



Comparative Gut Microbiota of 59 Neotropical Bird Species

Sarah M. Hird^{1,2*}, César Sánchez^{1,2}, Bryan C. Carstens³ and Robb T. Brumfield^{1,2}

¹ Museum of Natural Science, Louisiana State University, Baton Rouge, LA, USA, ² Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, USA, ³ Department of Evolution, Ecology and Organismal Biology, Ohio State University, Columbus, OH, USA

OPEN ACCESS

Edited by:

Boran Kartal,
Radboud University, Netherlands

Reviewed by:

David William Waite,
University of Auckland, New Zealand
Michael Roggenbuck,
University of Copenhagen, Denmark
Kevin Kohl,
Vanderbilt University, USA

*Correspondence:

Sarah M. Hird
shird@ucdavis.edu

† Present Address:

Sarah M. Hird,
UC Davis Genome Center, University
of California Davis, Davis, CA, USA

Specialty section:

This article was submitted to
Evolutionary and Genomic
Microbiology,
a section of the journal
Frontiers in Microbiology

Received: 23 April 2015

Accepted: 25 November 2015

Published: 21 December 2015

Citation:

Hird SM, Sánchez C, Carstens BC
and Brumfield RT (2015) Comparative
Gut Microbiota of 59 Neotropical Bird
Species. *Front. Microbiol.* 6:1403.
doi: 10.3389/fmicb.2015.01403

The gut microbiota of vertebrates are essential to host health. Most non-model vertebrates, however, lack even a basic description of natural gut microbiota biodiversity. Here, we sampled 116 intestines from 59 Neotropical bird species and used the V6 region of the 16S rRNA molecule as a microbial fingerprint (average coverage per bird ~80,000 reads). A core microbiota of Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria was identified, as well as several gut-associated genera. We tested 18 categorical variables associated with each bird for significant correlation to the gut microbiota; host taxonomic categories were most frequently significant and explained the most variation. Ecological variables (e.g., diet, foraging stratum) were also frequently significant but explained less variation. Little evidence was found for a significant influence of geographic space. Finally, we suggest that microbial sampling during field collection of organisms would propel biological understanding of evolutionary history and ecological significance of host-associated microbiota.

Keywords: Neotropical birds, gut microbiota, V6, host-associated microbiota, evolution

INTRODUCTION

Gut microbiota are an essential component of vertebrate health. Microbes provide many necessary functions for the host organism, including aiding digestion, vitamin synthesis, protection against pathogens, training the immune system and organ development (Qin et al., 2010; Diaz Heijtz et al., 2011; Al-Asmakh et al., 2014). The gut microbiota is one of the most densely populated natural environments known (Whitman et al., 1998), possibly composed of thousands of species (Xu and Gordon, 2003). Generally speaking, gut microbiota communities tend to be more similar between more similar hosts, although the specific members of the microbiota can vary significantly between hosts of the same species (Eckburg et al., 2005; Hird et al., 2014) and even between identical twins (Turnbaugh et al., 2010). The microbiota may have an influence above the level of the individual, as they can affect mate choice (Sharon et al., 2010) and cause hybrid inviability (Brucker and Bordenstein, 2013). Understanding the role of the microbiota in evolution is a major outstanding question and the subject of much ongoing research.

Birds are a globally distributed class of vertebrates. The bird lineage is thought to be approximately 150 million years old and has likely been in symbiotic relationships with microorganisms the entire time. Birds live on every continent and exhibit extreme morphological and ecological diversity, much of which is centered in the Neotropics (Jenkins et al., 2013). Pertinent to gut microbiota, bird diets range from robust and opportunistic to strictly carrion feeding (i.e., vultures) to nectar feeding (i.e., hummingbirds) to folivorous (i.e., hoatzin) with corresponding variation in their intestinal morphology. Most avian gut microbiota studies have

looked at one or a few host species; from these studies we know that bird gut microbiota are influenced by host genetics and evolutionary history (Banks et al., 2009; Dewar et al., 2013) as well as by ecological factors, such as dietary specialization (Roggenbuck et al., 2014). The gut communities can also change as a result of seasonal dietary fluctuations (Wienemann et al., 2011).

Modern molecular techniques provide an insight into the biodiversity contained within the guts of birds. Firmicutes, Proteobacteria, and Actinobacteria tend to dominate avian gut samples (summarized in Waite and Taylor, 2014), although captivity status can have a significant effect on microbial composition (Xenoulis et al., 2010; Wienemann et al., 2011; Kohl, 2012). Most gut microbiota studies have not included physical space as a variable that may be contributing to microbial variation in host gut communities. Geographic distance is associated with gut microbiota similarity (Yatsunenkov et al., 2012) and, generally speaking, bacterial communities that are geographically closer are more similar than communities more distant (Green and Bohannan, 2006). Avian gut microbiota have shown conflicting results regarding the importance of geographic distance; whereas some studies have found it to be associated with the gut microbiota (Hird et al., 2014), others have looked but found no such evidence (Banks et al., 2009).

Large comparative studies inform us about the relationship between higher taxonomic classes and broader ecological groups. In mammals, such studies have found that host taxonomy is strongly associated with gut microbiota communities (Ley et al., 2008a) as is dietary specialization (Ley et al., 2008b; Muegge et al., 2011). Diet seems to be the most important factor across insects (Anderson et al., 2012; Colman et al., 2012) and environmental variables have the most influence on gut microbiota in fishes (Sullam et al., 2012), although taxonomy may also have a role. A recent meta-analysis of all previously published bird gut microbiota studies found that taxonomy of the bird had a major influence on the composition of the gut microbes (Waite and Taylor, 2014). Understanding how various host and environmental factors interact to shape the gut microbiota is a major goal of modern microbial ecology and evolution.

A molecular survey of the gut microbiota is a basic biodiversity measure that is lacking for the vast majority of wild bird species. Our primary aim for the current study was to create a microbial catalog for the gut microbiota diversity found in 59 Neotropical bird species sampled in the wild. Second, we test for taxonomic and ecological associations between bird host and gut microbiota. Finally, we tested for a geographic signal in our dataset, which was gathered from 12 localities across Costa Rica and Peru.

MATERIALS AND METHODS

Sampling

The large intestine was extracted from 108 birds in Costa Rica and seven in Peru (Table 1, Figure 1, Supplemental Table S1) during fieldwork conducted between May and August 2010 [LSU IACUC protocol 09-001; Sistema Nacional de Areas de

Conservación (SINAC) permit number: 109-2010-SINAC; access to genetic material by Comisión Nacional para la Gestión de la Biodiversidad (CONAGEBio) under permit: R010-2010-OT-CONAGEBIO]. Immediately after euthanization, birds were dissected and the largest section of undisturbed large intestine tied off, cut out and deposited into CryoVials. Samples were frozen in liquid nitrogen, following the protocol of Godoy-Vitorino et al. (2010) and kept frozen at the LSU-MNS Collection of Genetic Resources until microbial DNA extraction (using Qiagen Power Soil extraction kits). We focused on bird species found in lowland forests across Costa Rica whose ranges extend south into the Peruvian Amazon.

Bird DNA Sequencing and Phylogenetic Tree Estimation

DNA was extracted from the liver, heart or pectoral muscle of birds using the standard tissue protocol from Qiagen DNeasy kits. The mitochondrial locus ND2 was sequenced using the L5215 (5' TAT CGG GCC CAT ACC CCG AAA AT 3') and H6313 (5' CTC TTA TTT AAG GCT TTG AAG GC 3') primers and a thermocycler program of 94°C for 2 min followed by 35 cycles of 94°C for 60 s, 45°C for 30 s, 72°C for 60 s and a final extension of 7 min at 72°C (Brumfield et al., 2007). Amplicons were cleaned using a BigDye Terminator kit and sequenced on ABI PRISM 3730xl at Beckman Coulter Genomics (Danvers, Massachusetts). The sequences were aligned with MUSCLE (Edgar, 2004) and we used BEAST (Drummond and Rambaut, 2007) to generate a sample from the posterior distribution of phylogenetic trees given the data (partitioned GTR + Γ model with estimated base frequencies). Because many of the internal branches of a multi-order bird phylogeny are short and several genomic-scale datasets have recently resolved some of these common conflicts, we also constrained the tree's internal nodes to reflect current genomic understanding of bird taxonomy (Klicka et al., 2007; Tello et al., 2009; Barker et al., 2013).

Microbial Fingerprint

Following Gloor et al. (2010) we used combinatoric primers and massive multiplexing of PCR amplicons for sequencing on an Illumina Hi-Seq. This method uses paired-end sequencing technology to generate pairs of sequences with 100% overlap across variable region 6 (V6) of the 16S component of rRNA; primer sequences align to positions 967–985 and 1078–1061 on *Escherichia coli* 16S rRNA (Gloor et al., 2010). We chose the V6 region of 16S because it is short enough that the sequencing technology was able to cover the entire region in both directions but variable enough to differentiate bacterial species (Chakravorty et al., 2007).

Several samples were extracted or amplified more than once to quantify differences along the digestive tract and/or determine how sensitive the methods are to differences. One sample was amplified and sequenced a second time from a single extraction; these replicates are *Cyanocompsa.cyanoides.1.1* and *Cyanocompsa.cyanoides.1.2*. Three birds had two extractions completed and were sequenced independently: *Attila.spadiceus.1* had one extraction from the posterior large intestine and the other from the anterior large intestine; *Trogon.rufus.2* had

TABLE 1 | Order, family, genus, species, sampling locality, and number of samples used in this study; sampling localities mapped on Figure 1.

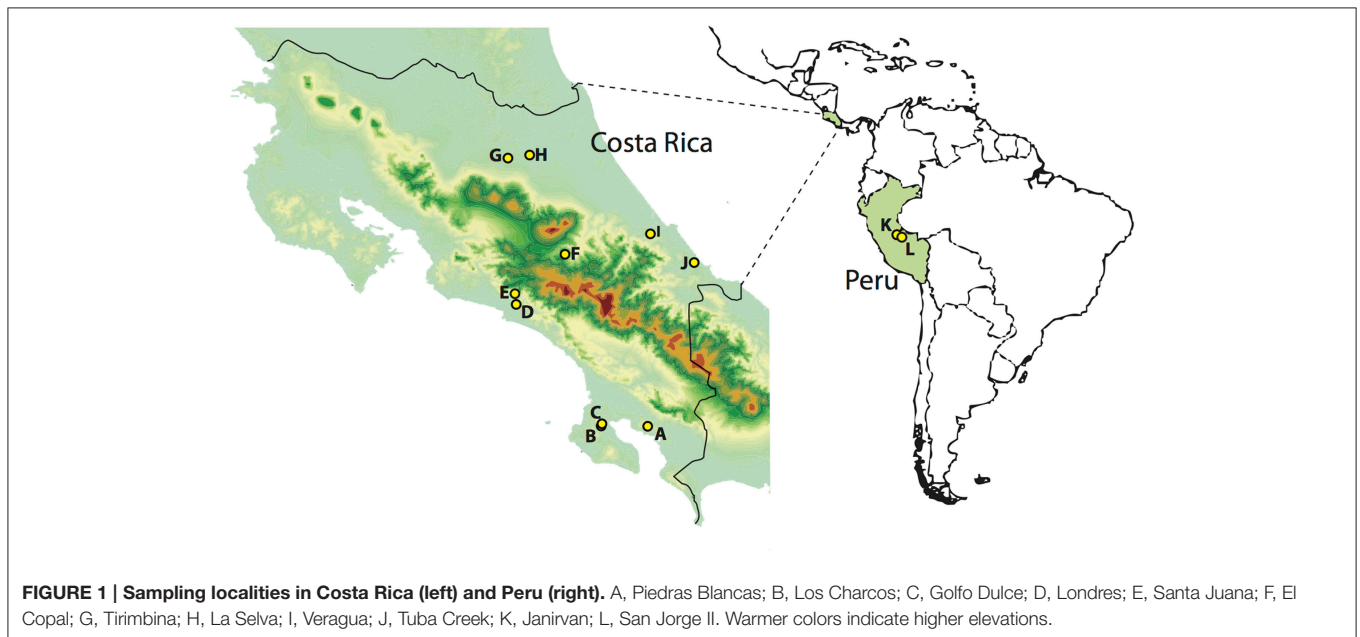
Order	Family	Genus	Species	Sampling locality												
				A	B	C	D	E	F	G	H	I	J	K	L	
Apodiformes	Trochilidae	<i>Amazilia</i>	<i>tzacatl</i>									1				
Apodiformes	Trochilidae	<i>Florisuga</i>	<i>mellivora</i>			2						1				1
Apodiformes	Trochilidae	<i>Phaethornis</i>	<i>longirostris</i>		2	1										
Apodiformes	Trochilidae	<i>Thalurania</i>	<i>colombica</i>													
Apodiformes	Trochilidae	<i>Threnetes</i>	<i>ruckeri</i>		1							3				
Caprimulgiformes	Caprimulgidae	<i>Nyctidromus</i>	<i>albicollis</i>											2		
Columbiformes	Columbidae	<i>Geotrygon</i>	<i>montana</i>				1									
Coraciiformes	Momotidae	<i>Baryphthengus</i>	<i>martii</i>									1				
Cuculiformes	Cuculidae	<i>Piaya</i>	<i>cayana</i>											1		
Passeriformes	Cardinalidae	<i>Cyanocompsa</i>	<i>cyanooides</i>				2					2				3
Passeriformes	Cardinalidae	<i>Habia</i>	<i>atrimaxillaris</i>		1	1										
Passeriformes	Cardinalidae	<i>Habia</i>	<i>fuscicauda</i>													
Passeriformes	Emberizidae	<i>Arremon</i>	<i>aurantiostrostris</i>					1				1				
Passeriformes	Emberizidae	<i>Arremonops</i>	<i>conirostris</i>													
Passeriformes	Formicariidae	<i>Formicarius</i>	<i>analís</i>													
Passeriformes	Furnariidae	<i>Automolus</i>	<i>ochrolaemus</i>	1												
Passeriformes	Furnariidae	<i>Dendrocincla</i>	<i>fuliginosa</i>													
Passeriformes	Furnariidae	<i>Glyphorhynchus</i>	<i>spirurus</i>										1		1	
Passeriformes	Furnariidae	<i>Xiphorhynchus</i>	<i>susurrans</i>													
Passeriformes	Icteridae	<i>Cacicus</i>	<i>uropygialis</i>													
Passeriformes	Incertae Sedis	<i>Saltator</i>	<i>maximus</i>													
Passeriformes	Parulidae	<i>Myiothlypis</i>	<i>fulvicauda</i>													
Passeriformes	Pipridae	<i>Manacus</i>	<i>aurantiacus</i>													
Passeriformes	Pipridae	<i>Manacus</i>	<i>candei</i>													
Passeriformes	Pipridae	<i>Ceratopipra</i>	<i>mentalis</i>													
Passeriformes	Thamnophilidae	<i>Cymbilaimus</i>	<i>lineatus</i>													
Passeriformes	Thamnophilidae	<i>Gymnopathys</i>	<i>leucaspis</i>													
Passeriformes	Thamnophilidae	<i>Hylophylax</i>	<i>naevioides</i>													
Passeriformes	Thamnophilidae	<i>Microrhopias</i>	<i>quixensis</i>													
Passeriformes	Thamnophilidae	<i>Myrmeciza</i>	<i>exsul</i>													
Passeriformes	Thraupidae	<i>Chlorophanes</i>	<i>spiza</i>													
Passeriformes	Thraupidae	<i>Oryzoborus</i>	<i>funereus</i>													
Passeriformes	Thraupidae	<i>Ramphocelus</i>	<i>costaricensis</i>													
Passeriformes	Thraupidae	<i>Ramphocelus</i>	<i>passerini</i>													
Passeriformes	Thraupidae	<i>Sporophila</i>	<i>corvina</i>													
Passeriformes	Thraupidae	<i>Tachyphonus</i>	<i>luctuosus</i>													
Passeriformes	Thraupidae	<i>Tangara</i>	<i>gyrola</i>													
Passeriformes	Thraupidae	<i>Tangara</i>	<i>larvata</i>													
Passeriformes	Thraupidae	<i>Thraupis</i>	<i>episcopus</i>													
Passeriformes	Thraupidae	<i>Volatinia</i>	<i>jacarina</i>													
Passeriformes	Tityridae	<i>Tityra</i>	<i>inquisitor</i>													
Passeriformes	Troglodytidae	<i>Cantorchilus</i>	<i>nigricapillus</i>													
Passeriformes	Troglodytidae	<i>Henicorhina</i>	<i>leucosticta</i>													
Passeriformes	Turdidae	<i>Turdus</i>	<i>grayi</i>													
Passeriformes	Tyrannidae	<i>Attila</i>	<i>spadiceus</i>	2												
Passeriformes	Tyrannidae	<i>Elaenia</i>	<i>flavogaster</i>													
Passeriformes	Tyrannidae	<i>Mionectes</i>	<i>oleagineus</i>													
Passeriformes	Tyrannidae	<i>Myiarchus</i>	<i>tuberculifer</i>													
Passeriformes	Tyrannidae	<i>Myiozetetes</i>	<i>granadensis</i>													

(Continued)

TABLE 1 | Continued

Order	Family	Genus	Species	Sampling locality											
				A	B	C	D	E	F	G	H	I	J	K	L
Passeriformes	Tyrannidae	<i>Myiozetetes</i>	<i>similis</i>			3									
Passeriformes	Tyrannidae	<i>Onychorhynchus</i>	<i>coronatus</i>								1				
Passeriformes	Tyrannidae	<i>Platyrinchus</i>	<i>coronatus</i>		1										
Passeriformes	Tyrannidae	<i>Tolmomyias</i>	<i>sulphurescens</i>								1				
Passeriformes	Vireonidae	<i>Hylophilus</i>	<i>flavipes</i>	1											
Piciformes	Galbulidae	<i>Galbula</i>	<i>ruficauda</i>								2				
Piciformes	Picidae	<i>Melanerpes</i>	<i>pucherani</i>								1				
Piciformes	Ramphastidae	<i>Pteroglossus</i>	<i>torquatus</i>							1					
Trogoniformes	Trogonidae	<i>Trogon</i>	<i>massena</i>										1		
Trogoniformes	Trogonidae	<i>Trogon</i>	<i>rufus</i>		1									2	

(A) COSTA RICA: Prov. Puntarenas, Golfito, Parque Nacional Piedras Blancas, elev 75 m. (B) COSTA RICA: Prov. Puntarenas, Osa, Los Charcos, elev 65–110 m. (C) COSTA RICA: Prov. Puntarenas, Osa, Reserva Forestal Golfo Dulce Rd. to Drake, elev 260 m. (D) COSTA RICA: Prov. Puntarenas, Quepos, Londres, elev 200 m. (E) COSTA RICA: Prov. San Jose, Tarrazu, Santa Juana, elev 400 m. (F) COSTA RICA: Prov. Cartago, Tucuttiqque, Reserva Biologica El Copal, elev 1050 m. (G) COSTA RICA: Prov. Heredia, Sarapiquí, Reserva Biologica, Tirimbina, elev 170 m. (H) COSTA RICA: Prov. Heredia, Sarapiquí, Estacion Biologica La Selva. (I) COSTA RICA: Prov. Limon, Teleferico Veragua, elev 260–430 m. (J) COSTA RICA: Prov. Limon, Talamanca, Tuba Creek, elev 80 m. (K) PERU: Pasco, Prov. Oxapampa, Dist. Puerto Bermudez, Comunidad San Juan, trail to Janirvan, waterfall, elev 325 m. (L) PERU: Junin, Prov. Satipo, Dist. Rio Tambo, Sector Yavyos, Fundo San Jorge II, elev 415 m.



two extractions in tandem from the posterior large intestine; *Nyctidromus.albicollis*.1 had one extraction from the posterior large intestine and a second from one of the ceca.

We used several measures of sequence quality control. First, both reads of a given pair had to match across 100% of the bases. The pairs also must have no errors in the individual tag or priming sequence. We used the BELLEROPHON (Huber et al., 2004) function within the mothur program (Schloss et al., 2009) to identify and discard potentially chimeric sequences. Finally, we used mothur to discard sequences that did not blast to the domain Bacteria. The reads passing these filters were included in the final dataset.

Subsampled Datasets

To assess patterns across different spatial, taxonomic and ecological scales, we subdivided the dataset eight ways.

1. Full dataset: all samples ($N = 116$).
2. >2 individuals: all individuals belonging to species sampled more than once ($N = 80$, removes singletons).
3. CFM: all individuals from the three species that were sampled from both Costa Rica and Peru: *Cyanocompsa cyanoides*, *Florisuga mellivora* and *Mionectes oleagineus* ($N = 17$, this dataset allows investigation of large-scale geographic differences between birds of the same species).

4. *Cyanoides*: all individuals belonging to the species *Cyanocompsa cyanoides* ($N = 8$, removes taxonomic variation).
5. Manakins: all individuals from two species that were sampled multiple times and belong to the same family: *Manacus candei* and *Ceratopipra mentalis* ($N = 13$, this dataset allowed us to look at the differentiation between closely related species).
6. Non-passerines: all the birds belonging to orders other than Passeriformes ($N = 27$, removes largest order).
7. Passerines: all the birds belonging to the order Passeriformes ($N = 88$, constrains taxonomic variation to a single order).
8. Tirimbina: all individuals from Tirimbina Biological Reserve, Sarapiquí, Costa Rica, the most densely sampled locality ($N = 45$, removes geographic variation).

Taxonomic Assignment and Clustering Analyses

The microbial ecology package QIIME (Caporaso et al., 2010b) was used for the following analyses. First, the *de novo* OTU picking protocol was used to assign the reads to phylotypes at 97% sequence similarity because 3% is frequently cited as the “species” level of microbial taxonomy (Schloss and Handelsman, 2005), hereinafter “phylotypes.” Next, we assigned taxonomies to phylotypes using the QIIME implementation of the RDP Classifier 2.2 Program (Wang et al., 2007), with the default confidence threshold of 80%. A “core microbiota” was calculated and included all phylotypes that were found in 100% of the samples.

A pairwise UniFrac (Lozupone and Knight, 2005) distance matrix (UDM) was constructed between each gut microbial community (i.e., each bird specimen). UniFrac distances are calculated based on the amount of branch length in a phylogenetic tree that is unique to either of two environments (vs. how much of the tree is shared by the environments). These distances can be based on presence-absence of OTUs (“unweighted”) or weighted by abundance and our analyses use both, as neither method is agreed to be more appropriate for multi-species microbiota studies. Our data were aligned using the QIIME implementation of PyNAST (Caporaso et al., 2010a) and a microbial phylogenetic tree was constructed with FASTTREE (Price et al., 2009). All individuals were randomly reduced to 3652 reads, equal to the lowest number of reads for any bird in the dataset. Despite this type of rarefaction being inappropriate for some questions (McMurdie and Holmes, 2014), we chose to normalize our data this way because we are comparing “whole microbiome” data across many individuals and highly variable sequencing depths can affect diversity estimates (Goodrich et al., 2014). We constructed UPGMA dendrograms based on both the unweighted UDM and weighted UDM to visually represent the relatedness of the gut microbiota for all datasets. Principal coordinates analysis (PCoA) was also performed on both the weighted and unweighted UniFrac distance matrices.

As a complement to the phylogenetic-based methods, we visualized the data with non-metric multidimensional scaling (NMDS). We square root-transformed the percentage of each sample that belonged to each bacterial phylum, then created a pairwise distance matrix using Bray-Curtis dissimilarity,

applied through the VEGDIST function of the VEGAN package (Oksanen et al., 2011) in R (R Development Core Team, 2010). The NMDS function of the ECODIST package (Goslee and Urban, 2007) was then used to calculate the two-dimensional positions of the samples (such that closer samples are more similar), the stress and R^2 value of the plot. Stress values >0.3 should not be considered valid whereas values <0.2 can be considered a good representation of the data (Quinn and Keough, 2002).

Categorical Variable Significance

To look for a relationship between categorical variables associated with each bird and the microbial communities, we used the statistical tools Adonis (McArdle and Anderson, 2001) and Anosim (Clarke, 1993) implemented in QIIME. The categorical variables included the American Ornithologists’ Union South American Classification Committee’s taxonomy, i.e., order, family, genus and species (Remsen et al., 2012), ecological variables, including dietary specialization and habitat (Bennett and Owens, 2002), spatial variables and individual properties, like age (based on percent of skull ossification), stomach contents (e.g., “insects” or “plant material”) and bacterial richness (bacterial taxa identified per bird). **Table 2** gives a detailed list of the variables and their sources. We calculated significance of all variables for both the weighted and unweighted UDMs with 999 permutations.

After testing the significance of each variable independently, we ran an additional Adonis test on the most frequently significant variables to quantify the amount of variation each variable was responsible for in the context of other variables. We used the full dataset’s unweighted and weighted UDMs as input, calculated 999 permutations, and permuted the order of the variables, which can affect the results of the test. Finally, we constrained the analyses to only permute the data within bird orders, as a measure of controlling for taxonomy. We then reran the weighted and unweighted UDMs.

RESULTS

After initial quality control steps, 9,897,718 pairs of reads remained with no errors in priming sequence, region of overlap or individual tags. Potentially chimeric sequences (1167, 0.01% of reads) were then discarded. A further 3,58,725 sequences that did not align to any sequence within the domain Bacteria (3.6% of reads) were removed; 75% of these discarded reads belonged to 11 individuals. The reads passing these filters were included in the final dataset, totaling 9,537,817 sequences and averaging 82,222 sequences per individual; reads/sample varied by over two orders of magnitude (range: 3652–853,078).

Four bacterial phyla were detected in all individuals: Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria comprising an average of 46.3, 37.3, 3.3, and 1.4% of each sample, respectively (**Figure 2**). An additional 16 phyla were identified: Acidobacteria, Chlamydiae, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Fusobacteria, Lentisphaerae, Nitrospira, OD1, OP10, OP11, Planctomycetes, Spirochaetes, TM7, Tenericutes, Verrucomicrobia, with an average of 10.6% of

TABLE 2 | Categorical variables tested for significance, including the number of categories within each variable (Cat) and a list of the possible designations (except taxonomic categories).

Variable	Cat	Description and source
Order	8	Bird order (Remsen et al., 2012)
Family	24	Bird family (Remsen et al., 2012)
Genus	53	Bird genus (Remsen et al., 2012)
Species	59	Bird species (Remsen et al., 2012)
Diet specific	11	Specific dietary specialization: nectar, generalist, insect, seed, arthropod, fruit, insect/fruit, fruit/insect, nectar/insect, arthropod/vertebrates, fruit/nectar/insects (C. Sanchez, pers. com.)
Diet broad	3	Broad dietary specialization: plant material, animal material, both
Diet B&O	4	Broad dietary specialization assigned by Bennett and Owens (2002): frugivore, nectarivore, insectivore, omnivore
Habitat	5	General habitat assigned by Bennett and Owens (2002): woodland, forest, forest/grassland, forest/grassland/scrub, all habitats
Foraging strata	9	Foraging strata assigned by Stotz et al. (1996): canopy, midstory, understory, terrestrial, under/midstory, midstory/canopy, terrestrial/understory, terrestrial/midcanopy, understory/canopy
Locality	12	Sampling locality (see Figure 1): Tirimbina, Londres, Los Charcos, Piedras Blancas, Golfo Dulce, El Copal, Janirvan, Tuba Creek, San Jorge II, La Selva, Veragua, Santa Juana
Country	2	Country of sampling: Costa Rica, Peru
NSEW	6	Relative location of sampling locality: north-east Costa Rica, midwest Costa Rica, southwest Costa Rica, middle Costa Rica, mideast Costa Rica, Peru
Elevation	13	Elevation of sampling locality: 65, 75, 80, 110, 170, 200, 250, 260, 325, 400, 415, 430, and 1050 m
Sex	3	Sex of the bird: male, female, unknown
Age	14	Percent of skull ossification (a proxy for age of bird): 0, 3, 5, 10, 15, 20, 25, 50, 70, 75, 90, 95, 100, unknown
Stomach contents	12	Contents of stomach at time of collection: insects, seeds, fruit, plant material, seeds/insects, insects/pollen, fruit/insects, insects/plants, seeds/plants, fruit/insects/seeds, empty, unknown
Phyla richness	10	Number of bacterial phyla identified in the gut microbiota fingerprint: 8, 9, 10, 11, 12, 13, 14, 15, 16, 17
Species richness	5	Which fifth the number of 97% OTUs identified in a gut microbiota fingerprint belongs to: 20%ile, 40%ile, 60%ile, 80%ile, 100%ile

sequences (from each individual) from unknown bacterial phyla. The core microbiota contained 56 phylotypes, 32 of which belonged to 26 known genera (**Table 3**). An additional 48 phylotypes were detected in >95% of the samples. The number of species-level phylotypes per bird varied between 109 and 288, with an average of 201 ($SD = 35$). Replicate samples were similar to one another in taxonomic composition (**Figure 2**) and generally clustered close to one another in multivariate space (**Figures 3, 4; Figure S2**). A heatmap of bacterial phylotypes vs. host taxonomy revealed little clustering and showed how specific phylotypes were found in high abundance in most individuals (**Figure S3**); most of these phylotypes belonged to the Firmicutes and Proteobacteria.

Clustering Analyses

The PCoA for the unweighted UDM displayed clustering by taxonomic order (**Figure 3**), but little obvious clustering by foraging stratum, diet or sampling locality. Host order displayed the most clustering in the weighted UDM PCoA and NMDS plot as well (**Figure S1**), although little correspondence is seen between the actual bird phylogeny and a dendrogram of the weighted UDM (**Figure 4**; see **Figure S2** for unweighted UDM and Bray-Curtis dendrograms).

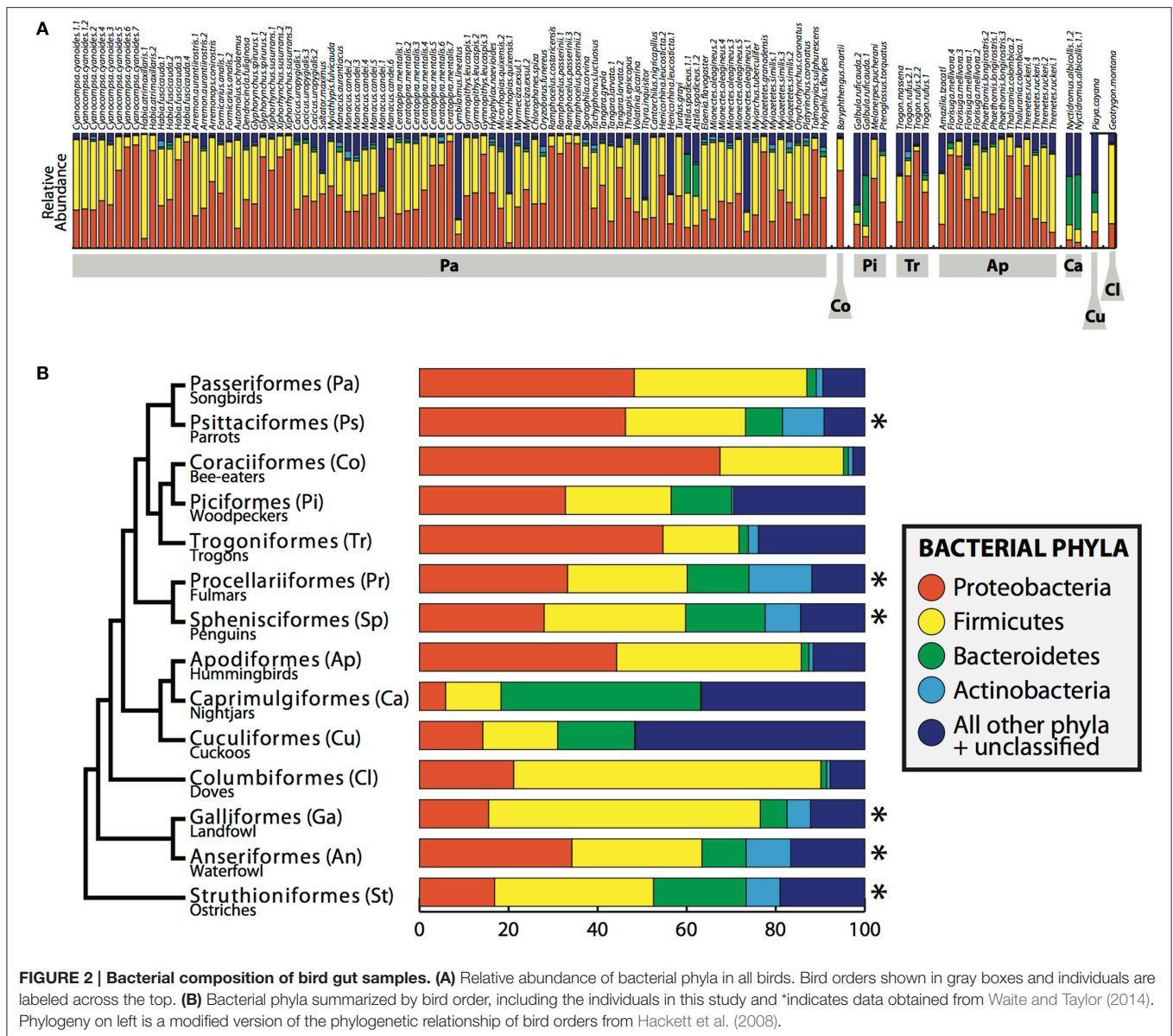
Categorical Variable Significance

To look for correlations between categorical variables and the gut microbiota, we conducted two statistical tests for significance on both the weighted and unweighted UDMs (**Table 4**). For the

full dataset, taxonomic categories explained the most amount of variation and were all significant at $p < 0.05$ for the Adonis statistical tests (**Figure 5A, Table 4**). All other variables explained much less variation although some were significant. The taxonomic categories were also significant in the Anosim test of the full dataset, whereas none of the ecological or spatial variables were (**Table 4**). Averaging across all 8 datasets, taxonomic variables were the most frequently significant; all four categories (order, family, genus, species) were significant in over 50% of the tests. The taxonomic categories also accounted for the most variation (**Figure 5B**). The ecological variables were significant in most datasets but generally explained much less variation than the taxonomic variables. The locality and individual variables had generally low R^2 values and were all significant in less than 33% of the tests.

Using the “Tirimbina” dataset, which included all individuals from a single locality, host order was still significant, with other taxonomic levels less so (**Table 4**). Conversely, in our most taxonomically restricted dataset including a single species, “*Cyanoides*,” geographic variables explained a large amount of variation but the effect was not significant (unweighted UDM $R^2 = 58\%$, $p = 0.23$; weighted UDM $R^2 = 74.9\%$, $p = 0.156$).

The multifactor Adonis tests yielded different results for the weighted and unweighted UniFrac distance matrices. Foraging stratum, host order and bacterial richness (at the phylum level) were all significant ($p < 0.05$) for the unweighted UDM and accounted for around 10% of the variation each (**Table 5A**). No variables were significant for the weighted UDM (**Table 5B**).



When data were permuted within the taxonomic orders (i.e., controlling for high-level host taxonomy), the significance of the variables did not change (Tables 5C,D), although the amount of variation that the host taxonomy explained increased.

DISCUSSION

Microorganisms are an essential component of biodiversity, and vertebrate evolutionary history is incomplete without an adequate understanding of our microbiota. This study greatly increases our basic understanding of what organisms live in the guts of wild Neotropical birds, spanning eight orders within Aves. We found taxonomic profiles similar to other avian gut studies with some exceptions. Compared to the meta-analysis performed by Waite and Taylor (2014), our samples appear to be enriched for Proteobacteria and have a deficit of Actinobacteria. If this

result is not a biological signal, the discrepancy may be due to the difference in marker choice or methods between our study and those in the meta-analysis or to an underlying methodological bias.

Several gut-associated taxa were defined as our “core microbiome.” The phyla Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes were found in all our samples and are often found in gut habitats across vertebrates (e.g., Turnbaugh et al., 2008; Roeselers et al., 2011); the same is true for the genera *Bacteroides*, *Clostridium*, and *Lactobacillus*. Additionally, some genera frequently found in avian guts specifically were identified in all our samples: *Streptococcus* and *Campylobacter* (Zhu et al., 2002; Xenoulis et al., 2010; Videnska et al., 2014). Although several known contaminants of microbiota studies (Salter et al., 2014) were found in our dataset (e.g., *Corynebacterium*, *Propionibacterium*, *Streptococcus*), the

TABLE 3 | Bacterial taxa identified in 100% of the bird samples (assigned by RDP Classifier Program).

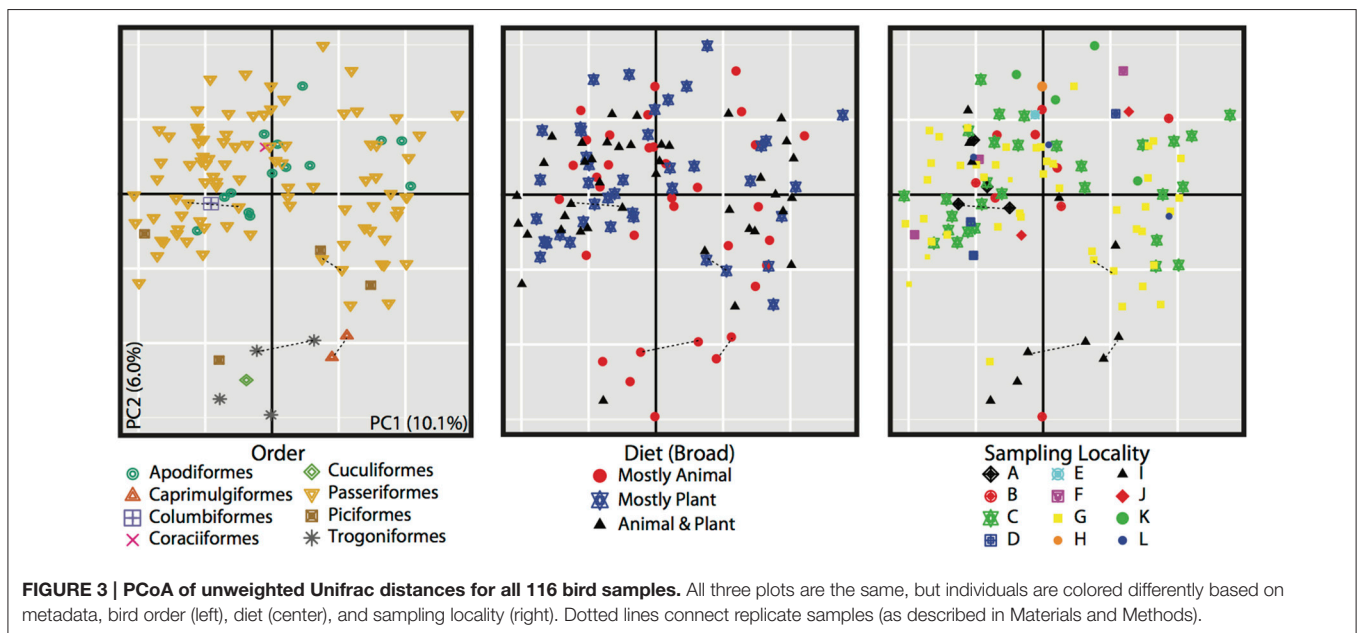
Phylum	Class	Order	Family	Genus							
Actinobacteria	5	Actinobacteria	5	Actinomycetales	4	Corynebacteriaceae	1	<i>Corynebacterium</i>	1		
						Microbacteriaceae	1				
						Propionibacteriaceae	1	<i>Propionibacterium</i>	1		
Bacteroidetes	5	Bacteroidia	1	Bacteroidales	1	Bacteroidaceae	1	<i>Bacteroides</i>	1		
		Flavobacteria	3	Flavobacteriales	3	Flavobacteriaceae	3	<i>Chryseobacterium</i>	1		
								<i>Planobacterium</i>	1		
Firmicutes	17	Bacilli	12	Bacillales	4	Bacillaceae	2	<i>Anoxybacillus</i>	1		
						Staphylococcaceae	1	<i>Staphylococcus</i>	1		
						Lactobacillales	7	Lactobacillaceae	1	<i>Lactobacillus</i>	1
								Leuconostocaceae	2	<i>Leuconostoc</i>	1
								<i>Weissella</i>	1		
						Streptococcaceae	3	<i>Lactococcus</i>	1		
								<i>Streptococcus</i>	1		
Clostridia	4	Clostridiales	4	Clostridiaceae	1	<i>Clostridium</i>	1				
						Veillonellaceae	2	<i>Veillonella</i>	1		
Proteobacteria	27	Alpha- proteobacteria	4	Rhizobiales	2	Methylobacteriaceae	1	<i>Methylobacterium</i>	1		
				Sphingomonadales	1	Sphingomonadaceae	1	<i>Sphingomonas</i>	1		
		Beta- proteobacteria	9	Burkholderiales	8	Burkholderiales	2	<i>Aquabacterium</i>	1		
						incertae sedis	1	<i>Tepidimonas</i>	1		
						Comamonadaceae	4	<i>Acidovorax</i>	1		
								<i>Diaphorobacter</i>	1		
								<i>Schlegelella</i>	1		
						Oxalobacteraceae	1	<i>Janthinobacterium</i>	1		
Epsilon- proteobacteria	2	Campylobacteriales	2	Campylobacteraceae	1	<i>Campylobacter</i>	1				
				Helicobacteraceae	1	<i>Helicobacter</i>	1				
Gamma- proteobacteria	11	Aeromonadales	1	Aeromonadaceae	1	<i>Aeromonas</i>	1				
		Enterobacteriales	4	Enterobacteriaceae	4	<i>Escherichia</i>	1				

(Continued)

TABLE 3 | Continued

Phylum	Class	Order	Family	Genus	
				<i>Kluyvera</i>	1
				<i>Yersinia</i>	1
		Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	1
				<i>Enhydrobacter</i>	1
			Pseudomonadaceae	<i>Pseudomonas</i>	1
		Xanthomonadales	Xanthomonadaceae	<i>Stenotrophomonas</i>	1

Gray boxes indicate the phylotype did not align to any named taxa at that taxonomic rank. Numbers in boxes are the total number of identified phylotypes in that group. Full length gray bar at bottom indicates a phylotype that did not align to any named phylum.



high biomass of our initial samples (intestinal contents) and the corroboration of many of these genera being found in other gut studies leads us to believe our results are not due to laboratory contamination. Overall, these results suggest that there may be a conserved set of microorganisms found in the guts of birds and perhaps all vertebrates.

The Importance of Host Taxonomy, Ecology, and Geographic Space

The statistical tests applied above indicate varying degrees of importance for taxonomy, ecology, geographic space, and individual traits for structuring the gut microbiota of birds. Generally speaking, it appears that *who* a bird is most important, *how* a bird lives is possibly important and *where* a bird lives may be of little importance.

Instead of phylogenetic distance, we used taxonomic categories as our evolutionary units, so that we could apply the same statistical methods to all metadata associated with the bird samples. Focusing on hierarchical taxonomic categories also allowed us to overcome the analytical problems associated with high individual variation in gut microbiota (e.g., Turnbaugh et al., 2008; **Figure 4**) while still learning about the effect of evolutionary history on the microbiota. Taxonomic categories were most frequently significant in our analyses and explained the most variation. There is clear evidence for associations between gut microbiota and host taxonomy, which has been widely noted in other systems and at varying scales (e.g., Ochman et al., 2010). It is important to note, though, that taxonomy is not the same as phylogeny; our results show that individuals of the same species/genus/family/order are more similar to one

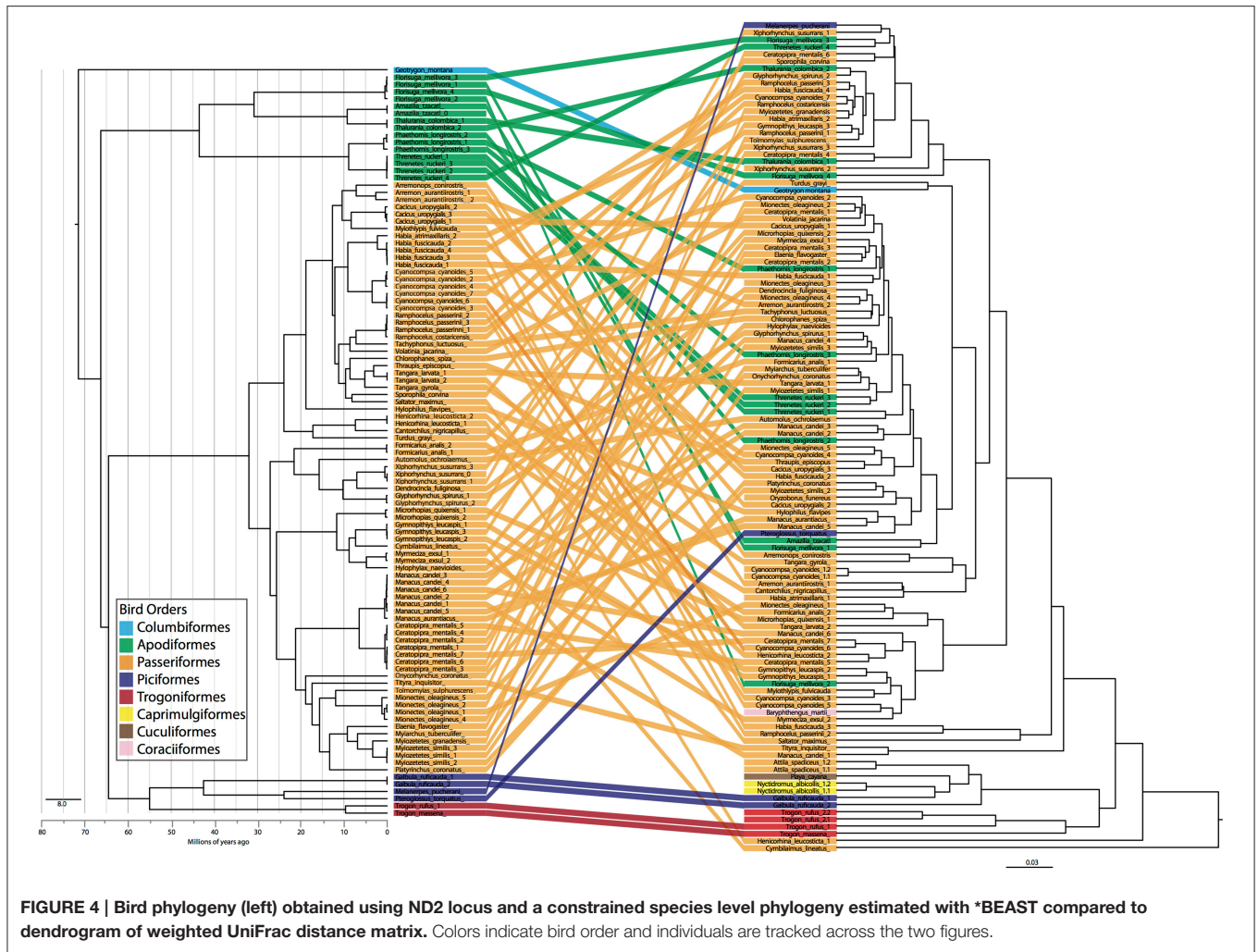


FIGURE 4 | Bird phylogeny (left) obtained using ND2 locus and a constrained species level phylogeny estimated with *BEAST compared to dendrogram of weighted UniFrac distance matrix. Colors indicate bird order and individuals are tracked across the two figures.

another than they are to individuals in other groups, not that more closely related species/genera/families/orders have more similar microbiota. In fact, little correlation was seen between the individual phylogeny and the gut microbiota dendrogram (Figure 4). Does this mean that phylogeny is not an underlying factor shaping the gut microbiota? We believe that it would be difficult to get significant signal at all taxonomic levels without phylogeny playing some role but this requires further investigation that may require parsing the microbiome into vertically vs. horizontally transmitted bacteria. It would not be surprising to find some vertical transmission of microbiota from parent to offspring occurring in birds, as all the birds in this study feed their young by regurgitating food into the mouth of nestlings. The exact role of evolutionary history on gut microbiota is an exciting next step.

We also found small but significant associations between the gut microbiota and host ecology. Broad dietary classifications (e.g., mostly plant, mostly animal, plant/animal) were more significant than specific dietary specializations and the literal stomach contents were only significant in two of the 32 tests. Perhaps long term habits or nutritional content have greater

influence on gut microbiota than day to day food intake, which is consistent with other studies showing the stability of the avian gut community once established (Benskin et al., 2010) and that diet can drive the convergence of gut microbiota in non-avian vertebrates (Ley et al., 2008a,b; Muegge et al., 2011). Of methodological note: the “stomach contents” variable contains a lot of variance particularly with respect to specificity and accuracy, as it is recorded in the field and only general data are taken, (i.e., “plant material” or “insects”).

Strata and habitat are important ecological aspects of avian biology. Foraging strata is associated with genetic divergence in Neotropical birds (Burney and Brumfield, 2009; Smith et al., 2014) because ecology affects dispersal ability. Our results reinforce the importance of foraging strata on avian biology and support an important role for ecology in differentiation of both host and microbiota. Microbes from the same ecological niche on the human body are able to share genes on a global scale (Smillie et al., 2011)—perhaps the microbiota of birds that share ecological niches are able to transfer genetic material as well. We could not decipher which aspect of host ecology is directly responsible for the signal we discovered in our current data—is

TABLE 4 | Full results for Adonis and Anosim tests of categorical significance.

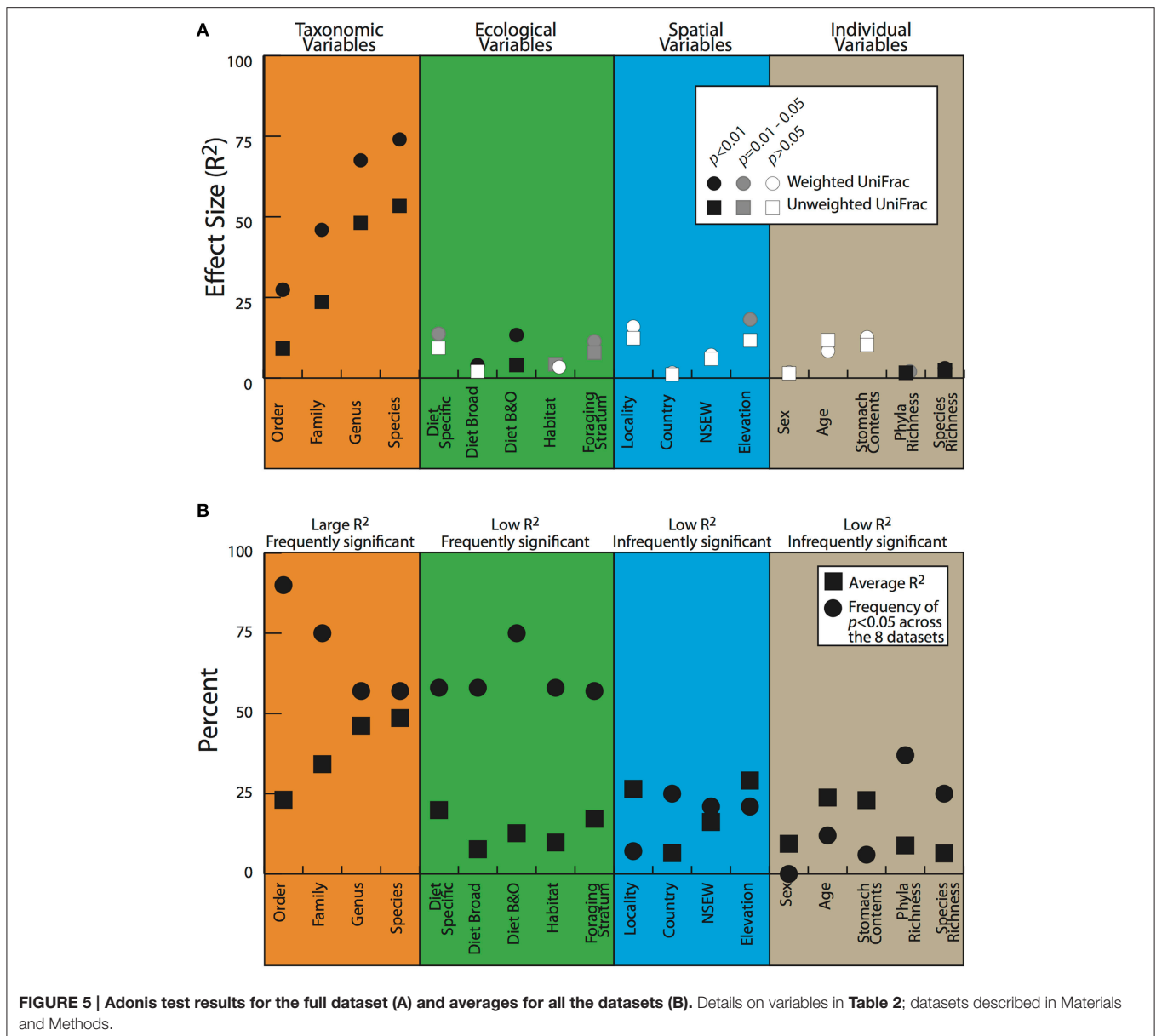
	Order						Family						Genus						Species							
	Adonis		ANOSIM		Adonis		ANOSIM		Adonis		ANOSIM		Adonis		ANOSIM		Adonis		ANOSIM		Adonis		ANOSIM			
	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P		
A.	Unweighted	9.2	0.001	0.136	0.027	23.63	0.001	0.117	0.001	48.12	0.001	0.122	0.022	53.4	0.001	0.133	0.022	53.4	0.001	0.133	0.022	53.4	0.001	0.133	0.022	
	Weighted	27.39	0.001	0.257	0.001	45.95	0.001	0.216	0.001	67.53	0.001	0.250	0.001	74.02	0.001	0.294	0.001	74.02	0.001	0.294	0.001	74.02	0.001	0.294	0.001	
	>2 individuals/species	8.11	0.001	0.117	0.058	20.53	0.001	0.121	0.009	33.31	0.001	0.121	0.019	34.65	0.001	0.143	0.01	34.65	0.001	0.143	0.01	34.65	0.001	0.143	0.01	
	Unweighted	35.33	0.001	0.268	0.002	47.33	0.001	0.239	0.001	61.74	0.001	0.258	0.001	62.92	0.001	0.273	0.001	62.92	0.001	0.273	0.001	62.92	0.001	0.273	0.001	
	Unweighted	8.08	0.03	0.342	0.016	14.71	0.027	0.317	0.004	14.71	0.027	0.317	0.003	14.71	0.02	0.317	0.003	14.71	0.02	0.317	0.003	14.71	0.02	0.317	0.003	
	Weighted	8.78	0.199	-0.066	0.599	15.05	0.272	-0.026	0.524	15.05	0.28	-0.026	0.559	15.05	0.277	-0.026	0.568	15.05	0.277	-0.026	0.568	15.05	0.277	-0.026	0.568	
	Cyanoides																									
	Manakins																									
	Non-passerines	31.9	0.001	0.504	0.001	39.5	0.001	0.520	0.001	56.22	0.001	0.718	0.001	59.86	0.001	0.707	0.001	59.86	0.001	0.707	0.001	59.86	0.001	0.707	0.001	
	Passerines	60.94	0.001	0.726	0.001	75.5	0.001	0.782	0.001	81.59	0.001	0.561	0.001	86.33	0.001	0.648	0.001	86.33	0.001	0.648	0.001	86.33	0.001	0.648	0.001	
B.	Unweighted	8.58	0.022	0.239	0.014	28.83	0.685	-0.042	0.711	64.05	0.394	0.029	0.396	64.05	0.386	0.108	0.103	64.05	0.386	0.108	0.103	64.05	0.386	0.108	0.103	
	Weighted	33.08	0.017	0.221	0.066	59.17	0.011	0.127	0.067	80.69	0.096	0.143	0.163	80.69	0.104	0.143	0.139	80.69	0.104	0.143	0.139	80.69	0.104	0.143	0.139	
	Diet specific																									
	Diet broad																									
	Diet B&O																									
	Habitat																									
	Foraging stratum																									
	Full dataset	9.36	0.127	0.010	0.401	0.13	0.020	0.082	4.06	0.001	0.000	0.463	0.017	0.034	0.089	7.85	0.026	0.050	0.050	0.026	0.050	0.050	0.026	0.050	0.050	
	>2 individuals/species	13.74	0.023	0.002	0.46	3.95	0.006	0.064	13.33	0.001	0.048	0.153	0.186	-0.012	0.613	11.39	0.019	0.103	0.103	0.019	0.103	0.103	0.019	0.103	0.103	
	CFM	13.17	0.001	0.106	0.007	3.72	0.007	0.076	6.24	0.001	0.092	0.016	0.001	0.077	0.009	7.33	0.021	0.021	0.021	0.009	0.021	0.021	0.009	0.021	0.021	
Cyanoides	16.28	0.008	0.051	0.13	5	0.031	0.146	19.77	0.001	0.050	0.166	0.029	0.025	0.196	16.95	0.001	0.132	0.132	0.001	0.132	0.132	0.001	0.132	0.132		
Manakins	14.71	0.016	0.317	0.004	8.08	0.022	0.342	14.71	0.021	0.317	0.003	0.023	0.317	0.002	14.71	0.027	0.317	0.317	0.002	0.317	0.317	0.002	0.317	0.317		
Non-passerines	15.05	0.311	-0.026	0.557	8.78	0.207	-0.066	0.59	15.05	0.286	0.536	0.308	-0.026	0.547	15.05	0.307	-0.026	0.537	0.307	-0.026	0.537	0.307	-0.026	0.537		
Passerines	33.77	0.001	0.428	0.001	13.43	0.001	0.359	19.11	0.001	0.445	0.001	0.001	0.475	0.001	27.87	0.001	0.285	0.285	0.001	0.285	0.285	0.001	0.285	0.285		
Tirimbina	53.27	0.003	0.451	0.001	29.07	0.001	0.358	45.14	0.002	0.642	0.001	0.001	0.5321	0.001	42.98	0.005	0.358	0.358	0.005	0.358	0.358	0.005	0.358	0.358		
(Continued)	8.95	0.073	0.012	0.336	2.55	0.201	0.002	1.59	0.046	0.043	0.085	0.067	0.033	0.105	8.76	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109	0.109		
	13.25	0.021	0.018	0.308	4.61	0.033	0.017	4.88	0.001	0.048	0.074	0.006	0.001	0.472	10.84	0.111	-0.036	0.111	0.006	0.111	-0.036	0.111	-0.036	0.111		
	21.88	0.131	0.048	0.214	5.31	0.113	0.048	4.61	0.387	-0.034	0.718	0.293	0.062	0.119	17.68	0.082	0.057	0.082	0.293	0.062	0.119	0.082	0.057	0.082		
	26.38	0.188	0.108	0.082	6.75	0.171	0.127	4.23	0.005	0.040	0.244	0.204	0.056	0.178	35	0.05	0.052	0.052	0.204	0.056	0.178	0.05	0.052	0.052		

TABLE 4 | Continued

C.		Locality						Country						NSEW						Elevation						
		Adonis		ANOSIM		P		Adonis		ANOSIM		P		Adonis		ANOSIM		P		Adonis		ANOSIM		P		
		R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	
Full dataset	Unweighted	12.36	0.068	0.019	0.324	1.12	0.067	-0.013	0.533	5.95	0.052	0.033	0.183	11.7	0.249	0.008	0.381									
	Weighted	15.94	0.072	0.091	0.049	1.64	0.09	-0.109	0.749	7.13	0.135	0.081	0.027	18.2	0.013	0.071	0.151									
>2 individuals/species	Unweighted	15.19	0.078	-0.023	0.611	2.17	0.011	0.156	0.09	7.89	0.014	0.070	0.07	15.48	0.356	-0.056	0.794									
	Weighted	18.8	0.092	0.055	0.211	1.57	0.253	-0.069	0.653	8.52	0.137	0.064	0.119	23.36	0.02	-0.006	0.521									
CFM	Unweighted	33.14	0.105	0.132	0.133	7.22	0.099	0.104	0.151	14.29	0.044	0.135	0.071	38.77	0.189	0.232	0.047									
	Weighted	30.15	0.52	-0.015	0.532	14	0.048	0.056	0.229	23.55	0.054	0.107	0.125	34.47	0.6	-0.089	0.725									
Cyanoides	Unweighted	58.83	0.238	0.096	0.368	15.47	0.206	0.146	0.169	29.59	0.253	-0.050	0.504	58.83	0.253	0.125	0.347									
	Weighted	74.9	0.156	0.165	0.26	26.9	0.172	0.031	0.382	43.34	0.213	0.063	0.293	74.9	0.17	0.250	0.243									
Manakins	Unweighted	16.38	0.397	0.202	0.206					16.38	0.399	0.169	0.207	23.93	0.532	0.202	0.215									
	Weighted	14.56	0.523	-0.096	0.652	14.56	0.508	-0.080	0.619	15.5	0.822	-0.096	0.636													
Non-passerines	Unweighted	22.93	0.008	0.163	0.026	3.38	0.909	-0.278	0.889	19.12	0.009	0.189	0.018	24.91	0.101	0.055	0.238									
	Weighted	26.36	0.125	0.094	0.121	1.47	0.671	-0.271	0.81	23.33	0.098	0.197	0.016	37.53	0.027	0.132	0.106									
Passerines	Unweighted	13.78	0.504	-0.036	0.755	1.8	0.019	0.218	0.039	6.53	0.749	-0.028	0.72	13.76	0.5	-0.049	0.77									
	Weighted	18.05	0.111	-0.011	0.536	2.13	0.133	-0.039	0.58	7.58	0.312	-0.029	0.676	17.13	0.151	-0.040	0.714									
Tirimbina	Unweighted																									
	Weighted																									

D.		Sex						Age						Stomach contents						Phyla richness						Species richness					
		Adonis		ANOSIM		P		Adonis		ANOSIM		P		Adonis		ANOSIM		P		Adonis		ANOSIM		P		Adonis		ANOSIM		P	
		R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P	R ²	P	R	P		
Full dataset	Unweighted	1.49	0.894	-0.009	0.651	11.75	0.223	-0.027	0.644	10.27	0.095	0.036	0.198	1.63	0.046	0.055	0.003	2.43	0.001	0.0541	0.003										
	Weighted	1.76	0.441	0.020	0.206	8.37	0.878	-0.133	0.966	12.64	0.118	0.114	0.016	2.52	0.187	0.030	0.011	3.03	0.001	0.025	0.016										
>2 individuals/species	Unweighted	2.36	0.644	-0.010	0.603	12.92	0.034	0.050	0.209	12.25	0.112	0.083	0.064	1.51	0.173	0.086	0.064	1.69	0.064	-0.007	0.621										
	Weighted	3.04	0.292	0.036	0.171	12.31	0.354	-0.070	0.772	15.82	0.104	0.095	0.084	2.24	0.024	0.002	0.463	2.33	0.095	0.011	0.287										
CFM	Unweighted	10.26	0.997	-0.173	0.975	46.68	0.029	0.300	0.047	31.49	0.415	0.154	0.168	6.56	0.303	-0.177	0.913	6.13	0.507	-0.010	0.487										
	Weighted	8.89	0.679	-0.071	0.715	58.21	0.071	0.156	0.194	31	0.45	-0.054	0.592	12.27	0.106	0.024	0.446	7.39	0.335	-0.065	0.707										
Cyanoides	Unweighted	32.11	0.086	0.275	0.152	30.75	0.163	0.059	0.312	42.3	0.546	0.200	0.25	13.37	0.652	0.577	0.117	15.81	0.18	0.167	0.321										
	Weighted	36.45	0.271	-0.076	0.558	50.63	0.076	0.241	0.132	56.88	0.223	0.183	0.266	34.31	0.088	0.346	0.239	16.72	0.317	-0.076	0.609										
Manakins	Unweighted	17.81	0.283	0.087	0.249	16.48	0.365	-0.240	0.879	15.82	0.53	-0.188	0.805	13.88	0.029	0.323	0.016	10.2	0.129	0.157	0.124										
	Weighted	8.73	0.874	-0.196	0.972	16.69	0.413	-0.135	0.676	23.17	0.202	0.301	0.072	27.23	0.015	0.179	0.015	10.77	0.253	0.123	0.186										
Non-passerines	Unweighted	8.43	0.18	0.073	0.153	33.04	0.058	0.114	0.155	32.32	0.149	0.033	0.318	4.46	0.178	0.048	0.119	4.65	0.119	0.038	0.291										
	Weighted	7.14	0.459	0.121	0.069	30.9	0.47	-0.163	0.925	38.05	0.166	0.173	0.055	8.12	0.099	0.068	0.083	8.61	0.083	-0.012	0.493										
Passerines	Unweighted	1.81	0.959	-0.043	0.926	11.4	0.523	-0.032	0.655	12.21	0.014	0.029	0.283	2.14	0.002	0.034	0.15	3.17	0.001	0.093	0.001										
	Weighted	2.05	0.531	-0.028	0.763	12.91	0.303	0.025	0.384	10.05	0.501	0.055	0.199	5.86	0.001	0.043	0.021	4.65	0.001	0.021	0.169										
Tirimbina	Unweighted	5.05	0.177	-0.076	0.905	17.02	0.81	-0.187	0.983	17.29	0.795	0.037	0.358	3.26	0.025	0.057	0.024	3.11	0.056	0.024	0.282										
	Weighted	4.43	0.444	-0.037	0.693	11.66	0.783	-0.057	0.7	7.8	0.93	0.118	0.132	3.69	0.156	-0.028	0.051	1.91	0.478	0.051	0.097										

Effect size (R² or R) and significance shown; results for inappropriate tests are grayed out. P-values less than 0.05 highlighted in pink and bolded.



it that birds in the same foraging stratum are exposed to the same environment microbes, that their behavior exposes them to particular microbes within the environment or that similar genes recruit specific microbes? Investigating how these aspects of host ecology affect gut microbiota is an exciting avenue of future research.

Despite ecological factors possibly being important for avian gut microbiota, (external) geographic space showed little effect on the microbiota. All the locality variables had poor correlations with the gut microbiota and our spatial tests revealed no statistical significance between space and microbiota. Thus, bird gut microbiota are likely not just a random assortment of the microbes available in a given environment. Can we further conclude that physical space has little effect on the microbiota? Possibly. None of our analyses found that gut communities

that are spatially closer are more closely related, both across Aves and within a single species (*Cyanocompsa cyanoides*). Alternatively, the apparent lack of effect of locality may be an issue of sampling. This dataset contains few species (or even genera) with multiple individuals. If locality is important, we might expect it to be working within species instead of across higher taxonomic levels. Hird et al. (2014) found that locality was significantly correlated with gut microbiota within 34 brown-headed cowbirds from two localities. Scale of analysis may be critical for detecting what factors are contributing to divergence and much more work is needed to define appropriate geographic, taxonomic and ecological sample sizes. Regardless, it appears that geographic space is much less important than taxonomy and ecology for structuring the gut microbiota in birds.

TABLE 5 | Multifactorial Adonis tests for categorical significance.

	R^2	p
A. UNWEIGHTED UDM		
Diet	0.01161	0.653
Foraging stratum	0.11111	0.007
Locality	0.08343	0.426
Host order	0.10287	0.003
Bacterial richness	0.10607	0.029
B. WEIGHTED UDM		
Diet	0.00688	0.764
Foraging stratum	0.09983	0.095
Locality	0.13718	0.064
Host order	0.07614	0.184
Bacterial richness	0.06742	0.497
C. UNWEIGHTED UDM—CONTROLLING FOR HOST ORDER		
Diet	0.01161	0.806
Foraging stratum	0.11111	0.008
Locality	0.08343	0.695
Host family	0.24009	0.014
Bacterial richness	0.10261	0.025
D. WEIGHTED UDM—CONTROLLING FOR HOST ORDER		
Diet	0.00688	0.766
Foraging stratum	0.09983	0.246
Locality	0.13718	0.093
Host family	0.21724	0.304
Bacterial richness	0.04818	0.833

P-values less than 0.05 are bolded.

Incorporating Microbiome Studies into Collection Based Research

Wild organisms have different microbiota than captive conspecifics (Wienemann et al., 2011) and microbial communities change once the host organism has died (Hauther et al., 2015). Thus, field preservation of the microbiota generates unique and irretrievable data. Studying the microbiota informs us about the host but in a comparative framework, and we can learn about evolutionary processes above the individual as well (e.g., Ley et al., 2008b). The data for this paper were obtained without additional sampling effort; every result represents information that would have been lost had we not collected the intestinal contents for this purpose. The methodologies herein can relatively easily be incorporated into field protocols, when vertebrate specimens are being prepared for preservation in research collections.

The addition of microbial fingerprints to studies of natural history and evolution provides a non-traditional glimpse into some very important biodiversity. Our protocol for field sampling added an average of 5–10 min to specimen preparation time and consisted of isolating the intact intestinal tract, tying an undisturbed portion off at both ends, clipping the intestines on the outside of the ties and storing in liquid nitrogen. Other field researchers may want to include microbiome components in “macrodiversity” studies, even if they cannot process the samples

immediately because doing so adds and preserves a novel and frequently entirely unknown dimension of data to vouchered museum specimens.

DATA ACCESSIBILITY

Sequence data, metadata file and an OTU table have been uploaded to FigShare available at figshare.com/s/fe202600737e11e58a2906ec4bbcf141.

AUTHOR CONTRIBUTIONS

Conception/design of study (SH, RB); field work (CS, SH); acquisition, analysis, and interpretation of data (SH, CS, BC, RB); writing and editing manuscript (SH, CS, BC, RB).

ACKNOWLEDGMENTS

The authors would like to thank NSF (DEB-0956069 and DEB-0841729), the AMNH Frank Chapman Grant (to CS) and Sigma Xi Grants-in-Aid-of-Research (to SH) for funding this project. The UC Davis Open Access Fund and the UC Davis Chancellor’s Postdoctoral Fellowship helped fund publishing of the manuscript. We thank Sistema Nacional de Areas de Conservación (SINAC) and Consejo Nacional para la Gestión de la Biodiversidad (CONAGEBIO) for permits to do field work and obtain the samples in Costa Rica. We thank Gilbert Barrantes for allowing us access to work at Museo de Zoología, Universidad de Costa Rica, Bernal Rodrigues for granting access to Reserva Biológica Tirimbina, and Reinaldo Aguilar at Los Charcos de Osa. We thank Michael Harvey, Gustavo Bravo, Diego Ocampo, and Karla Conejo for field assistance. We also thank the editors and reviewers at *Frontiers* for improving the manuscript with their suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fmicb.2015.01403>

Figure S1 | Principal coordinates analyses on unweighted (left column) and weighted (middle column) UniFrac distances and Non-metric Multidimensional Scaling (NMDS) analysis of bacterial composition of samples (right column). Samples are colored by bird order (top row), foraging strata (second row), diet (third row), and sampling locality (bottom row).

Figure S2 | Gut community dendrograms of (A) unweighted UniFrac distance and (B) Bray-Curtis distance. Colors indicate host bird taxonomic order.

Figure S3 | Heatmap of relative abundance of each identified phylotypes (columns) for each individual (rows). Taxonomic class of the bacteria and family of the bird are shown with gray bars. Horizontal black lines delimit taxonomic orders of the birds.

Supplemental Table S1 | Detailed information about each sample, including taxonomic information, dietary assignments, habitat, foraging stratum (FS), sampling locality (L), elevation (Elev), percent of skull ossification (Age), bacterial phyla identified (BRP), the percentage of individuals with fewer phylotypes identified (BRS) and LSUMNS bird tissue collection number (B#).

REFERENCES

- Al-Asmakh, M., Stukenborg, J.-B., Reda, A., Anuar, F., Strand, M.-L., Hedin, L., et al. (2014). The gut microbiota and developmental programming of the testis in mice. *PLoS ONE* 9:e103809. doi: 10.1371/journal.pone.0103809
- Anderson, K. E., Russell, J. A., Moreau, C. S., Kautz, S., Sullam, K. E., Hu, Y., et al. (2012). Highly similar microbial communities are shared among related and trophically similar ant species. *Mol. Ecol.* 21, 2282–2296. doi: 10.1111/j.1365-294X.2011.05464.x
- Banks, J. C., Cary, S. C., and Hogg, I. D. (2009). The phylogeography of Adelie penguin faecal flora. *Environ. Microbiol.* 11, 577–588. doi: 10.1111/j.1462-2920.2008.01816.x
- Barker, F. K., Burns, K. J., Klicka, J., Lanyon, S. M., and Lovette, I. J. (2013). Going to extremes: contrasting rates of diversification in a recent radiation of New World Passerine birds. *Syst. Biol.* 62, 298–320. doi: 10.1093/sysbio/sys094
- Bennett, P. M., and Owens, I. P. F. (2002). *Evolutionary Ecology of Birds: Life Histories, Mating Systems and Extinction*. New York, NY: Oxford University Press.
- Benskin, C. M. H., Rhodes, G., Pickup, R. W., Wilson, K., and Hartley, I. R. (2010). Diversity and temporal stability of bacterial communities in a model passerine bird, the zebra finch. *Mol. Ecol.* 19, 5531–5544. doi: 10.1111/j.1365-294X.2010.04892.x
- Brucker, R. M., and Bordenstein, S. R. (2013). The hologenomic basis of speciation: gut bacteria cause hybrid lethality in the genus *Nasonia*. *Science* 341, 667–669. doi: 10.1126/science.1240659
- Brumfield, R. T., Tello, J. G., Cheviron, Z. A., Carling, M. D., Crochet, N., and Rosenberg, K. V. (2007). Phylogenetic conservatism and antiquity of a tropical specialization: army-ant-following in the typical antbirds (*Thamnophilidae*). *Mol. Phylogenet. Evol.* 45, 1–13. doi: 10.1016/j.ympev.2007.07.019
- Burney, C. W., and Brumfield, R. T. (2009). Ecology predicts levels of genetic differentiation in Neotropical birds. *Am. Nat.* 174, 358–368. doi: 10.1086/603613
- Caporaso, J. G., Bittinger, K., Bushman, F. D., DeSantis, T. Z., Andersen, G. L., and Knight, R. (2010a). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* 26, 266–267. doi: 10.1093/bioinformatics/btp636
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010b). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303
- Chakravorty, S., Helb, D., Burday, M., Connell, N., and Alland, D. (2007). A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J. Microbiol. Methods* 69, 330–339. doi: 10.1016/j.mimet.2007.02.005
- Clarke, K. R. (1993). Non-parametric multivariate analysis of changes in community structure. *Aust. J. Ecol.* 18, 117–143. doi: 10.1111/j.1442-9993.1993.tb00438.x
- Colman, D. R., Toolson, E. C., and Takacs-Vesbach, C. D. (2012). Do diet and taxonomy influence insect gut bacterial communities? *Mol. Ecol.* 21, 5124–5137. doi: 10.1111/j.1365-294X.2012.05752.x
- Dewar, M. L., Arnould, J. P. Y., Dann, P., Trathan, P., Groscolas, R., and Smith, S. (2013). Interspecific variations in the gastrointestinal microbiota in penguins. *Microbiologyopen* 2, 195–204. doi: 10.1002/mbo3.66
- Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., et al. (2011). Normal gut microbiota modulates brain development and behavior. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3047–3052. doi: 10.1073/pnas.1010529108
- Drummond, A. J., and Rambaut, A. (2007). BEAST: bayesian evolutionary analysis by sampling trees. *BMC Evol. Biol.* 7:214. doi: 10.1186/1471-2148-7-214
- Eckburg, P., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., et al. (2005). Diversity of the human intestinal microbial flora. *Science* 308, 1635. doi: 10.1126/science.1110591
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340
- Gloor, G. B., Hummelen, R., Macklaim, J. M., Dickson, R. J., Fernandes, A. D., MacPhee, R., et al. (2010). Microbiome profiling by illumina sequencing of combinatorial sequence-tagged PCR products. *PLoS ONE* 5:e15406. doi: 10.1371/journal.pone.0015406
- Godoy-Vitorino, F., Goldfarb, K. C., Brodie, E. L., Garcia-Amado, M. A., Michelangeli, F., and Domínguez-Bello, M. G. (2010). Developmental microbial ecology of the crop of the folivorous hoatzin. *ISME J.* 4, 611–620. doi: 10.1038/ismej.2009.147
- Goodrich, J. K., Di Rienzi, S. C., Poole, A. C., Koren, O., Walters, W. A., Caporaso, J. G., et al. (2014). Conducting a microbiome study. *Cell* 158, 250–262. doi: 10.1016/j.cell.2014.06.037
- Goslee, S. C., and Urban, D. L. (2007). The ecodist package for dissimilarity-based analysis of ecological data. *J. Stat. Softw.* 22, 1–19. doi: 10.18637/jss.v022.i07
- Green, J., and Bohannan, B. J. M. (2006). Spatial scaling of microbial biodiversity. *Trends Ecol. Evol.* 21, 501–507. doi: 10.1016/j.tree.2006.06.012
- Hackett, S. J., Kimball, R. T., Reddy, S., Bowie, R. C. K., Braun, E. L., Braun, M. J., et al. (2008). A phylogenomic study of birds reveals their evolutionary history. *Science* 320, 1763–1768. doi: 10.1126/science.1157704
- Hauther, K. A., Cobaugh, K. L., Jantz, L. M., Sparer, T. E., and DeBruyn, J. M. (2015). Estimating time since death from postmortem human gut microbial communities. *J. Forensic Sci.* 60, 1234–1240. doi: 10.1111/1556-4029.12828
- Hird, S. M., Carstens, B. C., Cardiff, S. W., Dittmann, D. L., and Brumfield, R. T. (2014). Sampling locality is more detectable than taxonomy or ecology in the gut microbiota of the brood-parasitic Brown-Headed Cowbird (*Molothrus ater*). *PeerJ* 2:e321. doi: 10.7717/peerj.321
- Huber, T., Faulkner, G., and Hugenholtz, P. (2004). Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* 20, 2317–2319. doi: 10.1093/bioinformatics/bth226
- Jenkins, C. N., Pimm, S. L., and Joppa, L. N. (2013). Global patterns of terrestrial vertebrate diversity and conservation. *Proc. Natl. Acad. Sci. U.S.A.* 110, E2602–E2610. doi: 10.1073/pnas.1302251110
- Klicka, J., Burns, K., and Spellman, G. (2007). Defining a monophyletic Cardinalini: a molecular perspective. *Mol. Phylogenet. Evol.* 45, 1014–1032. doi: 10.1016/j.ympev.2007.07.006
- Kohl, K. D. (2012). Diversity and function of the avian gut microbiota. *J. Comp. Physiol. B* 182, 591–602. doi: 10.1007/s00360-012-0645-z
- Ley, R. E., Hamady, M., Lozupone, C., Turnbaugh, P. J., Ramey, R. R., Bircher, J. S., et al. (2008a). Evolution of mammals and their gut microbes. *Science* 320, 1647–1651. doi: 10.1126/science.1155725
- Ley, R. E., Lozupone, C. A., Hamady, M., Knight, R., and Gordon, J. (2008b). Worlds within worlds: evolution of the vertebrate gut microbiota. *Nat. Rev. Microbiol.* 6, 13. doi: 10.1038/nrmicro1978
- Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* 71, 8228. doi: 10.1128/AEM.71.12.8228-8235.2005
- McArdle, B. H., and Anderson, M. J. (2001). Fitting multivariate models to community data: a comment on distance-based redundancy analysis. *Ecology* 82, 290–297. doi: 10.1890/0012-9658(2001)082[0290:FMMTCD]2.0.CO;2
- McMurdie, P. J., and Holmes, S. (2014). Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput. Biol.* 10:e1003531. doi: 10.1371/journal.pcbi.1003531
- Muegge, B., Kuczynski, J., Knights, D., Clemente, J. C., González, A., Fontana, L., et al. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science* 332, 970–974. doi: 10.1126/science.1198719
- Ochman, H., Worobey, M., Kuo, C.-H., Ndjango, J.-B. N., Peeters, M., Hahn, B. H., et al. (2010). Evolutionary relationships of wild hominids recapitulated by gut microbial communities. *PLoS Biol.* 8:e1000546. doi: 10.1371/journal.pbio.1000546
- Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., et al. (2011). *Vegan: Community Ecology Package*. R package version 2.0-2. Available online at: <http://CRAN.R-project.org/package=vegan>
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). FastTree: computing large minimum-evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26, 1641–1650. doi: 10.1093/molbev/msp077
- Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., et al. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 464, 59–65. doi: 10.1038/nature08821
- Quinn, G., and Keough, M. (2002). *Experimental Design and Data Analysis for Biologists*. Cambridge: Cambridge University Press. doi: 10.1017/cbo9780511806384

- R Development Core Team (2010). *R: A Language for Statistical Computing*. Vienna: R Foundation for Statistical Computing.
- Remsen, J. V. Jr., Cadena, C. D., Jaramillo, A., Nores, M. A., Pacheco, J. F., Perez-Eman, J., et al. (2012). *A Classification of the Bird Species of South America*. American Ornithologists' Union. Available online at: <http://www.museum.lsu.edu/~Remsen/SACCBaseline.html>
- Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., Guillemin, K., et al. (2011). Evidence for a core gut microbiota in the zebrafish. *ISME J.* 5, 1595–1608. doi: 10.1038/ismej.2011.38
- Roggenbuck, M., Schnell, I., Blom, N., Bælum, J., Bertelsen, M. F., Pontén, T. S., et al. (2014). The microbiome of New World vultures. *Nat. Commun.* 5:5498. doi: 10.1038/ncomms6498
- Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., et al. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 12:87. doi: 10.1186/s12915-014-0087-z
- Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., et al. (2009). Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* 75, 7537–7541. doi: 10.1128/AEM.01541-09
- Schloss, P. D., and Handelsman, J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Environ. Microbiol.* 71, 1501–1506. doi: 10.1128/AEM.71.3.1501-1506.2005
- Sharon, G., Segal, D., Ringo, J. M., Hefetz, A., Zilber-Rosenberg, I., and Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20051–20056. doi: 10.1073/pnas.1009906107
- Smillie, C. S., Smith, M. B., Friedman, J., Cordero, O. X., David, L. A., and Alm, E. J. (2011). Ecology drives a global network of gene exchange connecting the human microbiome. *Nature* 480, 241–244. doi: 10.1038/nature10571
- Smith, B. T., McCormack, J. E., Cuervo, A. M., Hickerson, M. J., Aleixo, A., Cadena, C. D., et al. (2014). The drivers of tropical speciation. *Nature* 515, 406–409. doi: 10.1038/nature13687
- Stotz, D. F., Fitzpatrick, J. W., Parker, T. A., and Moskovits, D. K. (1996). *Neotropical Birds: Ecology and Conservation*. Chicago, IL: The University of Chicago Press.
- Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'Connor, M. P., Rosen, G. L., Knight, R., et al. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol. Ecol.* 21, 3363–3378. doi: 10.1111/j.1365-294X.2012.05552.x
- Tello, J., Moyle, R., Marchese, D., and Cracraft, J. (2009). Phylogeny and phylogenetic classification of the tyrant flycatchers, cotingas, manakins, and their allies (*Aves: Tyrannides*). *Cladistics* 25, 429–467. doi: 10.1111/j.1096-0031.2009.00254.x
- Turnbaugh, P., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., et al. (2008). A core gut microbiome in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540
- Turnbaugh, P. J., Quince, C., Faith, J. J., McHardy, A. C., Yatsunenko, T., Niaz, F., et al. (2010). Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7503–7508. doi: 10.1073/pnas.1002355107
- Videnska, P., Rahman, M. M., Faldynova, M., Babak, V., Matulova, M., Prukner-Radovic, E., et al. (2014). Characterization of egg laying hen and broiler fecal microbiota in poultry farms in Croatia, Czech Republic, Hungary and Slovenia. *PLoS ONE* 9:e110076. doi: 10.1371/journal.pone.0110076
- Waite, D. W., and Taylor, M. W. (2014). Characterizing the avian gut microbiota: membership, driving influences, and potential function. *Front. Microbiol.* 5:223. doi: 10.3389/fmicb.2014.00223
- Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/AEM.00062-07
- Whitman, W. B., Coleman, D. C., and Wiebe, W. J. (1998). Prokaryotes: the unseen majority. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6578–6583. doi: 10.1073/pnas.95.12.6578
- Wienemann, T., Schmitt-Wagner, D., Meuser, K., Segelbacher, G., Schink, B., Brune, A., et al. (2011). The bacterial microbiota in the ceca of Capercaillie (*Tetrao urogallus*) differs between wild and captive birds. *Syst. Appl. Microbiol.* 34, 542–551. doi: 10.1016/j.syapm.2011.06.003
- Xenoulis, P. G., Gray, P. L., Brightsmith, D., Palculict, B., Hoppes, S., Steiner, J. M., et al. (2010). Molecular characterization of the cloacal microbiota of wild and captive parrots. *Vet. Microbiol.* 146, 320–325. doi: 10.1016/j.vetmic.2010.05.024
- Xu, J., and Gordon, J. I. (2003). Honor thy symbionts. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10452–10459. doi: 10.1073/pnas.1734063100
- Yatsunenko, T., Rey, F. E., Manary, M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053
- Zhu, X. Y., Zhong, T., Pandya, Y., and Joerger, R. D. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Appl. Environ. Microbiol.* 68, 124–137. doi: 10.1128/AEM.68.1.124-137.2002

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2015 Hird, Sánchez, Carstens and Brumfield. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.