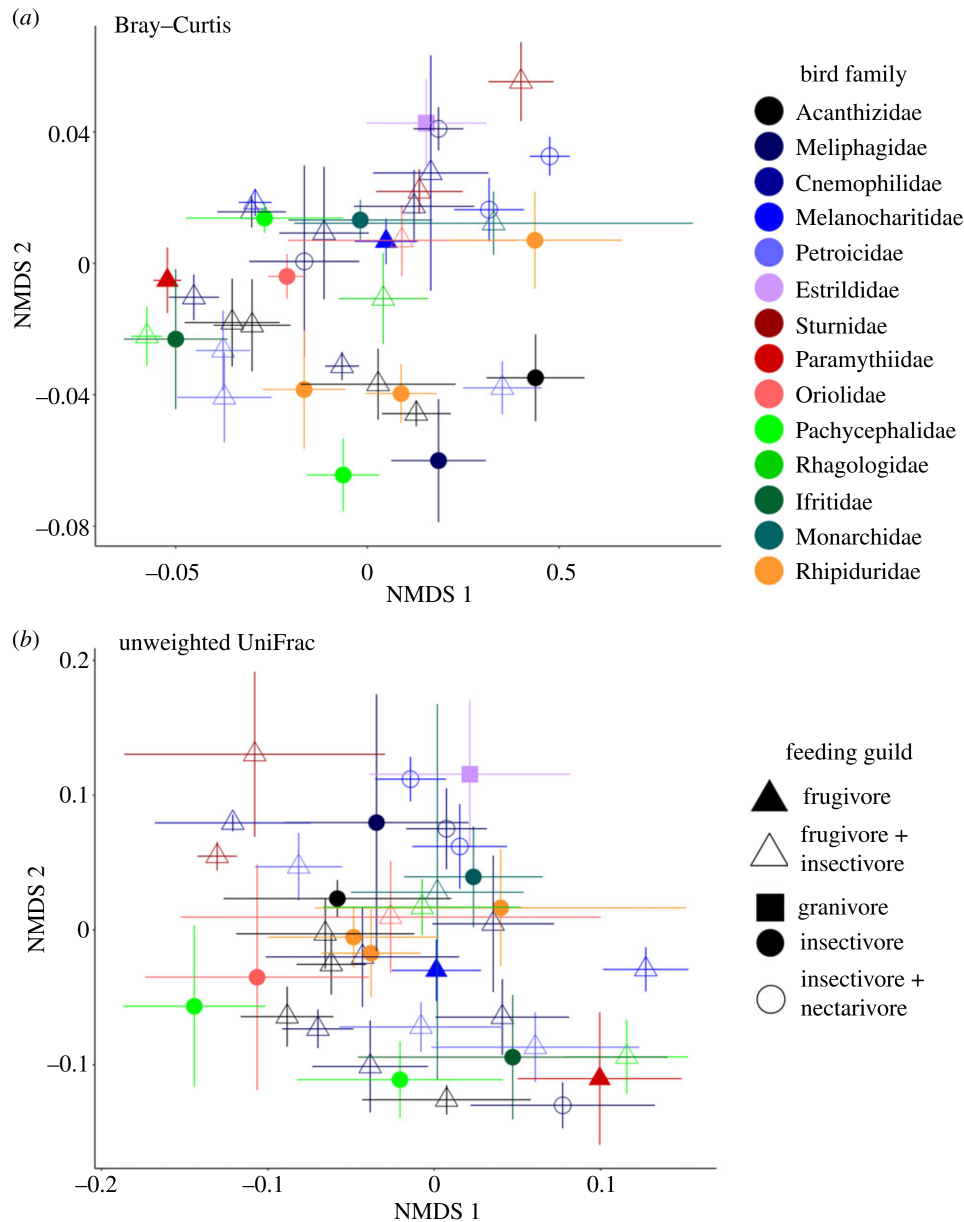

Figure 1. Passerine gut microbiomes are species-specific. Nonmetric multidimensional scaling (NMDS) plots demonstrating the centroids (±s.e.) of the gut microbial communities of different species. (*a*) Bray–Curtis dissimilarity–stress = 0.2096, (*b*) unweighted UniFrac distances–stress = 0.2613.

guild comparisons revealed that granivores had significantly lower richness and Shannon's diversity than other dietary guilds (electronic supplementary material, figure S8). However, we should acknowledge that only one granivorous species was included. Dietary guild only significantly affected gut bacterial compositions based on Bray–Curtis and Jaccard, not UniFrac distances (table 1), suggesting that the phylogenetic composition of microbiomes is not impacted by the feeding guild. The effect of the feeding guild was secondary to the host effect (figure 1 and table 1). Pairwise comparisons (conducted only using Bray–Curtis dissimilarity) demonstrated that microbial community composition significantly differed between all five dietary guilds (electronic supplementary material, table S10).

Relative abundances of the 30 most abundant genera indicated differences in the composition of some genera between the five dietary guilds, suggesting affiliation of some bacterial genera with host diets (figure 3). However, unique ASVs in different feeding guilds tend to be associated with the number of species sampled (electronic supplementary material, figure S9). Microbiome heterogeneity was significantly different between guilds (KW: $\chi^2 = 45.24$, d.f. = 4, $p < 0.0001$). *Post hoc* analyses indicated significant differences in community variation between multiple feeding guilds, with insectivorous species generally being more variable (figure 3). The effect of the feeding guild suggests that species-specific bacterial communities are secondarily shaped by diet and that diet-associated microbes vary between species from the same feeding guild, while phylogenetic beta diversities are unaffected by dietary guild (table 1).

(d) No strong species-specific effect of locality on the composition of passerine microbiomes

Overall, sampling locality significantly affected beta diversity measured with Bray–Curtis, Jaccard and βMNTD, but not UniFrac distances (table 1). For species-level analyses, we used thirteen bird species with three or more individuals from each of two or more localities and the beta diversity was measured with Bray–Curtis and βMNTD. The Bray–Curtis analyses indicated that only *Melanocharis versteri* and *Melilestes megarhynchus* were significantly influenced by

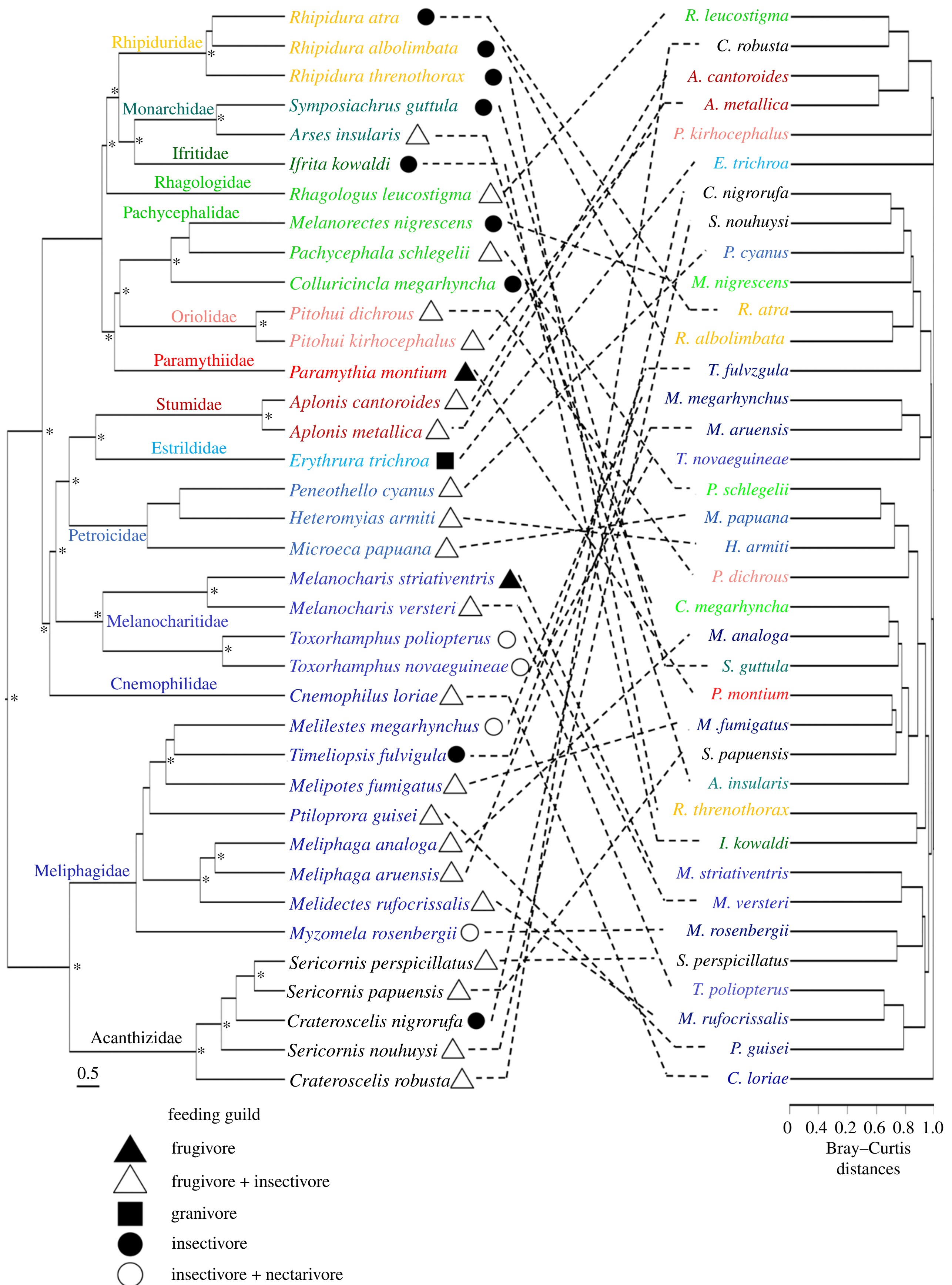

Figure 2. No phylosymbiosis between host phylogeny and microbial community similarity. Both the host phylogeny (posterior probability greater than 0.99 are indicated with asterisks) and the average species microbial community dissimilarity trees are ladderized. Bird species names are coloured by the family.

locality (electronic supplementary material, figure S10). There was only a marginally significant effect in *M. versteri* when evaluated using βMNTD and no effect in *M. megarhynchus* (electronic supplementary material, figure S10). Furthermore, pairwise comparisons of *M. versteri* microbiomes (Bray–Curtis) did not reveal significant differences between

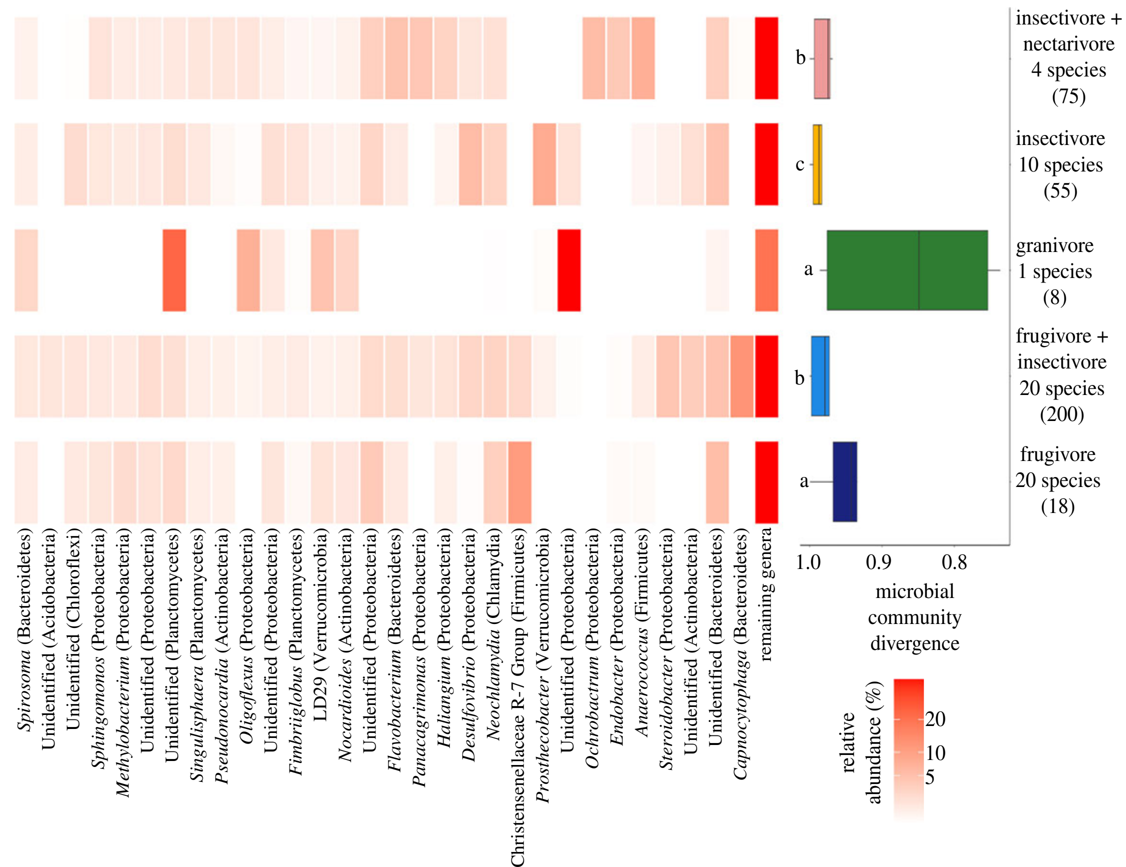

Figure 3. Abundance of bacterial genera and microbial heterogeneity differ by feeding guild. Heat map depicting the hierarchical clustering of 30 most abundant bacterial genera and their representation in each feeding guild (phylum is in parentheses). Box plots represent the microbial community divergence. Letters to the left of each box plot represent the results of Dunn's *post hoc* test. Numbers within parentheses indicate the sample sizes. (Online version in colour.)

localities (electronic supplementary material, figure S10*a*). By contrast, *M. megarhynchus* individuals from Wanang (200 m.a.s.l.) differed significantly from Dengenumbu (1700 m.a.s.l.) and Baitabag (200 m.a.s.l.) (electronic supplementary material, figure S10*b*). There was no significant effect of sampling locality for any of the remaining eleven species for either of the distance matrices (electronic supplementary material, figure S11), suggesting that locality does not strongly impact passerine gut microbiomes at the species-level, contradicting several previous studies [21,23,24].

(e) Bird microbiome heterogeneity is significantly associated with body mass

Average ASV richness per species demonstrated a marginally significant negative association with host body mass (figure 4*a*). We did not find an association between body mass and species-level PD (PGLS: $F = 0.5851$, $R^2 = 0.0164$, $p = 0.4495$). Overall, microbiome heterogeneity (divergence) was also significantly negatively associated with body mass (figure 4*b*). Heterogeneity measured with correction for sample sizes (three randomly picked individuals per species), demonstrated significantly negative associations with host body mass in three out of five random datasets (electronic supplementary material, figure S12). However, even the non-significant datasets demonstrated the tendency for negative associations between microbial divergence and body mass (electronic supplementary material, figure S12). Thus, average ASV richness and intraspecific variation are reduced in larger bird species, potentially associated with the slower gut retention time and more stable gut environments compared to smaller birds.

4. Discussion

Our findings demonstrate that the composition and intraspecific variation of species-specific, yet non-phylosymbiotic, passerine gut microbiomes are influenced by the combined effects of host diet and gut retention time. Despite the overall effect of locality on gut microbiomes, species-level analyses showed no effect of locality on the gut microbiome structure of bird species; likely as a result of similar habitats used by individuals from the same species across localities at similar elevations. This sheds new light on how diet, high individual variation, and flight-associated digestive tract modifications may be causal to the lack of/weak phylosymbiosis observed in bird microbiomes [10,13,14,16,17].

(a) Gut microbiomes are species-specific and secondarily influenced by host diet

The significant effect of host species on microbiomes, along with the lower PD than expected by chance in many species, suggests that passerine microbiomes are species-specific, consistent with other studies [13,23,25]. However, the extent of species specificity is compromised by extremely small core gut microbiomes, a positive association between sample size and total ASV richness, and high intraspecific variation. Differences in the diet of the individual bird may explain this,

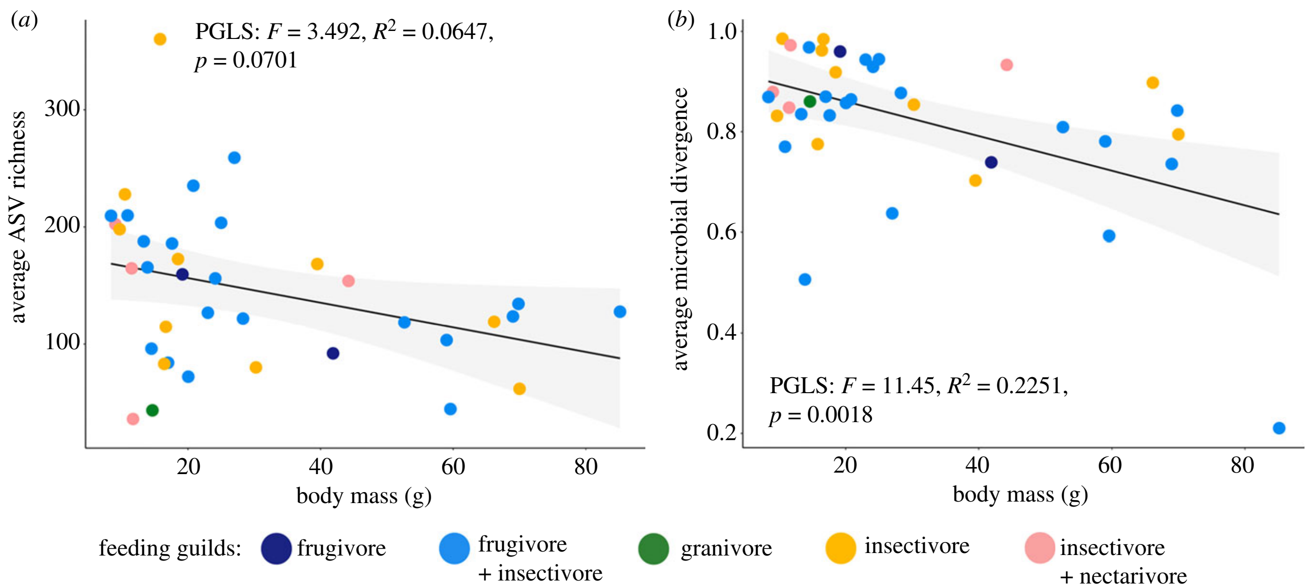

Figure 4. Host species body mass is negatively associated with (*a*) average ASV richness and (*b*) microbial heterogeneity. PGLS results are given within each plot and grey background indicates s.e.

as microbiomes (when bacterial phylogeny is not considered) are secondarily influenced by host diet, which is consistent with previously documented flexible responses in gut communities to diet changes [17,19,22,26].

Individual dietary intake of wild New Guinean birds varies greatly within a species [32], suggesting broader dietary niches than often assumed (e.g. previously designated insectivores are now often known to also feed on plant material [32]). The effects of this are also evident from the presence of multiple clusters in ordination plots of individuals from different localities (electronic supplementary material, figures S10 and S11). These PCoA clusters thus probably represent individuals consuming similar diets and consequently end up with similar gut microbial communities, despite the differences in their location. We only observed a strong effect of locality in *M. megarhynchus*, which is the only species in our dataset that occurs from lowlands to mid elevations. Food availability can differ markedly between elevations [32], potentially driving this effect (electronic supplementary material, figure S10b). This suggests that individual bird species have a large pool of potential gut microbial symbionts, but that variation in diet serves as a filter so that individuals only harbour a subset of these microbes [17].

(b) Gut retention time influences average amplicon sequence variant richness and microbiome heterogeneity

Flight-affiliated adaptations (short intestinal tracts and faster gut retention) [18] have been hypothesized to drive avian gut microbial community structure [10]. Fast gut passage potentially implies a less stable gut environment and higher microbial turnover, ultimately leading to high individual variation and small core microbiomes. In support of this, our results show that larger birds with longer intestinal tracts and hence longer retention times have reduced average gut microbiome richness and relatively lower intraspecific variation than smaller bird species. This appears comparable to other ecological systems. Herein, microbiomes of smaller birds represent smaller communities that will tend to experience stronger ecological drift leading to faster divergence in community composition and higher extinction rates, ultimately leading to higher and faster community turnover compared to larger communities/birds [54–56].

The association between gut physiology and microbiome stability is also evident in flight-less bird species, which tend to have more specific and stable microbiomes [10]. The link between microbial heterogeneity and body mass (even after controlling for sample sizes) implies that associations between gut bacterial symbionts and passerine bird hosts are likely to be less strict than those observed in other animal taxa [5–9,57]. These more fluid associations may explain the marked influence of habitat-associated diet differences on bird microbiomes [23,24]. Therefore, having a microbiome that can perform all the necessary and important functions in digestion, development and immunity probably depends little on the taxonomic composition of gut bacteria, but more on physiological capabilities—potentially served by a myriad of microbial taxa—that provide hosts with symbiont-derived benefits despite a less stable gut environment [58].

(c) Implications for host–symbiont evolution in passerines

Variable and malleable microbiomes of passerine birds imply the absence of tight long-term associations between specific bacterial taxa and specific host species. This is in line with previous suggestions that birds rely less on gut microbes [10]. However, this flexibility may have been important in facilitating the colonization of new niches during the radiation of the Passeriformes. Further, even if core microbiomes are small, species specificities imply non-random associations, so disentangling functional host–symbiont complementarity is needed to understand gut microbial roles in wild birds. Thus, while it is most likely that beneficial impacts exist [59], the fluid nature of the microbiomes are markedly different from the tighter long-term associations

seen for example in multiple clades of mammals [1,57,60,61]. Mammals, with some exceptions, tend to harbour more gut-adapted microbes, reduced transmission from the environment [1,60], more specialized digestive tracts [1,62] and more frequent vertical transmission from parents to offspring [63]. By contrast, high levels of transient microbes [1,10] and reduced complexity of digestive tracts in birds [18] appear to lead to fluctuating and variable microbiomes. The reduced stability of microbiomes, even within an individual's lifetime, would increase the variability over bird generations, ultimately resulting in no or weak phylosymbiosis. Notably, however, the gradient of microbiome stability driven by species-specific variation in gut retention time could predictably lead to a gradient in the level of adaptation in bird host–microbe associations [62]. This could ultimately lead to a gut physiology-driven gradient in the potential for coevolution or co-speciation between passerine hosts and specific gut bacterial symbionts.

Ethics. Birds were handled as gently as possible and released immediately after sample collection. Samples were collected under research visas 99902260244 (2017, 2018), 99902749307 (2019) to K.A.J., 99902341112 to K.H.B. and 9902077829 to K.S., and exported from Papua New Guinea under export permits 018208 (2017), 019067, 019069 (2018), 019362, 019422, 019423, and 019152 (2019).

Data accessibility. Microbiome data is available from the GenBank SRA archive (PRJNA673614, PRJNA673602, PRJNA673580, PRJNA673591) and the accession number of each sample is given in electronic supplementary material, tables S3 and S4. Supplementary file 1 in the electronic supplementary material provides the R scripts along with doi:10.5281/zenodo.4298695 to main data tables and phylogenetic trees.

Authors' contributions. K.H.B., K.A.J. and M.P. developed the idea; K.H.B., K.A.J., B.K., G.M. and K.S. collected samples; K.H.B. carried out the molecular work and data analyses; all authors critically revised the manuscript.

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