

Research



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A bird's-eye view of phylosymbiosis: weak signatures of phylosymbiosis among all 15 species of cranes

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In numerous animal clades, the evolutionary history of host species drives patterns of gut microbial community structure, resulting in more divergent microbiota with increasing phylogenetic distance between hosts. This phenomenon, termed phylosymbiosis, has been observed in diverse evolutionary lineages, but has been difficult to detect in birds. Previous tests of phylosymbiosis among birds have been conducted using wild individuals, and thus interspecific differences in diet and environment may have masked a phylogenetic signal. Therefore, we tested for phylosymbiosis among all 15 species of cranes (family Gruidae) housed in the same captive environment and maintained on identical diets. 16S rRNA sequencing revealed that crane species harbour distinct gut microbiota. Overall, we detected marginally significant patterns of phylosymbiosis, the strength of which was increased when including the estimates of absolute microbial abundance (rather than relative abundance) derived from microbial densities determined by flow cytometry. Using this approach, we detected the statistically significant signatures of phylosymbiosis only after removing male cranes from our analysis, suggesting that using mixed-sex animal cohorts may prevent the detection of phylosymbiosis. Though weak compared with mammals (and especially insects), these results provide evidence of phylosymbiosis in birds. We discuss the potential differences between birds and mammals, such as transmission routes and host filtering, that may underlie the differences in the strength of phylosymbiosis.

1. Introduction

There is mounting evidence that gut microbial community structure is strongly influenced by the evolutionary history of the host, resulting in more divergent microbiota with increased interspecific phylogenetic distance. To date, interspecific differences in gut microbial communities have been correlated with host phylogenetic relationships among natural populations of sponges [1], corals [2], arthropods [3], mammals [4] and primates [5,6]. Importantly, this phenomenon, known as phylosymbiosis, occurs even when host species are maintained in a common laboratory environment [7–9], suggesting that gut microbial communities assemble deterministically in accordance with evolutionarily divergent host phenotypes independent of extrinsic environmental variables. Therefore, a thorough investigation of phylosymbiosis across a variety of host lineages, especially those in which phylosymbiotic signatures may be subtle, is necessary to fully understand mechanistic underpinnings driving the correlation between gut microbial community composition and host evolutionary history.

Despite widespread evidence of phylosymbiosis across most major taxonomic groups, this phenomenon has yet to be observed in birds (class Aves). Birds are a diverse group of organisms that provide numerous ecological services [10], yet studies into their gut microbial ecology are extremely limited [11]. To date, there have been several attempts to link gut microbial community composition

to avian phylogenetic relationships [12–18], the most extensive of which was conducted using individuals from 491 species (representing 34 orders) of birds [18]. While most of these studies detected a statistically significant effect of taxonomy (e.g. species identity) [12–17], none reported congruence between the gut microbial community composition and host phylogeny. Notably, one study found a statistically significant correlation between interspecific differences in gut microbiota and passerine phylogenetic distances, but did not explicitly test for patterns of phyllosymbiosis [16]. While this lack of support for avian phyllosymbiosis could be a product of avian evolutionary history (e.g. weight-saving digestive adaptations) [18], it may also be a product of their sensitivity to extrinsic environmental factors or species-specific differences in natural feeding strategies across several avian orders [11,19]. Therefore, studies that reduce the influence of these variables using captive birds on identical diets may increase the chances of detecting subtle phyllosymbiotic signatures [7], thereby enabling the identification of the mechanisms and host factors driving this phenomenon [20,21].

The aim of this study was to characterize differences in gut microbiota across several closely related avian taxa in a controlled environment to determine whether some birds exhibit phyllosymbiosis. As noted above, previous attempts to detect phyllosymbiosis in birds may have been unsuccessful due to pronounced differences in habitat and diet across a taxonomically broad cohort spanning many avian orders. It is for these reasons that the use of computational or laboratory methods has been identified as a major goal for future phyllosymbiosis studies [22]. Therefore, we sought to control for these potentially confounding factors by characterizing the gut microbiota of all 15 species of cranes (all within the family Gruidae) maintained on identical diets in a shared captive environment. We further improved upon previous investigations of phyllosymbiosis by applying two recent methodological advances: (i) the correction of relative abundance values using microbial density estimates derived from flow cytometry analysis [23,24] and (ii) eco-evolutionary analysis of sequence variants via cladal taxonomic units [25]. Using these approaches, we tested whether evolutionary relationships between crane species were associated with changes in the gut microbiota, with the explicit prediction that dendrograms representing interspecific differences in gut microbial beta diversity would be more congruent with crane phylogeny than trees drawn at random.

2. Material and methods

(a) Animal husbandry and sample collection

We focused our study on a captive population of all 15 species of cranes (family Gruidae; electronic supplementary material, S1) at the International Crane Foundation (Baraboo, Wisconsin, USA). Cranes in this population are intended for education, conservation and captive breeding purposes, but offer a unique opportunity to test for patterns of phyllosymbiosis in the absence of confounding environmental factors. With the exception of two individuals, all cranes were born in captivity (electronic supplementary material, S1). Some cranes in this population have a history of antibiotic treatment, but not within two months of faecal sample collection (electronic supplementary material, S1). Captive cranes were housed individually (or in breeding pairs) in 15 × 18 m outdoor pens with chain-link fencing along each side and grass covered soil as a substrate. Each pen was covered by flight netting and included a 4.2 × 4.2 m indoor enclosure. Cranes are naturally

omnivorous and received identical diets in the form of pelleted food (Zeigler Crane Breeder or Maintenance Diet, Gardners, PA, USA; electronic supplementary material, S2), and fresh water was provided *ad libitum* in buckets within the enclosures. The birds were exposed to ambient temperature and natural photoperiod. None of the cranes had a history of chronic infectious disease, malnutrition or husbandry-related problems, and each bird was visually normal according to experienced keepers in the weeks preceding sample collection.

Because previous research has shown that faeces provides the best non-invasive approximation of the avian microbiome [26], we collected faecal samples from a total of 44 individuals across 15 crane species over a one-week period in October 2017 (range of 2–4 individuals per species; electronic supplementary material, S1). Cranes in this population varied in age (ranging from 1 to 55 years old) and sex (electronic supplementary material, S1). All samples consisted of a single, fresh-appearing voided faecal mass collected into sterile Whirl-Pak collection bags (Nasco, Fort Atkinson, WI) with a sterile cotton swab or sterilized tongue depressor. Faecal samples were immediately frozen at –80°C. All faecal samples were collected over the same 10-day period (electronic supplementary material, S1).

(b) Microbial density via flow cytometry

For flow cytometry, 200–500 mg of faecal sample was fixed in 4% paraformaldehyde in DMSO at a final dilution of 1 : 100 000. Samples were then syringe filtered (5 µm) to remove large particulates and eukaryotic host cells. Then, 949 µl of the filtered sample was stained with 1 µl SYBR green and spiked with 50 µl of CountBright counting beads (ThermoFisher product number C36950) as an internal reference, bringing the total volume of each sample to 1 ml. The flow cytometry analysis of the microbial cells present in each faecal sample was performed using a BD Biosciences LSR II Flow Cytometer with FACSDiva software (version 8). We used three gating parameters to exclude non-fluorescent, eukaryotic and clustered cell complexes: forward-angle light scatter (FSC), linear side scatter (SSC) and log fluorescein isothiocyanate (FITC). FITC versus event counting data plots were gated to distinguish SYBR-positive fluorescent events from non-fluorescent events. FSC-area versus SSC-area data plots were gated to distinguish CountBright internal reference beads from all other recorded events. FSC-height versus FSC-width data plots were gated to identify single prokaryotic cell events. These settings allowed the software to count 10 000 SYBR-positive (at emission wavelength of 530 nm) counting events that were distinguishable as single-cell bacteria and internal reference beads. Instrument, gating and event recording settings were identical for all samples. Raw flow cytometry data were used to estimate microbial density of faecal samples using the number of internal reference bead counts according to the manufacturer's instructions.

(c) Molecular analyses and bioinformatics

We isolated DNA from approximately 250 mg of crane faeces using the Qiagen PowerFecal DNA Kit with an overnight incubation in lysis buffer at 65°C to increase extraction yields [27]. Additionally, we conducted four 'blank' extractions alongside the faecal extractions to account for possible microbial DNA contaminants within commercial kits [28]. We used polymerase chain reaction (PCR) to amplify a portion of the bacterial 16S rRNA gene for Illumina sequencing using the primers 515F and 806R targeting the V4 region of microbial small subunit ribosomal RNA gene [29]. Amplified products were pooled in equimolar concentrations and loaded onto an Illumina MiniSeq mid-output flow cell (2 × 153 base paired-end reads). All library preparation, pooling and sequencing was performed at the DNA Services facility at the University of Illinois at Chicago. Sequence reads have been deposited in the NCBI SRA database under PRJNA553772.

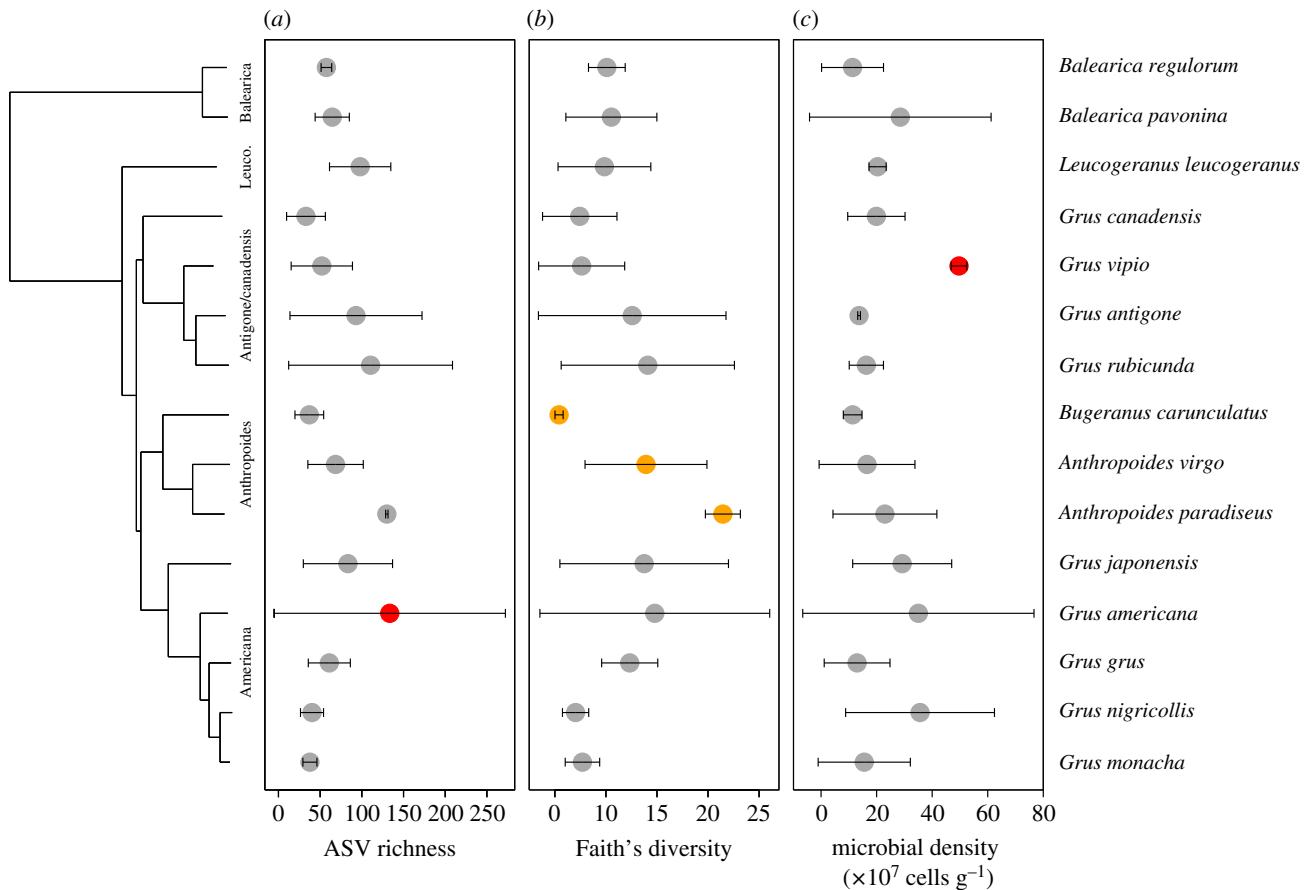


Figure 1. Phylogenetic patterns of crane gut microbial alpha diversity. (a) Observed bacterial ASV richness. (b) Faith's bacterial phylogenetic diversity. (c) Microbial density in cells/gram of faeces. Points represent means and error bars represent standard deviation. Red points indicate statistically significant ($p < 0.05$) local Moran's Index (I_i) values and orange points indicate trending I_i values ($0.05 \leq p < 0.1$). Sample size ranged from 2 to 4 individuals per species. (Online version in colour.)

Illumina sequencing reads were filtered and processed using the DADA2 pipeline [30] in QIIME2 version 2018.8 [31]. Bacterial 16S rRNA sequence variants (hereafter ASVs) were identified using the Greengenes reference database version 13.8 [32]. Illumina sequencing generated a total of 1.75 million reads (mean of 39 860 per sample) and 1339 ASVs after DADA2 processing. These sequences were further processed by removing non-bacterial ASVs (archaea, chloroplasts and mitochondria) in QIIME2, reducing our total number of reads to 1.6 million (mean of 36 104 per sample ± 2393 SE) and 1300 ASVs. We detected a total of 29 ASVs in four negative controls. Overall, the bacterial communities of these negative controls exhibited little overlap with crane faecal samples (electronic supplementary material, S3). Based on previous evidence suggesting that computationally removing contaminant ASVs may strongly influence our results and interpretation [33,34], we simply summarized the occurrence of these reads among kit controls and crane faecal samples (electronic supplementary material, S3).

Next, we generated two types of ASV tables for downstream comparisons of diversity metrics and tests of phylosymbiosis. First, we generated an evenly sampled ASV table using the standard approach of rarefying to the lowest sequence return among all samples (in our case, 8838) in QIIME2. This table was used to calculate metrics of alpha diversity (ASV richness and Faith's phylogenetic diversity) in QIIME2 and beta diversity (Bray–Curtis, unweighted UniFrac, weighted UniFrac) in the R package *Phyloseq* (v. 1.22.3) [35]. As a final step, we averaged ASV abundances by host species of origin for downstream analysis in CllaTU [25] and tests of phylosymbiosis. Second, we generated an unrarefied ASV table that accounted for differences in absolute microbial abundances across samples by correcting the

number of reads assigned to each ASV using flow cytometry derived microbial densities [23,24]. This was accomplished by first calculating the relative abundance of each ASV within a given sample, then multiplying the relative abundance value by the microbial density as estimated by flow cytometry. Again, we averaged ASV abundances by host species of origin for later tests of phylosymbiosis.

(i) Phylogenetic and statistical analyses

Using the molecular phylogeny of cranes [36], we investigated whether gut microbial ASV richness, Faith's phylogenetic diversity and microbial density were associated with interspecific crane phylogenetic distances using Moran's index of autocorrelation (I) [37,38] in the R package *Phylosignal* (v. 1.2) [39]. We then tested whether crane species deviated from expected (based on 999 random permutations) ASV richness, Faith's phylogenetic diversity and microbial density values using the local indicator of phylogenetic association (I_i) [40] in *Phylosignal* [39]. For both I and I_i , values range from -1 (perfect clustering of dissimilar samples) to 1 (perfect clustering of similar samples), with 0 indicating a perfect random association between microbial communities and host phylogeny.

For the metrics of beta diversity, we visualized differences in gut microbial community structure via principal coordinate analysis (PCoA) of Bray–Curtis distances using both rarefied and microbial density-corrected ASV relative abundances in the R package *Phyloseq* [35]. Additionally, we implemented this same approach using unweighted and weighted UniFrac distances, which incorporate microbial phylogeny into analyses [41]. We then used permutational analysis of variance (PERMANOVA)

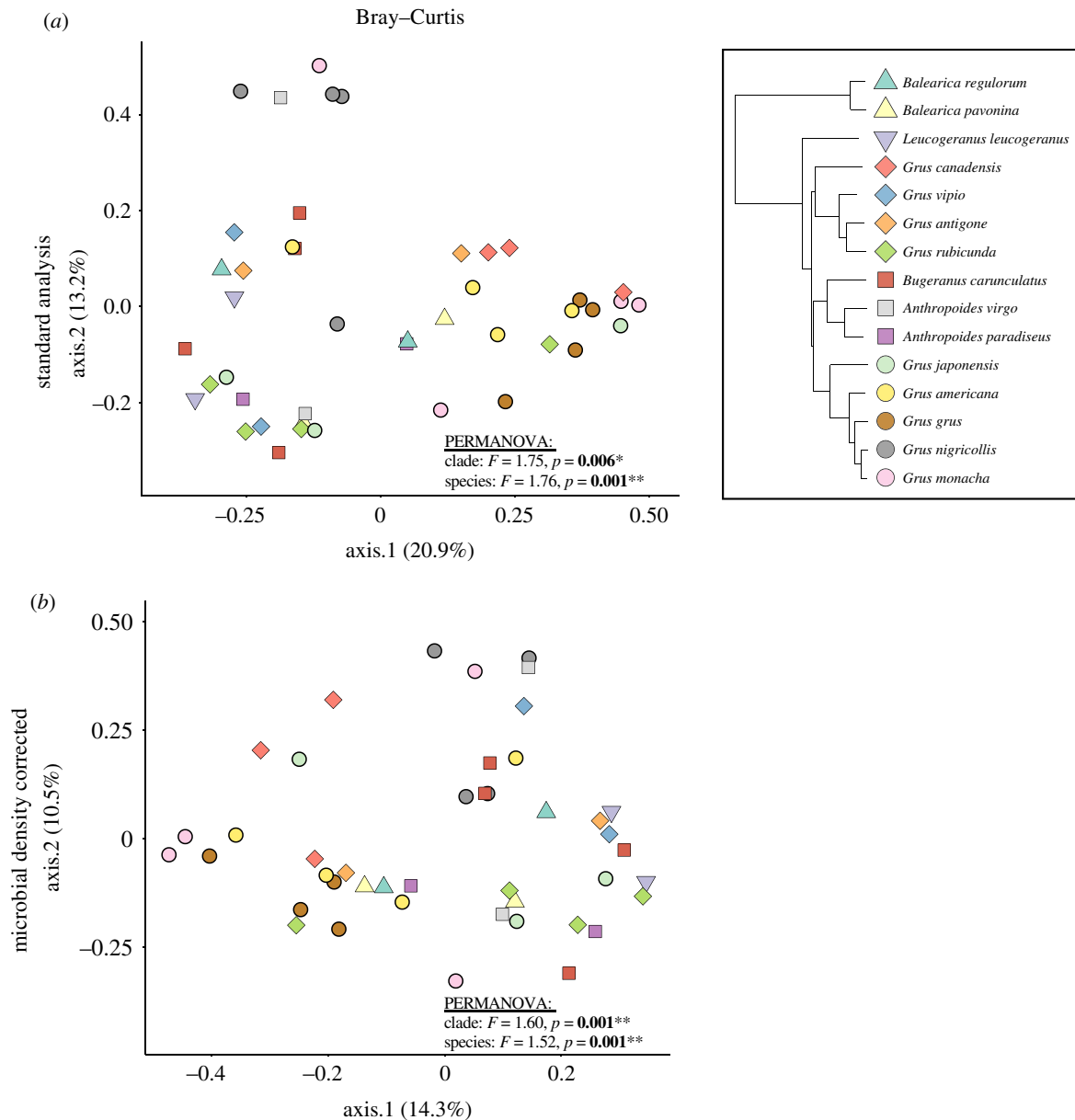


Figure 2. Taxonomic- and clade-level patterns of crane gut microbial beta diversity using Bray–Curtis distances. (a) PCoA plot based on the relative abundance of ASVs between crane species and clades. (b) PCoA ordination-based microbial density-corrected ASV tables. (Online version in colour.)

with random effects for sex in PRIMER with PERMANOVA+ v. 7.0.13 to determine whether gut microbial communities differed across (i) crane species and (ii) the five currently recognized clades within the crane molecular phylogeny (as defined in [36,42]): Balearica, Leucogeranus, Antigone/Canadensis, Anthropoides and Americana.

We used two approaches to investigate the distribution of microbial taxa across the crane phylogeny. First, we used linear discriminant analysis effect size (LEfSe version 1.0) [43] on rarefied ASV tables to identify microbial taxa with statistically significant differences in relative abundances across crane species. LEfSe was conducted using default settings ($LDA \geq 2.0, p \leq 0.05$) at the bacterial phylum and genus levels in Galaxy (<http://huttenhower.sph.harvard.edu/galaxy/>). However, this approach is limited by Linnaean taxonomic classifications and thus may not fully capture differences in gut microbial communities that occur at intermediate levels of taxonomy [25]. To address this limitation, we investigated the distribution of microbial taxa across the crane phylogeny using rarefied ASV tables and cladal taxonomic units with ClaaTU (v. 0.1) [25], which groups microbial lineages into ecologically relevant taxonomic units. In our case, we used ClaaTU to identify microbial

taxa that were more prevalent among crane clades (as defined above) than expected by chance. We corrected p -values for multiple comparisons in R using a relatively permissive false-discovery rate [44] threshold of $p \leq 0.2$ to identify microbial taxa that may explain the patterns of phylosymbiosis among cranes in this dataset.

Finally, we constructed microbial dendrograms from both rarefied and microbial density-corrected ASV tables to test whether host microbial community dendrograms exhibited the patterns of phylosymbiosis with the crane phylogeny. Using averaged ASV tables (see details above), we calculated Bray–Curtis, unweighted UniFrac and weighted UniFrac distances in the R package *Phyloseq* [35]. Beta diversity distance matrices were then clustered using the UPGMA method in R (function: `hclust()`, `method = 'average'`) [45]. We tested for patterns of phylosymbiosis by comparing gut microbiota dendrograms with the crane phylogeny via the Robinson–Foulds and matching cluster metrics with 100 000 random trees using a previously published Python script [7]. While both of these methods are acceptable for assessing topological congruency between trees, the matching cluster method accounts for incongruences between closely related branches and is therefore considered a more refined approach for detecting phylosymbiosis

[7]. Both of these approaches produce a normalized score between 0 (complete congruence) and 1 (complete incongruence). p -values were determined by the probability of 100 000 randomized dendrogram topologies yielding equivalent or more congruent phyllosymbiotic patterns than the actual microbiota dendrogram.

3. Results

First, we investigated whether interspecific variation in metrics of crane gut alpha diversity richness was correlated with host phylogenetic distances using Moran's index [37]. We observed a non-significant correlation between host interspecific phylogenetic distances and gut bacterial richness ($I = -0.069$, $p = 0.423$; figure 1a), Faith's phylogenetic diversity ($I = -0.055$, $p = 0.354$; figure 1b) and microbial density ($I = -0.165$, $p = 0.934$; figure 1c). We then tested whether individual crane species exhibited gut microbial richness, diversity and density that differed from random permutations using the local indicator of phylogenetic association (I_i). Using this approach, *G. americana* exhibited higher gut microbial richness than expected at random ($I_i = -0.521$, $p = 0.013$; figure 1a). For Faith's phylogenetic diversity, we observed a trend ($0.05 < p < 0.1$) in local phylogenetic signal for all three members of the *Anthropoides* clade (figure 1b): *G. carunculatus* ($I_i = -0.297$, $p = 0.059$), *A. virgo* ($I_i = 0.133$, $p = 0.072$) and *A. paradiseus* ($I_i = -0.298$, $p = 0.083$). Only one species, *G. vipio*, exhibited higher microbial density than expected at random ($I_i = -0.696$, $p = 0.017$; figure 1c). Within a crane species, males and females harboured similar gut microbiota (electronic supplementary material, figure S1).

Next, we investigated whether gut bacterial communities were distinct among crane species and phylogenetic clades. Using the standard rarefaction-based analysis and Bray–Curtis distances, we found a statistically significant effect of species (PERMANOVA pseudo- $F_{44,15} = 1.76$, $p = 0.001$) and clade (PERMANOVA pseudo- $F_{44,15} = 1.75$, $p = 0.006$; figure 2a). When correcting the relative abundance of sequencing reads by microbial density estimates (derived from flow cytometry analysis), we were able to detect both species- (PERMANOVA pseudo- $F_{44,5} = 1.52$, $p = 0.001$) and clade-level (PERMANOVA pseudo- $F_{44,5} = 1.60$, $p = 0.001$) differences in gut microbial community structure (figure 2b). Additionally, we performed this same analysis using phylogenetically aware metrics of beta diversity (i.e. unweighted and weighted UniFrac). For unweighted UniFrac, we found a statistically significant effect of species on crane gut microbiota using both standard rarefaction-based (PERMANOVA pseudo- $F_{44,5} = 1.37$, $p = 0.010$) and microbial density analyses (PERMANOVA pseudo- $F_{44,5} = 1.35$, $p = 0.015$; electronic supplementary material, figure S2). By contrast, there was no effect of crane clade using either approach ($p > 0.05$; electronic supplementary material, figure S2). For weighted UniFrac, there was no effect of species or clade in any case ($p > 0.05$; electronic supplementary material, figure S2).

From a total of 1300 ASVs, we identified a total of 19 bacterial phyla and 215 genera across all crane species. Regardless of host species, crane gut microbiota was almost entirely composed of the phyla Firmicutes (mean \pm standard error: $76 \pm 3\%$) and Proteobacteria ($15 \pm 2\%$; figure 3a), which translated to an average of 17×10^7 (± 2.55 SE) and 3.6×10^7 (± 0.55 SE) microbial cells/gram of faecal material, respectively (figure 3b). Despite these broad-scale similarities based on

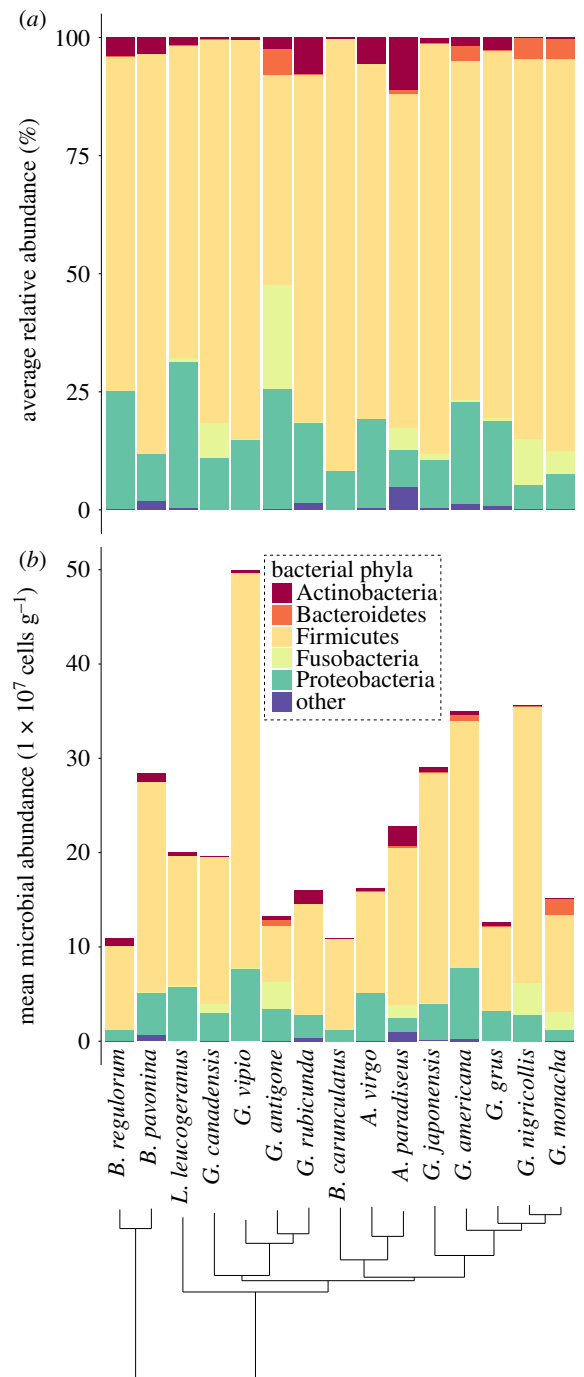


Figure 3. Relative versus absolute abundances of microbial phyla in the crane gut microbiota. Phylum-level gut microbial community composition among all 15 species of cranes ($n = 2$ to 4 individuals per species). (a) Standard approach using rarefied ASV tables to estimate the relative abundance of bacterial phyla. (b) Mean microbial abundance estimates based on un-rarefied, microbial density-corrected ASV tables. Only the five most abundant microbial phyla are presented, with less abundant phyla pooled into 'other'. (Online version in colour.)

Linnaean taxonomy, clades within the crane phylogeny exhibited statistically significant differences in the presence of eco-phylogenetic groups of bacteria using the ClaaTU workflow (figure 4). Among the 484 eco-phylogenetic groups that were conserved in at least one crane clade, we found 87 instances where a group occurred within a specific crane clade more than expected at random (FDR adjusted $P \leq 0.2$; figure 4). Most of these groups were classified as Firmicutes (70%), specifically those in the orders Clostridiales (33%) and Lactobacillales (17%). At the genus level, LEfSe analysis

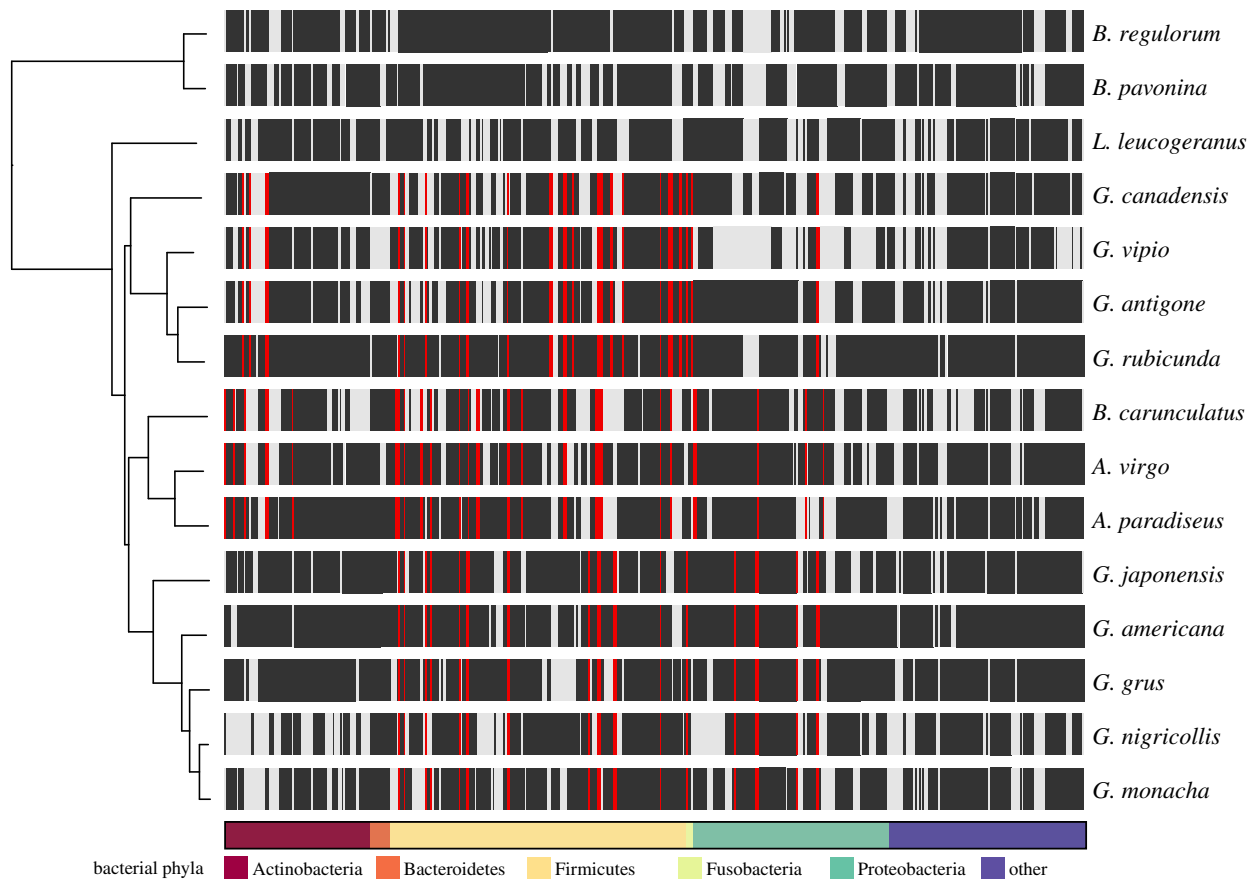


Figure 4. Patterns of conserved bacterial eco-phylogenetic groups across the crane phylogeny. The 484 eco-phylogenetic groups that were conserved in at least one crane clade are plotted as columns in a heatmap that illustrates their occurrence across the crane phylogeny. Black ticks indicate an eco-phylogenetic group was detected in a given crane species. Red ticks indicate an eco-phylogenetic group with a statistically significant (FDR adjusted $p \leq 0.2$) association with a crane clade. (Online version in colour.)

revealed that *G. canadensis* exhibited significantly higher relative abundances of the bacterial genus *Lactobacillus* compared with other crane species (LDA = 5.50, $p = 0.040$). By contrast, the *A. paradiseus* exhibited a significantly lower relative abundance of the bacterial genera *Corynebacterium* (LDA = 5.14, $p = 0.020$) and *Streptococcus* (LDA = 4.90, $p = 0.017$). There were no differentially abundant phyla across crane species (LDA > 2.0, $p > 0.05$).

Finally, we investigated for the patterns of phyllosymbiosis by comparing microbial community dendrograms and the crane phylogenetic tree. Because phyllosymbiosis predicts that phylogenetic relatedness will correlate with beta diversity relationships of microbial communities [7], we expected that microbiota and crane dendrograms would exhibit more congruence than expected at random. Using the UPGMA trees constructed using the standard rarefaction pipeline and Bray–Curtis distances, we found no statistically significant congruence between crane phylogeny and microbiota dendrograms using normalized Robinson–Foulds (nRF = 1.0, $p = 1.0$) and a non-significant trend when using the matching cluster method (nMC = 0.518, $p = 0.086$; table 1). UPGMA trees based on weighted and unweighted and UniFrac distances produced similar non-significant results (table 1). When using ASV relative abundances that were corrected for microbial density, we detected a slightly stronger signal of phyllosymbiosis using both normalized Robinson–Foulds (nRF = 0.917, $p = 0.193$) and matching cluster methods (nMC = 0.506, $p = 0.063$; table 1, figure 5). Again, weighted and unweighted and UniFrac distances produced similar non-significant results (table 1). As a final test, we also conducted an analysis using only female

cranes to determine if sex affected our ability to detect phyllosymbiosis. Using this approach, we detected a statistically significant topological congruence using normalized Robinson–Foulds with uncorrected Bray–Curtis distances (nRF = 0.470, $p = 0.011$) as well as matching cluster method using weighted UniFrac distances (nMC = 0.494, $p = 0.017$; table 1).

4. Discussion

The aim of this study was to characterize differences in gut microbiota across crane species to determine whether the gut communities of birds exhibit phyllosymbiosis. Indeed, we detected the effects of crane species and clade on gut microbial community structure using standard rarefaction-based analysis and when correcting relative abundances with estimates of microbial density. These interspecific differences in gut microbial community structure yielded a statistically significant pattern of phyllosymbiosis in this dataset containing all 15 species of cranes, but only once all male cranes were removed from our analysis. Below, we contextualize these findings with respect to previous research on the avian gut microbiome and phyllosymbiosis in general, as well as discuss the potential differences between birds and mammals that may underlie the differences in the strength of phyllosymbiosis.

When comparing across host species, we found that the crane gut microbiota exhibited major similarities at the phylum level while harbouring distinct gut microbial communities at finer taxonomic scales. In terms of similarities, we found that the crane gut microbiota, regardless of host species,

Table 1. Summary of phylosymbiosis test statistics using Robinson–Foulds and matching cluster methods on rarefaction-based and microbial density-corrected distance matrices. Both of these approaches produce a normalized score between 0 (complete congruence) and 1 (complete incongruence). p -values were determined by the probability of 100 000 randomized dendrogram topologies yielding equivalent or more congruent phylosymbiotic patterns than the actual microbiota dendrogram. * $p < 0.05$; ** $0.05 \leq p < 0.1$.

		distance metric	host-microbe score	max congruence	normalized score	p	
Robinson–Foulds	all cranes	Bray–Curtis	12	12	1.000	1.000	
		unweighted UniFrac	12	12	1.000	1.000	
		weighted UniFrac	12	12	1.000	1.000	
		corrected Bray–Curtis	11	12	0.917	0.193	
		corrected unweighted UniFrac	12	12	1.000	1.000	
		corrected weighted UniFrac	11	12	0.916	0.194	
	females only	Bray–Curtis	12	12	1.000	1.000	
		unweighted UniFrac	12	12	1.000	1.000	
		weighted UniFrac	12	12	1.000	1.000	
		corrected Bray–Curtis	12	12	1.000	1.000	
		corrected unweighted UniFrac	11	12	0.916	0.194	
		corrected weighted UniFrac	12	12	1.000	1.000	
	matching cluster	all cranes	Bray–Curtis	44	85	0.518	0.086**
			unweighted UniFrac	44	85	0.518	0.086**
weighted UniFrac			46	79	0.582	0.171	
corrected Bray–Curtis			43	85	0.506	0.063**	
corrected unweighted UniFrac			50	81	0.617	0.485	
corrected weighted UniFrac			51	85	0.600	0.592	
females only		Bray–Curtis	39	83	0.470	0.011*	
		unweighted UniFrac	51	83	0.615	0.591	
		weighted UniFrac	48	81	0.593	0.306	
		corrected Bray–Curtis	44	83	0.530	0.086**	
		corrected unweighted UniFrac	46	85	0.541	0.170	
		corrected weighted UniFrac	40	81	0.494	0.017*	

was primarily composed of taxa in the phylum Firmicutes (76%). The dominance of Firmicutes is consistent with previous descriptions of crane gut microbiota [46,47] and the avian gut microbiome in general [11]. However, cranes in our study harboured a higher relative abundance of Firmicutes compared with the previous studies of crane gut microbiota [46,47] and was similar to that reported in mammals [48,49]. While the potential effects of Firmicutes on crane physiology are currently unknown, studies in mammals [50] and chickens [51] have demonstrated that Firmicutes are important to host metabolism and digestive health, most likely through their ability to produce short-chain fatty acids through the breakdown of dietary carbohydrates and polysaccharides [52]. In terms of differences in gut

microbial communities, ClaaTU analysis revealed that taxa within the phylum Firmicutes and the order Lactobacillales were differentially conserved among crane clades. Further investigation using LEfSe revealed that *Lactobacillus* (phylum Firmicutes) was differentially abundant across crane species, which is notable since bacteria in this genus are associated with weight gain and increased concentrations of short-chain fatty acids in chickens [51,53]. Further, the bacterial genus *Corynebacterium* (phylum Actinobacteria) was less abundant in the short-range migrant *A. paradiseus*. This is notable since previous research has demonstrated that non-migratory resident individuals exhibit a reduced relative abundance of *Corynebacterium* compared with migratory conspecifics [54]. While crane

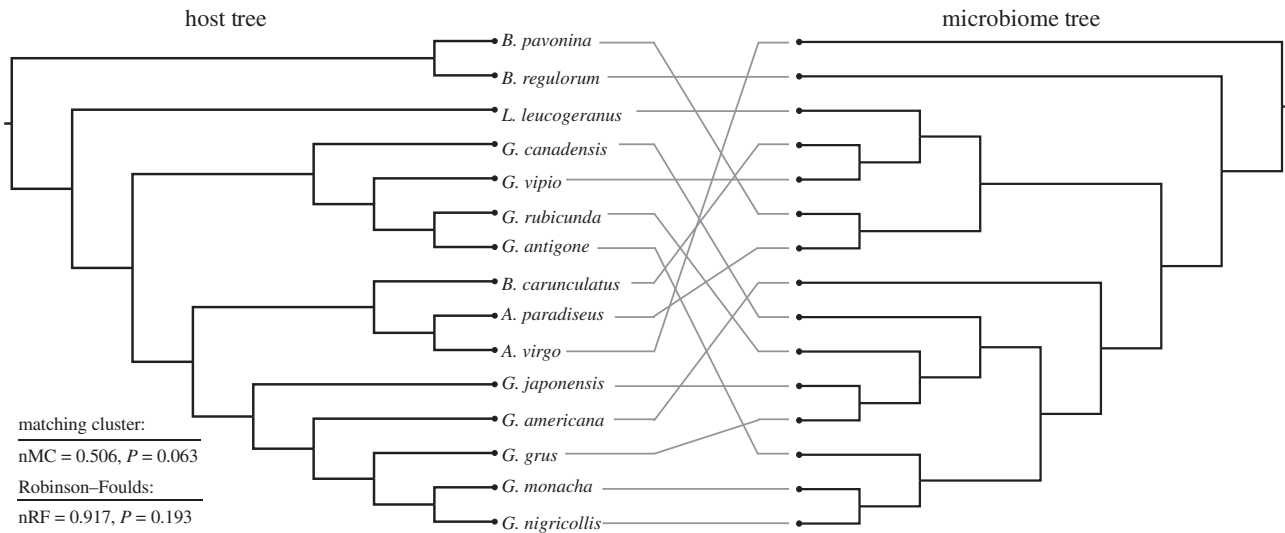


Figure 5. Comparison of host and gut microbiome dendrograms. Host phylogenetic tree is from Krajewski *et al.* [36] and microbiome tree was constructed by UPGMA clustering of microbial density-corrected ASV tables and Bray–Curtis distances.

migratory behaviours vary across species and populations, the reduced relative abundance of this bacterial genus in a short-range migrant suggests that the microbiome could contribute to some aspect of migratory physiology, though these functional roles are currently unknown and warrant further investigation.

Another major finding of our study is that correcting ASV relative abundances using microbial densities improved the distinguishability of gut microbial communities across host species and enabled the detection of a statistically significant signature of phylosymbiosis. To date, the qualitative and quantitative investigations of phylosymbiosis have focused solely on the relative abundances of microbial taxa [7,21]. However, there have been numerous calls to incorporate cell quantification techniques into gut microbiome studies [23,24,55], as differences in absolute abundances could confound results based on relative abundances. Consistent with previous studies in mammals [56], we observed statistically significant interspecific differences in microbial density across crane species with substantial variation among conspecifics (figure 1). Analysing microbial densities separately for males and females greatly reduced intraspecific variation (electronic supplementary material, figure S1), suggesting that host sex may affect microbial carrying capacity. The incorporation of absolute abundance data into our analyses increased the distinguishability and revealed a statistically significant signal of phylosymbiosis among female cranes. This result is noteworthy since previous research has been unable to demonstrate that avian lineages exhibit patterns of phylosymbiosis [12–16]. One caveat to these findings is that a given bacterial species can possess anywhere between 1 and 15 copies of the 16S rRNA gene [57], and thus 16S rRNA relative abundance values should be interpreted with caution [58]. While correcting 16S rRNA relative abundance values by absolute microbial abundance cannot address this fundamental limitation, the substantial variation in microbial densities across crane species provide sufficient justification for this approach. Therefore, we argue that the future studies of phylosymbiosis should incorporate the measurements of microbial density and possibly corrections for 16S rRNA gene copy number.

Overall, the phylosymbiotic pattern of cranes was weak compared with those observed in mammals (and especially insects) [7]. These differences may be due to the unique life-history traits exhibited by avian host species [11,19]. For example, mammals are thought to inherit their gut microbiota through

contact with vaginal and faecal microbes during the birth process [59]. Vertical transmission of the microbiota in birds may occur through parental care, though this has not been explicitly demonstrated, especially in birds like cranes that do not regurgitate food to their young. Additionally, some evidence exists for potential maternal microbial transmission through eggs [27]. Theoretical simulations suggest that ecological filtering or selection of microbes by the host underlies the emergent property of phylosymbiosis [21]. Thus, physiological differences between mammals and birds may explain the differences in the strength of phylosymbiosis across these groups. For example, the aspects of ‘oral tolerance’, or the immune non-responsiveness to ingested antigens varies between mammals and birds, with birds having a limited developmental window where oral tolerance occurs [60]. Additionally, birds (especially those in the order Gruiformes) exhibit a much more acidic stomach than mammals [61], thereby acting as a microbial filter that may limit the degree to which host species can differ in gut microbial communities. Understanding the mechanisms by which mammals and birds differentially structure their gut microbiome according to host evolutionary history is an open question in the field.

In this study, we provide the most explicit and well-controlled test of phylosymbiosis in birds and demonstrate statistically significant (but overall weak) patterns of phylosymbiosis among a distinct avian clade. Because phylosymbiosis has been demonstrated to have a functional role in other host groups [7], these patterns likely influence the health and performance of crane species, and thus may have implications for crane conservation. More broadly, this study demonstrates that even weak patterns of phylosymbiosis may be detected with controlled experimental design and inclusion of microbial densities into analyses. These findings represent a substantial contribution to the microbiome research community, as the detection of even weak patterns of phylosymbiosis may be necessary to understand mechanisms driving the correlation between gut microbial community composition and host evolutionary history.

Data accessibility. Raw sequence reads have been deposited in the NCBI SRA database under PRJNA553772. R code and associated data files are available as part of the electronic supplementary material.

Authors' contributions. B.K.T. conducted DNA extractions, analysed data and wrote the paper. J.S. conducted flow cytometry experiments.

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