Microbial Ecology

A Molecular Comparison of Plumage and Soil Bacteria Across Biogeographic, Ecological, and Taxonomic Scales

Isabelle-Anne Bisson[1], Peter P. Marra[1], Edward H. Burtt Jr.[2], Masoumeh Sikaroodi[3] and Patrick M. Gillevet[3]

(1) Smithsonian Environmental Research Center, 647 Contees Wharf Road, Edgewater, MD 21037, USA
(2) Department of Zoology, Ohio Wesleyan University, Delaware, OH 43015, USA
(3) Department of Environmental Sciences and Policy, George Mason University, Manassas, VA 22030, USA

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Abstract

We used molecular methods to determine the microbial community of soil and avian plumage across biogeographic, ecological, and taxonomic scales. A total of 17 soil and 116 feather samples were collected from five avian species across multiple habitat types within one Neotropical and one temperate locality. Hypotheses regarding patterns of microbial composition relative to acquisition and dispersal of plumage bacteria in the ecosystem were tested by comparing microbial communities within and between soil and plumage. Samples from the plumage of American Redstarts (*Setophaga ruticilla*) were collected across both habitat types and geographic scales for intraspecific comparisons. The microbial diversity in avian plumage was moderately diverse and was dominated by *Pseudomonas* species. Despite a highly significant individual bird effect on microbial composition of the plumage, we detected significant biogeographic and type of habitat effects. *Pseudomonas* species were more abundant on the temperate site when all avian species were included in the analysis, and *Bacillus subtilis* and *Xanthomonas* groups were more abundant on the Neotropical site for redstarts alone. However, 16S rDNA sequence libraries were not significantly different between Jamaican and Maryland redstarts. Biogeographic and habitat effects were significant and more pronounced for soil samples indicating lower dispersal of soil microbiota. We detected a significant difference between soil and plumage microbial communities suggesting that soil plays a small role in plumage bacterial acquisition. Our results suggest bacterial communities on the plumage of birds are dynamic and may change at different stages in a bird's annual cycle.

Introduction

Microorganisms account for more than half of the total biomass on the planet, yet we have barely begun to quantify the species diversity, structure, and functioning of microbial ecosystems [42]. Recent improvements and availability of molecular techniques (e.g., [56]) enable us to explore unusual microbial ecosystems (e.g., [8]) and to include unculturable as well as culturable microorganisms [39, 42]. Such microbial explorations are important because microorganisms, particularly bacteria, are key contributors to the health of most ecosystems on the planet [36]. Given the ubiquity [17] and importance of bacterial communities, the need to document their diversity, ecology, and dispersal across all ecosystems and biogeographic regions is critical to the global understanding of the biosphere.

The plumage of migratory birds forms an especially fascinating ecosystem. Avian plumage is a diverse ecosystem that harbors a complex community of ectoparasites such as feather lice (Phthiraptera: Ischnocera) and mites (e.g., *Ornithonyssus bursa*), and microorganisms such as feather-degrading bacteria (e.g., *Bacillus licheniformis*) and fungi (e.g., *Arthroderma* spp.). Although the plumage invertebrates are well studied (e.g., [9, 12, 13, 47]), very few studies have focused on plumage microorganisms [6]. In a pioneering effort, Burtt and Ichida [8] assayed more than 1600 birds using culture-dependent techniques and found that the frequency of birds with feather-degrading bacilli varied with the behavioral ecology of the birds. For

Present address of Isabelle-Anne Bisson: Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ 08544, USA
Present address of Peter P. Marra: Smithsonian Migratory Bird Center, National Zoological Park, 3001 Connecticut Ave, Washington, DC 20008, USA
Correspondence to: Isabelle-Anne Bisson; E-mail: ibisson@princeton.edu

example, feather-degrading bacilli were more often isolated from water birds and ground foragers than from species that typically forage for flying insects. Recently, Burtt and Ichida [7] showed that the feather-degrading ability of *B. licheniformis* varies geographically and may select for color variation in the plumage of Song Sparrows (*Melospiza melodia*). Such variation in feather-degrading bacilli associated with geographical, ecological, and behavioral differences in songbirds generally and Song Sparrows in particular suggests that structure and function of the entire microbial ecosystem in plumage may be influenced by a bird's ecology (e.g., migration, habitat type) or behavior (e.g., maintenance, feeding behavior).

The mode of acquisition of plumage bacteria by birds is poorly understood. The greater abundance of feather-degrading bacilli, common soil bacteria [35], on ventral feathers and in ground foraging species [8] suggests that soil is an important source of plumage microorganisms (see also [52]). Birds may also acquire their plumage microbial community through contact with vegetation, or through unrelated birds in the community (horizontal acquisition), or from parent to offspring (vertical acquisition). Because migratory birds may act as key dispersal agents of microbial organisms [46], including emerging infectious diseases such as West Nile virus [37] and avian influenza [33], and because evidence suggests that some diseases may be dispersed from the plumage [28], understanding more about the diversity and acquisition of plumage bacteria can help assess this possibility further. Therefore, an assay of the diversity and abundance of bacteria in the plumage of birds and their local environment across biogeographic, ecological, and taxonomic scales is a fundamental first step. Microorganisms in the plumage of Neotropical migratory birds provide a unique model because these microorganisms are exposed to multiple and very different habitats within a single annual cycle as the bird migrates between temperate breeding and Neotropical nonbreeding sites. Yet, we do not know how long-distance movement of the plumage ecosystem affects microbial dispersal or how microbial diversity within the plumage is affected by geographic and environmental changes associated with avian migration.

Microbial surveys across large spatial scales have primarily been conducted within one ecosystem type at a time (e.g., soil [22], aquatic [63]). Our assay of bacterial diversity and community composition in soil as well as plumage ecosystems, which differ in their dispersal mechanisms, is a unique test of how dispersal influences the biogeography of microbial communities. Our objective is to describe, identify, and compare the diversity and abundance of bacteria in plumage and associated local soil across geographic, ecological, and taxonomic scales. Studies on microorganisms inhabiting plumage ecosystems thus far have been limited to culture-dependent, feather-degrading species in temperate-zone birds (e.g., [8, 52]). In this study, we used culture-independent techniques to determine the bacterial community in the plumage of a migratory species, the American Redstart (*Setophaga ruticilla*), and associated migrant avifauna between linked, but separate nonbreeding (tropical) and breeding (temperate) locations. These sites are linked in that we have used hydrogen isotopes to show that the Jamaican and Maryland sites represent local samples of a larger, linked redstart population (δD; Norris et al., unpublished data). Such linkage allows comparison across geographic regions that are connected through the very ecosystem under study: the plumage. We also compare the diversity and abundance of the plumage bacterial community to that of the soil in the bird's immediate environment using the same culture-independent techniques. Finally, we compare the microbial community within soil and plumage samples across habitat types within the nonbreeding and breeding sites. We specifically test the following three hypotheses: (1) the plumage bacterial community is acquired locally and differs between Neotropical (nonbreeding) and temperate zone (breeding) locations and between habitat types within each breeding and non–breeding site. If plumage bacteria are acquired locally, we also expect that bacterial composition does not differ among species and individual birds captured within the same locality; (2) birds acquire their plumage bacterial community from the soil and similar microbial groups will be detectable in both ecosystems; (3) the bacterial composition of the plumage shows less biogeographic patterns than that for the soil ecosystem because high vagility of the plumage ecosystem in migratory birds enables the bacterial soil community from different biogeographic regions to colonize the plumage ecosystem.

Materials and Methods

Study System A total of 29 individuals from five avian species were captured by using mist nets, song recordings, and decoys at one Neotropical nonbreeding site (Black River, Jamaica) in March 2004 and one temperate breeding site (Maryland, USA) in June 2004. Avian species included the focal study species, the American Redstart, which was sampled at both nonbreeding ($n = 10$) and breeding ($n = 7$) locations. Three associated migratory species that were sampled only at the breeding site included the Red-eyed Vireo (*Vireo olivaceus*, $n = 5$), the Hooded Warbler (*Wilsonia citrina*, $n = 1$), and the Common Yellowthroat (*Geothlypis trichas*, $n = 1$). Additionally, the Black-throated Blue Warbler (*Dendroica caerulescens*) was sampled at the nonbreeding site only ($n = 5$).

The nonbreeding site was located at the Font Hill Nature Preserve (18°02′N, 77°57′W) in St. Elizabeth Parish, Black River, Jamaica. Two habitat types/study plots were considered in the nonbreeding site: mangrove forest and second growth scrub. Study sites in mangrove forest were dominated by black mangrove (*Avicennia germinans*) with less extensive presence of white mangrove (*Languncularia recemosa*) and red mangrove (*Rhizophora mangle*). Study sites in second growth scrub contained trees ranging from 2 to 5 cm in diameter at breast height and 2–8 m in height forming a dense understory and ground layer of vegetation. The temperate breeding site was located in the Patuxent River Park (38°45′N, 76°42′W) in Maryland, USA. Birds were captured in two different 5-ha plots that were located 1800 m apart. Plots differed in ground water levels with the "wet" plot mostly covered by water of 1 m in depth and little to no ground vegetation, whereas the "dry" plot was moist with heavy ground vegetation 2–5 m in height. However, differences in habitat between the plots in Maryland were not as pronounced as between mangrove and dry scrub in Jamaica.

Field Sampling of Plumage and Soil Microorganisms Before removing each captured bird from the net, we sterilized our hands using quaternary disinfectant or store-bought hand sanitizer [8]. These disinfectants reduced microbial contamination on our hands to barely detectable levels (Burtt and Ichida, pers. comm.). Gloves were not used because of the difficulty they posed when removing birds from the net. We sterilized and dried our hands after each bird was handled. We marked each American Redstart with a unique combination of US Fish and Wildlife Service band and color bands. Other species were marked by cutting the tip of the third left rectrix to ensure that we did not resample the same bird. Shortly after capture and removal, we plucked 10 feathers each from the breast, dorsum, and head regions along with one tail feather using sterile forceps. Feathers from each body region were placed in individual sterile envelopes and stored at 4°C until processed in the laboratory. After sampling, we sexed and aged each bird before release. Additionally, eight soil samples were collected from the same sites where avian plumage was sampled in both the mangrove ($n = 5$) and dry scrub ($n = 3$) habitats in Jamaica and nine from the two plots in Maryland (dry, $n = 5$; wet, $n = 4$). The top 1 cm of soil was collected using a sterile spoon, placed in sterile 50-mL tubes, and stored at 4°C until processed in the laboratory.

DNA Extraction, LH-PCR Fingerprinting, and 16S rDNA Sequencing. We used length heterogeneity-polymerase chain reaction (LH-PCR), a culture-independent method, to assay the plumage microbial community present in the five avian species and 17 soil samples. LH-PCR is a PCR technique that distinguishes different organisms based on natural variations in the length and composition of the hypervariable regions of the 16S ribosomal DNA sequence. LH-PCR is robust and highly reproducible for different environmental samples when compared to other methods such as terminal restriction fragment length polymorphism (T-RFLP; [32, 38]) and therefore provides a consistent measure of microbial community composition. Cloning and sequen-cing of the 16S rDNA gene can subsequently be conducted for a subset of samples to describe the community at more definitive taxonomic levels (e.g., species).

We extracted DNA directly from feather samples (three feathers for each breast, dorsal, and head body regions, and one tail feather) and soil samples (~500 mg) using a FastDNA spin kit for soil (QBiogene, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. One modification was performed for feathers only. The first two steps of the extraction procedure involved the sonication of feather samples for 15 min in 978 μL phosphate buffer saline (PBS and 122 μL MT buffer to dislodge attached bacteria [34] followed by 10 min of vortexing. The extraction method is highly reproducible in microbial DNA extractions from difficult environmental samples. The extracted DNA was quantitated on agarose gels and the first two variable regions of the 16S rDNA was amplified using about 10 ng DNA in a PCR reaction with fluorescently labeled (6FAM) forward primer (27F 5′-FAM-AGA GTT TGA TCM TCG CTC AG-3′) and unlabeled reverse primer (335R 5′-GCT GCC TCC CGT AGG AGT-3′). Both primers are universal bacterial primers [30].

Before PCR preparation, tubes and pipette tips were UV-irradiated (254 nm) by using a Spectroline Ultraviolet Transilluminator for 30 min [45]. UV irradiation produces pyrimidine dimer adducts that prevent the effective amplification of DNA contamination, which may be present in laboratory equipment or PCR reagents [45]. PCR reactions were prepared by using pureTaq Ready-To-Go PCR beads (Amersham Biosciences Inc., Piscataway, NJ, USA). Each bead is reconstituted in a 25-μL (final volume) mixture containing bovine serum albumin, 2.5 mM $MgCl_2$, each deoxynucleotide triphosphate at a concentration of 200 μM in 10 mM Tris–HCl (pH 9.0), 50 mM KCl buffer, each primer at a concentration of 10 μM, and 5 units/μL of *Taq* DNA polymerase. Before adding the FAM-labeled 27F primer, the PCR reaction was UV-irradiated by using the same transilluminator for 5 min to minimize DNA contamination of samples. A typical PCR run using a PTC 200 Thermal Cycler includes an initial denaturation step at 95°C for 11 min, followed by 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 48°C for 30 s, and extension at 74°C for 2 min. There is a final extension step at 72°C for 45 min to ensure the proper extension of all fragments.

Each PCR reaction included two negative control samples in which no DNA was added to verify the lack of contaminating DNA and a positive control using *Escherichia coli* DNA. LH-PCR samples are stored at 4°C in the dark until used (usually less than 1 week). We duplicated PCR amplifications for a subset of the samples ($n = 98$ feathers; $n = 12$ soil) to assess reproducibility and replicate PCR products that were subsequently processed for LH-PCR profiling. Dilution of PCR products were made based on the quantization on 1% agarose gel and ethidium bromide and the diluted product was mixed with ILS-600 (Promega) size standard (1/20 in Hi Di Formamide) and run on a SCE9610 (Spectrumedix LLC) capillary sequencer. The raw data were then analyzed with Genospectrum software (Spectrumedix LLC), which performs color deconvolution and size interpolation of the resulting electropherogram peaks. The peaks of the electropherograms represent different operation taxonomic units (OTU) of microbiota and generally indicate different genera/species of bacteria. A custom PERL script was used to calculate relative peak areas (Normalized Abundance) and interleave the profiles from various samples.

The plumage of one redstart sampled at the nonbreeding site and one sampled at the breeding site were chosen for cloning and sequencing. Feathers collected from the breast, head, and back body regions and one tail feather were pooled for each bird and used in a PCR reaction with the same primers that were used for LH-PCR fingerprinting (nonlabeled L27F and 355R). After visual verification of the PCR products on a 1% agarose gel with ethidium bromide, the products were cloned using TOPO TA Cloning (Invitrogen Life Technologies) kit following the manufacturer's protocols. White colonies containing cloned PCR products were picked and lysed in TE buffer (pH 8.0). The cloned PCR products were amplified with M13 primers [M13F (–40) 5′-GTTTTCCCAGTCACGAC-3′ and M13R 5′-CAGGAAACAGCTATGAC-3′] and visualized by ethidium bromide in 1% agarose gel. Products of the right size were purified by using Ampure kit (Agencourt) and quantitated by running on 1% agarose gel using ethidium bromide. The sequencing reactions were done on purified PCR products using Big Dye Terminator v3.0 kit (Applied Biosystems Inc.). We used standard sequencing reaction conditions of one cycle of 96°C for 1 min, and 45 cycles of 96°C for 30 s, 45°C for 15 s and 60°C for 4 min. The sequencing reactions were then purified using Sephedex G-50 (Sigma-Aldrich) and run on SCE9610 (SpectruMedix LLC). The sequences were analyzed using BaseSpectrum software (SpectuMedix LLC) and imported to Sequencher 4.1 software (GeneCode Corporation) to make the final base calls. The 300- to 350-bp amplicons (16s rDNA sequence PCR-amplified) are adequate to identify bacterial components in environmental communities [16, 20, 21, 25, 29, 31, 49, 57, 63, 64].

Statistical Analyses and Clone Identification

Multidimensional scaling ordination and analysis of similarity We used the Bray–Curtis [3] index to construct similarity matrices for subsequent nonparametric statistical tests in Primer v.5.2.9 [11]. We tabulated the relative abundance (normalized abundance calculated from the relative peak areas using PERL scripts) of each amplicon from the LH-PCR analyses into a data matrix and then applied a first square-root transformation of the data. We then used the Bray–Curtis similarity index to measure bacterial community similarity. Multidimensional scaling ordination (MDS) analyses are performed and plotted to assess similarities among samples in low-dimensional space (2D or 3D) without *a priori* grouping. MDS plots represent relative distances among samples in relation to the rank order of their relative similarities. In the MDS plots, sample points that are close together are more similar in their bacterial composition than those that are far apart. The goodness-of-fit of the plot to the similarity matrix is assessed with a stress formula. Stress values <0.2 typically indicate a good representation of the data in a 2D space [11].

One-way and two-way nested analysis of similarity (ANOSIM) tests were subsequently performed to compare the plumage bacterial composition and diversity at three scales: (1) taxonomic, i.e., among avian species with sample size >1; (2) ecological, i.e., between habitats within the nonbreeding (Jamaica) and breeding (Maryland) sampling sites; and (3) geographic, i.e., between the nonbreeding and breeding sites. ANOSIM uses similarity matrices to conduct an approximation of the standard univariate analysis of variance testing for among-group differences. ANOSIM tests provide the R test statistic together with a probability value. R values are equally, if not more, important to consider when evaluating the outcome of ANOSIM analyses. Specifically, the R test statistic can be defined as

$$\mathrm{R} = \frac{(\overline{r}_{\mathrm{B}} - \overline{r}_{\mathrm{W}})}{\frac{1}{2}M}$$

where $\overline{r}_{\mathrm{B}}$ is the average of all rank similarities among samples within groups and $\overline{r}_{W}$ is the average of rank similarities from all sample pairs between groups, $M = n(n-1)/2$. The R value represents the absolute value of how similar or dissimilar the groups are. If the similarities among samples between and within groups are the same, $R = 0$ and we accept the null hypothesis. If the similarities among samples are greater between groups than within groups, $R = 1$ and we reject the null hypothesis. When significant differences ($P < 0.05$) were found at any scale, we used similarity percentages (SIMPER) to determine which amplicons contributed most to the similarities within groups and dissimilarities among groups.

Because Burtt and Ichida [8] found differences in bacterial composition among different body regions in 32 avian species, we first performed a two-way crossed ANOSIM to test for body region and individual bird effect to determine whether analyses should additionally be performed separately for each morphological region. The community of plumage bacteria differed significantly among body regions (two-way crossed ANOSIM, $R = 0.270$, $P = 0.001$) and individuals ($R = 0.487$, $P = 0.001$), with the individual effect producing a higher global R value. The same was true when we conducted the analyses for American Redstarts separately (body region effect, $R = 0.240$, $P = 0.001$; individual effect, $R = 0.473$, $P = 0.001$). Further plumage bacterial composition comparisons among groups (species, plots within sites, between sites, and between soil and feather samples) were therefore conducted as a one-way design using all body regions per individual, as a one-way design for each body region separately using individuals as replicates, and when significant among-group differences were detected, as a two-way nested design using individuals nested within each group to account for individual effect. We did not sample the tail feather from one Redstart in Jamaica and that individual was omitted from analyses conducted on body regions separately. In addition, we used a one-way ANOSIM to test for differences between soil and feather bacterial compositions.

All other analyses including diversity graphs were conducted using SPSS statistical package v. 11.0 [54].

Clone identification and analyses Sequences from the clones were compared to sequences in the Ribosomal Database Project database (version 8.0) to assess patterns in the microbial community using a custom PERL script. We compared the community clone library sequences using LIBSHUFF v. 1.22 [53]. The LIBSHUFF program provides a statistical framework to test observed differences between two or more 16S rDNA libraries. To compute the difference between libraries (ΔC), the program applies an approximation of the Cramér–von Mises statistic,

$$\Delta C_{XY} = \sum_{D=0.00}^{0.5} |C_X(D) - C_{XY}(D)|^2$$

where D is the evolutionary distance among sequences, C (coverage) describes the extent to which the 16S rDNA library represents the bacterial population, and $C_{XY} = 1 - (N_{XY}/n)$, where N_{XY} is the number of sequences in the library of sample X that are not found in the library of sample Y and n is the total number of sequences in sample X. The same is computed for sequences in the library of sample Y (ΔC_{YX}). LIBSHUFF further applies a Monte Carlo procedure to shuffle the samples between the two libraries to obtain p values. When ΔC value of the original libraries is greater than 95% of the ΔC values of the randomized shuffle of sequences, the libraries are considered significantly different at a p value of 0.05.

Each amplified 16S rDNA sequence, or amplicon, is hereafter referred to as operational taxonomic unit (OTU). All unique 16S rDNA sequences were submitted to GenBank (accession numbers DQ856126–DQ856301).

Results

A total of 66 LH-PCR OTUs were amplified in both plumage and soil samples. We performed paired-sample t tests for a subset of the plumage data ($n = 98$ over all body regions) and soil data ($n = 12$) to assess the repeatability of LH-PCR duplicates. We used the Bonferroni correction to adjust for multiple comparisons. Duplicates were not significantly different at the 99% confidence level for all OTUs amplified from plumage samples ($|t_{97}| \leq 1.967$, $p \geq 0.052$) and soil samples (($|t_{11}| \leq 2.477$, $p \geq 0.031$). LH-PCR duplicates were therefore highly repeatable as shown in previous reports [38].

Bacterial Composition of the Plumage in All Species Sampled LH-PCR analyses of the plumage bacterial community revealed a total of 48 OTUs across all feather samples ($n = 115$). Three of the 48 amplicons were amplified in most plumage samples and therefore represented the dominant bacterial OTUs in the plumage of birds. Average amplicon similarity among feather samples (57.8%), including all body regions, indicated moderate similarity among plumage samples. The most abundant OTU was 336 bp in length contributing 74.7% of the average OTU similarity among all feather samples (hereafter referred to as OTU 336), followed by one at 344 bp (7.5% of average similarity) and one at 333 bp (5.82% of average similarity).

No a priori grouping Axis one of the two-dimensional MDS plot (Fig. 1) separated three of the five Black-throated Blue Warbler individuals sampled at the non-breeding site from all other individuals of all species. These clusters (group one and two, Fig. 1) differed significantly ($R = 0.948$, $p = 0.001$) in microbial composition. OTU 336, which was the most important contributor (13.5% contribution) to the average among-group dissimilarity (78.4%, Fig. 2), was less abundant in the cluster including the three Black-throated Blue Warbler individuals (0.07) than in the first cluster including all other plumage samples (0.64).

Geographic site effect We detected a significant geographic site effect using a two-way nested ANOSIM controlling for individual effect and including all avian species (Table 1). OTUs 336, 344, 357, and 333 cumulatively contributed 41% of the average dissimilarity be-

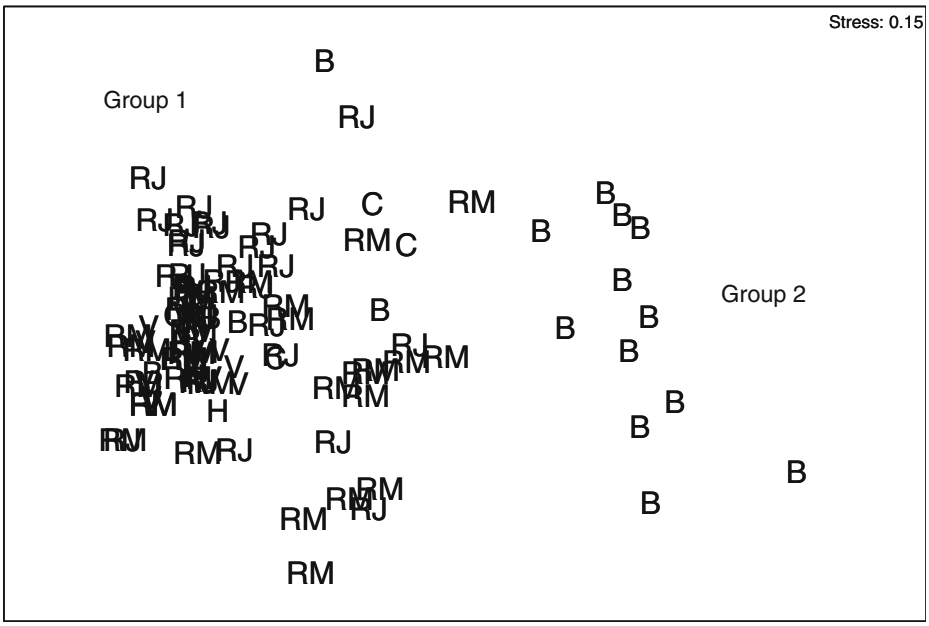

Figure 1. Two-dimensional ordination of plumage bacterial composition sampled at the nonbreeding site (Jamaica) and breeding site (Maryland) including all body regions for five avian species: Black-throated Blue Warblers (B), American Redstarts in Maryland (RM), American Redstarts in Jamaica (RJ), Common Yellowthroat (C), Hooded Warbler (H), and Red-Eyed Vireo (V). The MDS shows two distinct clusters, one of which (group 2) included three of the five Black-throated Blue Warbler individuals sampled.

tween sites (9.3%, 8.9%, 8.4%, and 7.2% contribution, respectively). We therefore tested for species effect on the plumage bacterial composition in both sites separately.

Avian species effect A one-way ANOSIM including all body regions detected a significant avian species effect on the plumage microbial composition sampled at both sites. Differences were greater between American Redstarts and Black-throated Blue Warblers on the nonbreeding site than between American Redstarts and Red-eyed Vireos on the breeding site (Table 1). OTU diversity (Shannon–Wiener index [50]) was highest for Black-throated Blue Warblers (Fig. 3), followed by Jamaican redstarts, Maryland redstarts, and finally, Red-eyed Vireos had the lowest

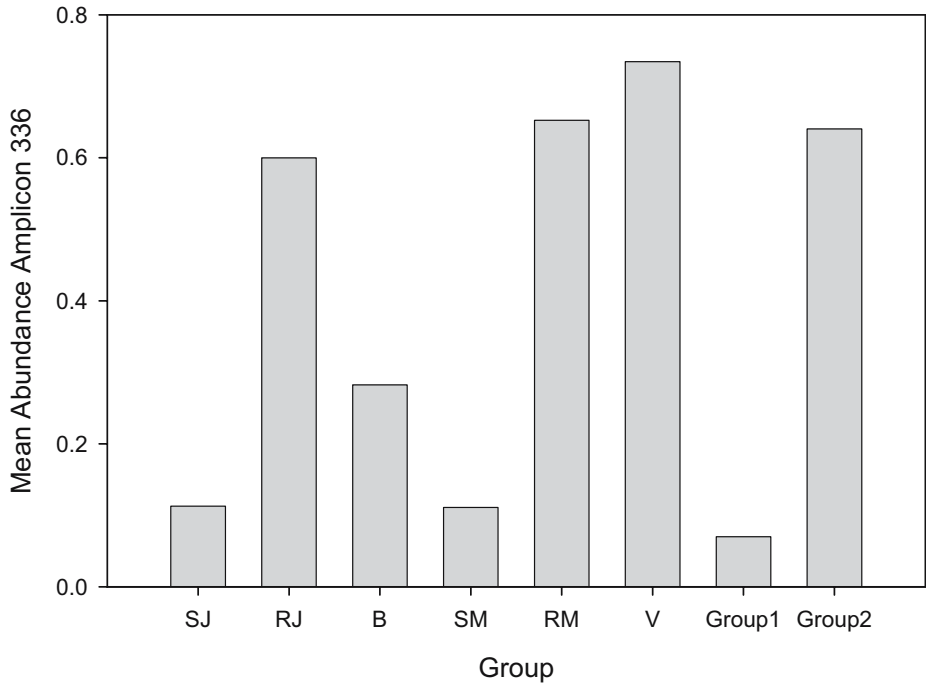

Figure 2. Mean normalized abundance of OTU 336 for each group compared in this study: soil in Jamaica (SJ), American Redstarts in Jamaica (RJ), Black-throated Blue Warblers (B), soil in Maryland (SM), American Redstarts in Maryland (RM), Red-eyed Vireos (V), and groups one and two from the cluster analysis (Fig. 1).

Table 1. ANOSIM and SIMPER (%) comparisons of plumage bacterial composition sampled in Black-throated Blue Warblers (B), American Redstarts (R), and Red-eyed Vireos (V) at the breeding (MD) and nonbreeding sites (JA) 2004

	One-way ANOSIM	*Two-way nested ANOSIM accounting for individual effect*	*Average dissimilarity (%)*
Main effect	*Global R*[a]	*Global R*	
I. Site			
JA vs. MD (all body regions)	0.076**	0.177*	48.0
JA vs. MD (per body region)			
Breast	0.041[ns]		46.8
Dorsal	0.040[ns]		45.3
Head	0.055[ns]		45.2
Tail	0.078[ns]		54.0
I. Species in MD			
R vs. V (all body regions)	0.079*	–0.252[ns]	39.0
R vs. V (per body region)			
Breast	0.147[ns]	–	39.4
Dorsal	–0.012[ns]	–	40.8
Head	0.018[ns]	–	37.4
Tail	0.047[ns]	–	39.3
I. Species in JA			
B vs. R (all body regions)	**0.480****	–0.104[ns]	62.2
B vs. R (per body region)			
Breast	0.377*	–	59.4
Dorsal	0.421*	–	58.2
Head	**0.652****	–	62.1
Tail	0.384*	–	68.0

[a]*R* values are significant at $*P<0.05$, $**P<0.01$, ns = not significant. Bold *R* values indicate the highest levels of differentiation detected among samples for each level of analysis. Dash lines (–) indicate that tests were not performed due to small sample sizes.

mean diversity. OTU 336 was the most important contributor to the dissimilarity among species (11.3% contribution), where redstarts had a higher abundance of this OTU (0.60) than Black-throated Blue Warblers (0.28) in Jamaica but a lower abundance of OTU 336 than Red-eyed Vireos in Maryland (0.05 and 0.13, respectively). However, the species effect was no longer significant when we controlled for individual effect (Table 1). The negative *R* value indicates greater variability in the bacterial composition among individuals than among species.

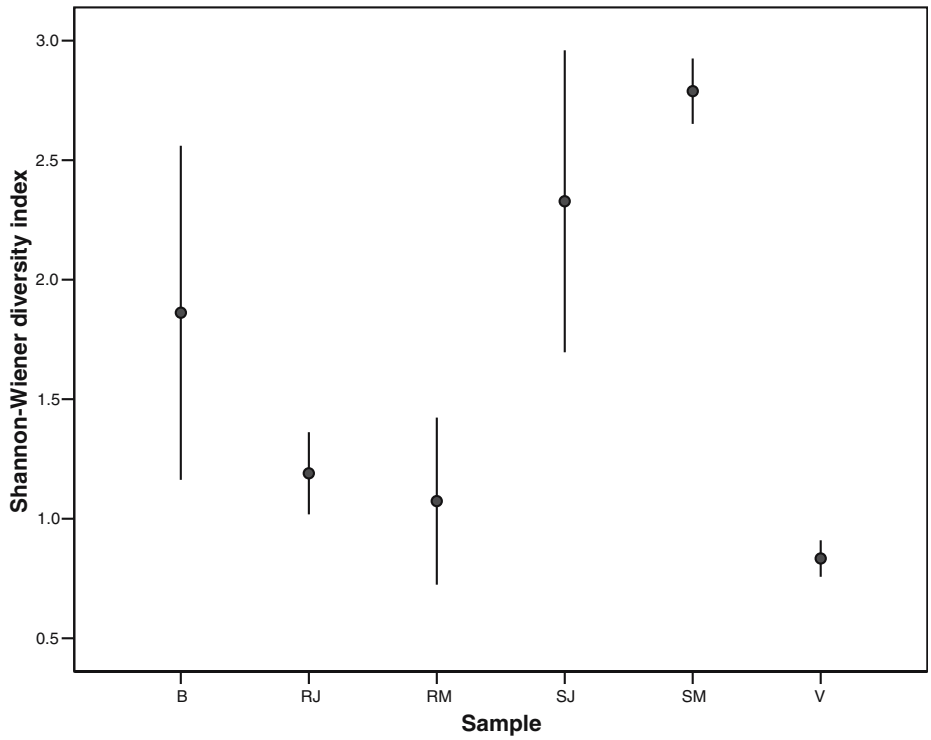

Figure 3. Means and 95 confidence intervals for Shannon–Wiener diversity index calculated for Black-throated Blue Warblers (B), American Redstart sampled in Jamaica (RJ), American Redstart sampled in Maryland (RM), Red-eyed Vireos (V), Jamaica soil samples (SJ), and Maryland soil samples (SM).

When analyses were repeated for each body region separately, we found significant differences in the plumage microbial composition among avian species sampled on the nonbreeding ground only (Table 1). Redstarts and Black-throated Blue Warblers differed primarily in the microbial composition of the head plumage (highest *R* value). Again, OTU 336 was the most important contributor to the dissimilarity in head feathers between redstarts and Black-throated Blue Warblers (9.4% contribution) closely followed by OTU 342 (8.29% contribution). Redstarts had a higher abundance of OTU 336 in their head plumage (0.60) than Black-throated Blue Warblers (0.28, Fig. 2) and did not have OTU 342.

Bacterial Composition of American Redstart Plumage

Geographic site effect LH-PCR analyses revealed 30 OTUs in Redstart feathers ($n = 67$). When all body regions were included, we detected a significant difference in the plumage bacterial composition between Jamaica and Maryland redstarts even when controlling for individual effect (Table 2, Fig. 4a). However, the low *R* value and the lack of clear separation of Jamaican vs Maryland redstarts (Fig. 4a) indicated within-site dissimilarities in the bacterial composition of redstart plumage (Figs. 4b and 5c). On average, the plumage microbial composition was more similar among Jamaican redstarts (63.3%) than among Maryland redstarts (55.8%). In this comparison, OTU 336 only contributes 6.7% of the average among-group dissimilarity (Fig. 2). OTUs 357, 344, 345, and 342 (in order of importance) cumulatively contributed 41.0% of the average among-group dissimilarity. Redstart plumage sampled in Jamaica had a higher average abundance of OTUs 357, 344, and 345 compared to redstarts sampled in Maryland (0.07 vs 0.02 for 357; 0.08 vs 0.05 for 344; 0.06 vs 0.02 for 345). Jamaican redstarts had a lower abundance of OTU 342 when compared to Maryland redstarts (0.02 vs 0.06; Fig. 2). The microbial composition of redstart head and dorsal plumage differed significantly between the nonbreeding and breeding site, with greater between-site differences (higher *R* value) found among redstarts' head feathers (Table 2).

Habitat effect We detected a significant habitat type effect (mangrove vs dry scrub) in bacterial composition of redstarts' plumage sampled at the nonbreeding site including all body regions (Table 2, Fig. 4b). In fact, greater differences were detected between habitat types than between geographic sites. The same was true for habitat types within Maryland (Table 2, Fig. 4c). However, the composition of plumage bacteria was more similar among redstarts in mangrove and dry scrub in Jamaica than in wet and dry habitats in Maryland (Table 2, Fig. 5b and c). OTUs 357 (15.6% contribution to average dissimilarity) and 344 (12.4% contribution) were more abundant in the plumage of redstarts in mangrove habitat than in the plumage of redstarts in dry scrub habitat (0.09 and 0.08 vs 0.02 and 0.04, respectively). In turn, OTU 344 (13.1% contribution to average dissimilarity) was more abundant in wet habitat (0.09) than in dry habitat (0.02) in Maryland, followed by OTU 342 (11.2%), which was also more abundant in the dry habitat (0.09 vs 0.02 for wet). OTU 336 contributes a small percentage to the dissimilarity between habitats within both Maryland (6.9) and Jamaica (5.8). Samples sizes were not large enough to conduct analyses for each body region separately. If we account for the individual effect, differences in plumage bacterial composition between habitats within Maryland were no longer significant but were still significant between habitats within Jamaica (Table 2).

Table 2. ANOSIM and SIMPER (%) comparisons of plumage bacterial composition for American Redstarts only sampled at the breeding (MD) and nonbreeding site (JA) 2004

	One-way ANOSIM	*Two-way nested ANOSIM accounting for individual effect*	*Average dissimilarity (%)*
Main effect	*Global R*[a]	*Global R*	
I. Site			
JA vs. MD (all body regions)	0.158**	0.177*	43.7
JA vs. MD (per body region)			
Breast	0.054[ns]		40.9
Dorsal	0.195*	–	43.8
Head	**0.210***	–	42.3
Tail	0.006[ns]		48.0
II. Habitat in MD			
Dry vs. wet (all body regions)	0.242**	–0.704[ns]	41.0
Dry vs. wet (per body region)			
Breast	**0.407**[ns]		42.8
Dorsal	0.167[ns]		50.7
Head	–0.037[ns]		47.3
Tail	0.074[ns]		47.8
III. Habitat in JA			
Mangrove vs. scrub (all body regions)	**0.300****	0.345*	47.0
Mangrove vs. scrub (per body region)			
Breast	**0.389***	–	43.5
Dorsal	0.357*	–	36.6
Head	0.171[ns]		33.4
Tail	0.241[ns]		52.2

[a]*R* values are significant at $*P < 0.05$, $**P < 0.01$, ns = not significant. Bold *R* values indicate the highest levels of differentiation detected among samples included in the specific analysis. Dash lines (–) indicate that tests were not performed due to small sample sizes.

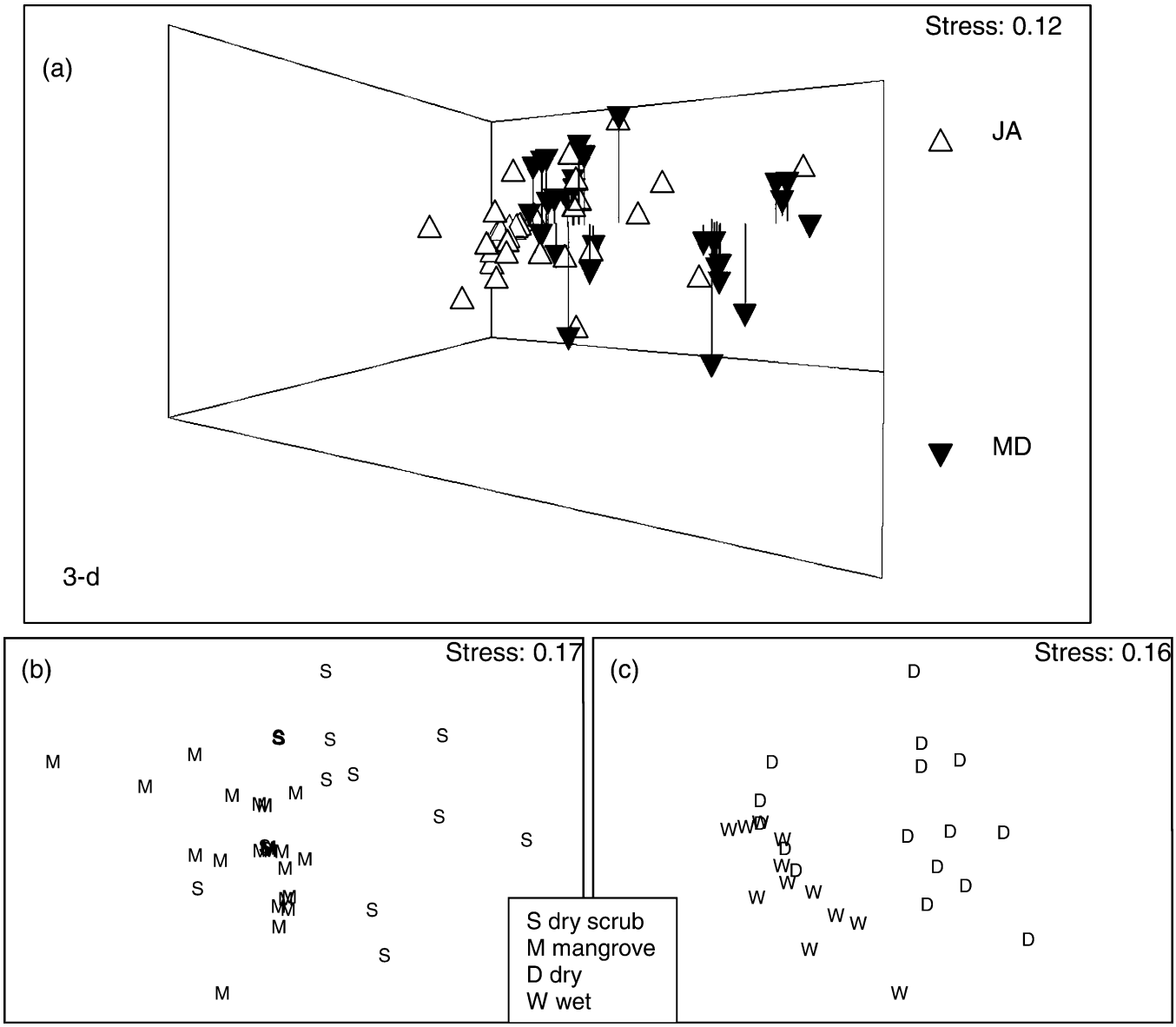

Figure 4. Three- and two-dimensional ordination of plumage (all body regions) bacterial composition sampled in American Redstarts in Jamaica (JA) and Maryland (MD). Sample points are coded for site (a) and habitat within Jamaica (b) and Maryland (c). Stress values are measures of the goodness of fit of the ordination plot to the data (see text for further details).

Bacterial Composition of Soil

Geographic site and habitat effects LH-PCR revealed 60 OTUs across all soil samples ($n = 17$). The microbial composition of soils sampled at the tropical site was significantly different from the composition of soils sampled at the temperate site (Table 3, Fig. 5a). Furthermore, the microbial composition in Maryland soil showed greater average similarity among samples (49.0%) than in Jamaican soil samples (33.9%), suggesting more heterogeneous microbial composition in Jamaican soils. OTU 341 was the most important contributor (12.6% contribution) in differentiating soils between geographic regions (average abundance 0.21 in Jamaica and 0.07 in Maryland), followed by OTU 336 (8.1%), 342 (5.7%), and 333 (5.2%) adding up to a 31.5% cumulative contribution. OTU 341 was the dominant OTU in Jamaica soil followed by OTU 336, which was the dominant OTU in Maryland soil. We detected significant habitat type effects (dry vs wet) at the breeding site only (Table 3).

Soil vs Plumage We found higher OTU diversity in soil than in feather samples (Fig. 3). The bacterial composition sampled in soil differed significantly from that sampled in plumage across all body regions in both

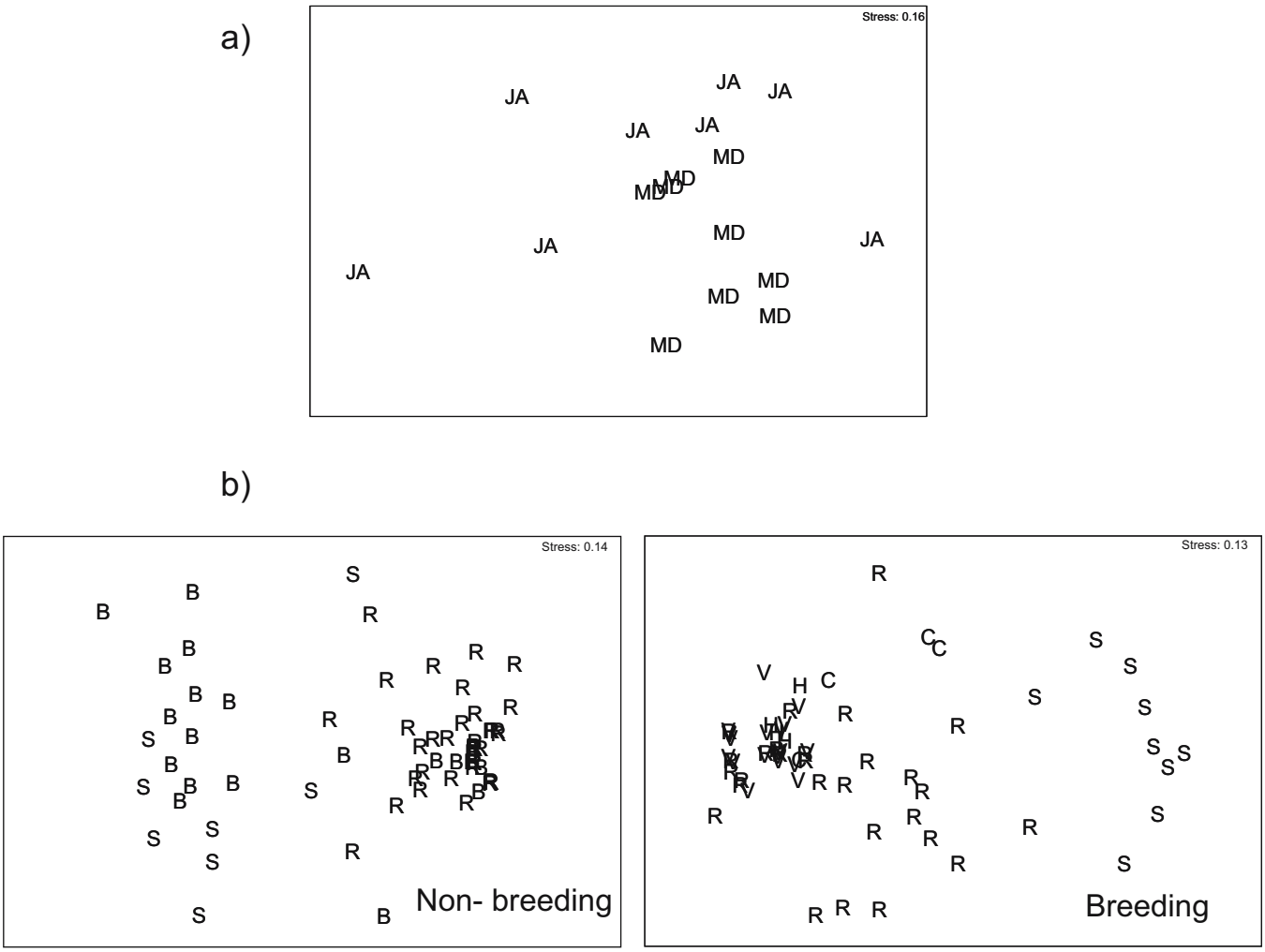

Figure 5. Two-dimensional ordination of the bacterial composition sampled in (a) soil on the nonbreeding (Jamaica, JA) and breeding (Maryland, MD) sites, 2004, and (b) in soil (S) and avian plumage at the nonbreeding and breeding sites. Sample points represent Black-throated Blue Warblers (B), American Redstarts (R), Common Yellowthroat (C), Hooded Warbler (H), Red-eyed Vireo (V).

Maryland and Jamaica (Table 3). Differences between plumage and soil samples were greater in Maryland than in Jamaica (Table 3) due to the greater similarity of bacterial communities in the plumage of Black-throated Blue Warblers and soil collected in Jamaica (Fig. 5b). When a one-way ANOSIM test was repeated for Black-throated Blue Warblers separately, we did not detect significant differences between plumage and soil bacterial composition within the Jamaican site ($R = 0.090$, $P = 0.133$; Fig. 5b). OTU 336 was an important contributor in the average dissimilarity between soil and plumage bacterial composition in both Jamaica (10.1% contribution) and Maryland (10.7% contribution). However, in Jamaica, OTU 341 contributed equally to the average dissimilarity among feather and soil samples (10.1%). At both sites, the plumage had a higher abundance of OTU 336 than soil samples (Fig. 2). Analyses conducted on redstarts separately showed significant differences between bacteria sampled in the plumage and those sampled in soil for both Jamaica and Maryland sites (Table 3).

OTU Identification Members of the genus *Pseudonomas* dominate the plumage community in all avian species sampled in both the temperate breeding and Neotropical nonbreeding site (Fig. 6). Most *Pseudomonas* spp. sequenced were 336 bp in length (Fig. 6), which is the length of the OTU that primarily contributes to between-group differences in plumage bacterial diversity and abundance and to differences among soil and plumage bacterial diversity and abundance. Other important contributors, such as those that contributed to differences among redstarts sampled in Maryland vs Jamaica correspond to the base pair lengths of *Bacillus subtilis* (357 bp), *Xanthomas* group (344 and 345 bp), *B. megaterium* (345 bp), one *Pseudonomas* (342 bp), and *Propionibacterium* group (342 bp) (Fig. 6).

Table 3. ANOSIM and SIMPER (%) comparisons of plumage bacterial composition between soil and plumage samples from the nonbreeding (JA) and breeding (MD) sites, 2004

Main effect	*Global R*[a]	*Average dissimilarity (%)*
I. MD vs. JA soil	0.300**	64.7
Mangrove vs. dry scrub in JA	0.005[ns]	59.4
Dry vs. wet in MD	0.394*	51.1
II. Plumage vs. soil (all avian species)		
Within MD (all body regions)	**0.940****	75.5
Within MD (per body region)		
Breast	**0.945****	73.5
Dorsal	0.932**	75.6
Head	0.924**	77.7
Tail	**0.939****	75.1
Within JA (all body regions)	0.508**	72.0
Within JA (per body region)		
Breast	0.495**	73.5
Dorsal	0.528**	75.6
Head	**0.574****	72.2
Tail	0.357**	75.1
III. Plumage vs. soil (redstarts only)		
Within MD (all body regions)	0.905**	72.8
Within MD (per body region)		
Breast	**0.924****	71.2
Dorsal	0.881**	74.2
Head	0.807**	74.1
Tail	0.901**	71.8
Within JA (all body regions)	0.939**	75.5
Within JA (per body region)		
Breast	0.812**	74.6
Dorsal	0.853**	77.1
Head	**0.859****	76.9
Tail	0.711**	73.3

[a] *R* values are significant at $*P < 0.05$, $**P < 0.01$, ns = not significant. Bold *R* values indicate the highest levels of differentiation detected among samples included in the specific analysis. Dash lines (–) indicate that tests were not performed due to small sample sizes.

Clones sequenced from the LH-PCR OTU libraries showed differences between American Redstart feathers sampled from one individual in Jamaica and one individual in Maryland (Fig. 6). The *Arthrobacter* subdivision, *Caulobacter*, *Saccharopolyspora*, and *Staphylococcus* groups were absent from the Maryland redstart plumage microbial community, whereas the *Alteromonas macleodii*, *Azoarcus*, *Bacillus megaterium*, *Francisella* groups, and the PPS *Tetradius* subgroup were absent from the Jamaican redstart plumage microbial community (Fig. 6). Interestingly, the presence of one Chloroplast sequence in the Maryland sample suggests that algae may be growing on redstarts at temperate sites. However, differences observed between both 16S rDNA libraries were not statistically significant (LIBSHUFF, $\Delta C_{XY} = 0.188$, $p = 0.121$; $\Delta C_{YX} = 0.051$, $p = 0.404$), which correlates with the LH-PCR fingerprint analysis.

Discussion

Previous studies showed that *Bacillus* species were frequent members of the plumage microbial community of birds [8, 41, 59], of which many demonstrated feather-degrading properties [61]. However, these findings were based solely on culture-dependent assays. Our study, which used molecular techniques exclusively, revealed a plumage microbial community in which *Pseudomonas* spp. predominated, followed by members of the *Xanthomonas* and *Fancisella* groups. Bacilli belonging to the *B. subtilis* and *B. megaterium* groups represented only a small percentage (Fig. 6) of the plumage community. Members of the *B. subtilis* group, also known to degrade keratin in feathers [55], were present in the plumage of redstarts sampled at both breeding and nonbreeding sites, whereas members of the *B. megaterium* group were only present in the plumage of redstarts sampled at the temperate location. The prevalence of *Pseudomonas* spp. in the feather community reported here may be related to the differences in our survey method. Shawkey and colleagues [52] found that culture-dependent methods isolated *Bacillus* spp. as the dominant bacterial group in the plumage, whereas culture-independent methods, such as restriction fragment length polymorphism and cloning, revealed *Pseudomonas* spp. as the dominant group. Clearly, differences in the methods preferentially isolated one group of bacteria over another. However, our results may support other findings that have shown that culture-independent techniques capture a broader spectrum of the microbial species that persist in the environment [15, 40].

Biogeographic Patterns in the Microbial Community of Plumage Until recently, microbial communities were thought to be globally ubiquitous [2, 18] with no biogeograhic differences in microbial composition. However, recent studies have demonstrated biogeographic differentiation for some bacterial taxa [10, 60]. Our research uses a unique model to test this hypothesis: the plumage of migratory birds. We found marked biogeographic differences in the plumage microbial community. LH-PCR analyses indicated that the plumage bacterial community in birds sampled at the Neotropical nonbreeding site was significantly different from the plumage bacterial community in birds sampled at the temperate breeding site (Table 1), which supports hypothesis 1. The same biogeographic difference was evident intraspecifically when we compared American Redstarts (Table 2) at breeding and nonbreeding sites. Despite a high *R* value ($R = 0.487$), individual variation did not drive the observed differences between biogeographic regions (Tables 1 and 2). However, we also found a degree of ubiquity in plumage bacterial composition across biogeographic regions as predicted from the high vagility of the plumage ecosystem (hypothesis 3). Although plumage microbial

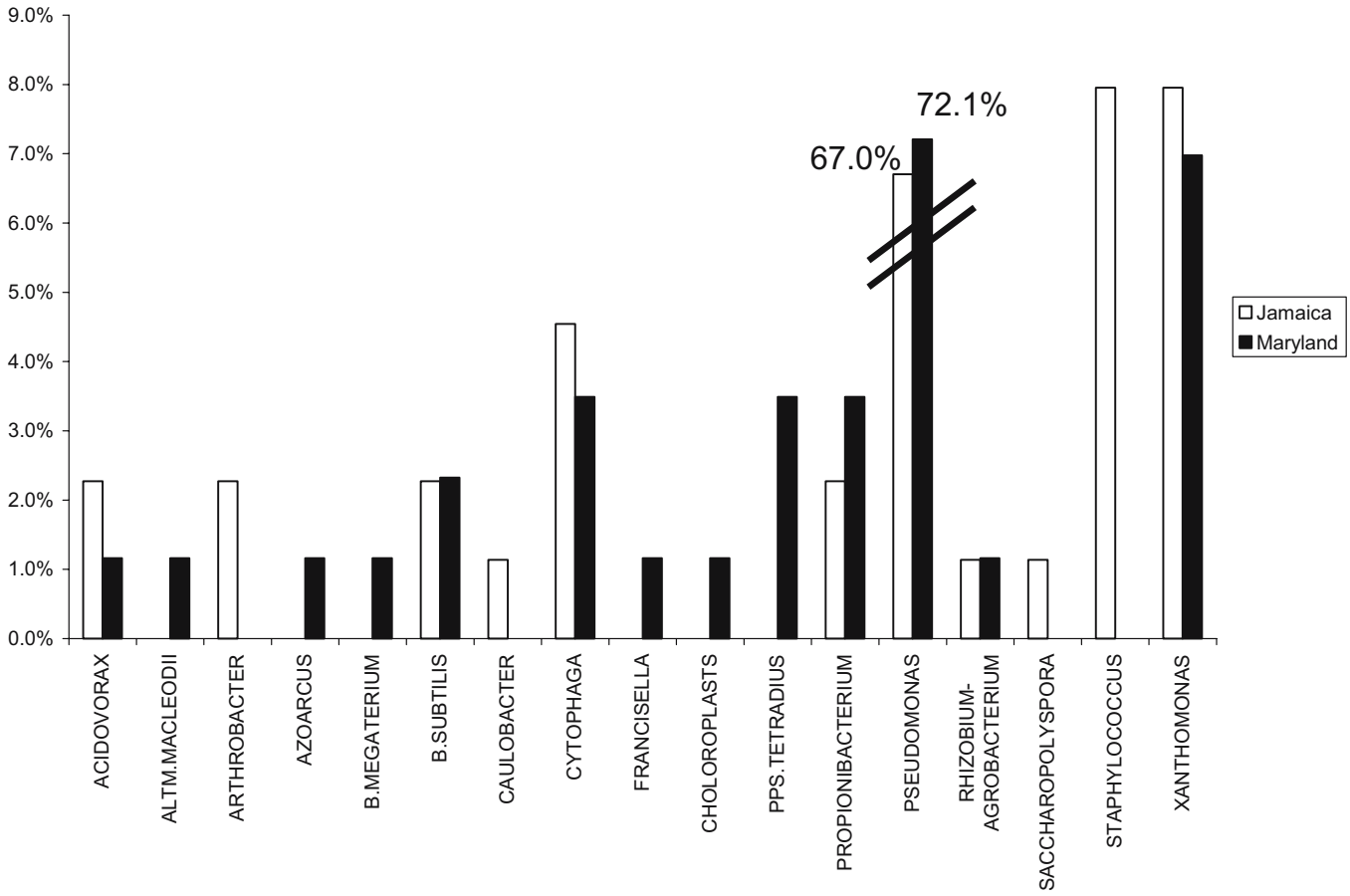

Figure 6. Ribosomal Database Project (RDP) phylotypes from 16S rRNA cloning of American Redstarts plumage sampled on the nonbreeding (Jamaica, $n = 1$) and breeding (Maryland, $n = 1$) sites, 2004.

composition was significantly different between the Neotropical and temperate sites, *R* values and average dissimilarities were generally low (Tables 1 and 2), indicating within-site variation. Similar biogeographic patterns were observed with 16S rDNA cloning and sequencing, which gives finer taxonomic resolution than LH-PCR. Our study shows differences in the 16S rDNA sequences cloned from one American Redstart's plumage sampled in Jamaica and one in Maryland (Fig. 6). Both libraries differed in their presence and absence of bacteria belonging to eight separate RDP classes (phylotypes). However, the differences were not significant.

Our results suggest that avian plumage sampled at breeding and nonbreeding sites host bacterial communities that differ in the abundance of similar types of bacteria, which supports hypothesis 1. Redstarts from Maryland and Jamaica differed primarily in the abundance of OTUs corresponding to the sequence lengths of the *B. subtilis*, *Xanthomonas*, and *Propionibacterium* groups (Fig. 6). Generally, these bacteria were more abundant in plumage sampled at the Neotropical nonbreeding site with the exception of OTU 342. The opposite was found when all avian species were included in among-site comparisons. The avian plumage microbial community sampled at the temperate breeding site harbored a greater abundance of OTUs corresponding to the sequence lengths of the *Pseudomonas*, *Xanthomonas*, *B. subtilis*, and *Fancisella* groups, with the exception of OTU 357. It therefore appears that although *Pseudomonas* species dominate the plumage microbial community in general, different bacterial species are more abundant in the plumage ecosystem at different stages of a bird's annual cycle. For redstarts specifically, this occurs during the nonbreeding stage in the Neotropics. We cannot comment on the annual changes in bacterial abundance of the plumage of other birds sampled because they were sampled only during one stage of their life cycle.

Avian behavior and/or geographic location may play important roles in driving the composition of the plumage microbial community. Humidity, climate, temperature, or other environmental factors may influence bacterial growth on feathers as it would in any other environmental ecosystem such as soil or water (e.g., [58]). Burtt and Ichida [7] suggested that the dark

plumage of Song Sparrows (*M. melodia*) from the humid forests of northwestern North America is more resistant to bacterial degradation and may have evolved as an adaptation to minimize damage caused by feather-degrading *B. licheniformis*, which degrades feathers more rapidly and completely in humid than in arid regions. Furthermore, birds often face trade-offs that vary during each stage of their life cycle, which may compromise their ability to control the growth of plumage microbes. As part of their self-maintenance behavior, birds coat their feathers with preen oil produced by the uropygial gland at the base of the tail, which inhibits bacterial growth ([51]; Reenerkens et al., unpublished data). Time constraints during the breeding season may limit self-maintenance behavior and result in an increase in plumage bacteria during this time. For example, European Starlings (*Sturnus vulgaris*) with experimentally augmented brood sizes showed higher numbers of free-living plumage bacteria [34], presumably as a result of less time dedicated to self-maintenance activities. Alternatively, molt sequence may play a more significant role in the plumage microbial composition. Burtt and Ichida [8] found a dramatic decrease in the occurrence of *B. licheniformis* in the plumage following molt. Most birds, including those in our study, undergo a complete molt at the end of the breeding season or shortly thereafter [43, 48]. Therefore, the microbial community in the plumage of nonbreeding birds sampled in the Neotropics will have colonized the plumage only recently (3–4 months in our study) and may not have reached the abundance of bacteria on feathers sampled from birds on the breeding grounds. Bacteria in the plumage of breeding birds have been present during the 8–10 months since the last molt. Further studies are needed to determine the influence of molt on the plumage microbial community and on the ecological or behavioral factors that result in differences among biogeographic regions that we have observed.

Ecological Patterns in the Plumage Microbial Community We detected ecological patterns in the plumage bacterial community. The microbial community in the plumage of American Redstarts differed among habitat types in both Jamaica and Maryland, suggesting that plumage bacteria are acquired from the local environment as suggested under hypothesis 1. OTUs corresponding to the sequence lengths of *B. subtilus* and members of the *Xanthomonas*, *Pseudonomas*, and/or *Propionibacterium* groups were the most important contributors to the differences between habitats. At both sites, members of the *Xanthomonas* group were more abundant in plumage sampled in wet habitats (mangrove in Jamaica and wet forest in Maryland). Interestingly, *Xanthomonas* spp. are well known plant pathogens (e.g., [19]) and have been isolated almost exclusively from plants. Plants may therefore play an important role in the environmental transmission of bacteria to plumage. Furthermore, redstarts in dry scrub habitat forage more frequently on the ground and may therefore come equally into contact with soil and plants.

Few studies have been conducted on the microbial community of wild bird feathers but, to date, most [7, 8, 34, 52] have proposed that plumage microbes are primarily transmitted through colonization from environmental substrates, particularly from microbes present in soil. Burtt and Ichida [8] found more feather-degrading bacilli in the ventral plumage and on ground-foraging birds, which come more often into contact with soil than the dorsal plumage, head plumage, or birds that forage in trees or the air. Lucas *et al.* [35] documented a diverse community of feather-degrading bacteria in soil. However, we found significant differences between the soil and plumage bacterial communities at both the breeding and nonbreeding sites with the exception of three Black-throated Blue Warblers sampled on the nonbreeding site (Table 3). These differences were still significant when breast feathers were analyzed separately, and furthermore, breast feathers showed greater differentiation between feather and soil composition (higher *R* values) than other body regions sampled from redstarts and Red-eyed Vireos sampled on the temperate breeding site (Table 3). Our results suggest that other environmental substrates may play more important roles in plumage bacterial acquisition than soil, contra hypothesis 2.

Individual and Species Effect We found significant differences in the plumage bacterial composition at the smallest scales of comparison. Plumage microbial composition differed significantly and strongly among individual birds and less strongly among body regions of individuals revealing a stronger bird effect. Whitaker *et al.* [59] report similar individual variation in *B. licheniformis* strains isolated from plumage. For example, one of nine individual Northern Saw-whet Owls (*Aegolius acadicus*) carried four genetically differentstrains while the remaining eight birds carried only one strain. We found a significant difference in the composition of plumage bacteria among species (Table 1). But when we controlled for individual variance, our results no longer show significant interspecific differences in plumage bacterial composition (Table 1). Individual variation therefore clearly accounts for much of the difference observed in the plumage bacterial community in our study. Furthermore, interspecific patterns detected in the microbial composition of Black-throated Blue Warblers (Fig. 1) were mostly driven by the composition found in the plumage of specific individuals. The microbial composition sampled in three of the five Black-throated Blue Warblers showed high average dissimilarity (78.4%) with all remaining birds sampled. Interestingly, the plumage microbial community of these three individuals did not

differ significantly from that of the soil sampled in the same location. Factors related to these observed differences and similarities are not clear. Differences observed in these individuals were not related to demographic or ecological factors. Of the three individuals, one adult male and one adult female were sampled in the dry scrub habitat, and one juvenile female was sampled from the mangrove habitat in Jamaica. Furthermore, Black-throated Blue Warblers are foliage gleaners and only infrequently come into contact with soil microbial organisms during foraging [26]. Larger sample sizes of Black-throated Blue Warblers sampled on their nonbreeding and breeding grounds are needed to clarify this pattern.

Patterns in Soil Microbial Community Interestingly, we found a significant biogeographic effect in comparisons of soil microbial communities. The microbial composition of soil samples collected in the Neotropics was significantly different from that sampled in the temperate site (Table 3). The Shannon–Wiener diversity indices (Fig. 3) suggest a trend toward higher microbial diversity in Maryland soil, although given the variability in the indices, this is inconclusive. However, soil sampled in Maryland showed greater within-site similarity than soil sampled in Jamaica (Fig. 5a). Our results provide an important contribution to ongoing efforts to understand the factors that drive latitudinal diversity gradients at the microbial level. The increase in biodiversity from northern latitudes to the equator has been well documented, is well accepted [24], and has been demonstrated on a variety of organisms ranging from plants to mammals (e.g., [23, 62]). To date, patterns of biodiversity for microscopic organisms such as bacteria and fungi remain understudied [14]. A limited number of studies, including ours, suggest opposite trends in which bacterial diversity does not increase toward the equator [27].

Although the average dissimilarity among Jamaica soil sample was higher—suggesting a heterogeneous distribution of soil bacteria at the Neotropical site, this pattern was unrelated to habitat type. The community of soil microorganisms in mangroves was not significantly different from that in dry scrub habitat. The similarity is surprising considering that mangrove and dry scrub are very different ecosystems (mesic vs xeric, respectively) that differ greatly in plant composition, and also considering that habitat and land use are the predominant ecological factors associated with variation in soil microbial composition [4, 5, 44].

Conclusions

Our study has revealed variation in composition of the microbial community in the soil and plumage of migratory birds at taxonomic, ecological, and geographic scales. The limited dispersal of soil microbial communities may drive the greater differentiation observed among soil bacterial communities from different geographic locations compared to the largely similar bacterial communities of the vagile plumage ecosystem of migratory birds. Alternatively, these differences may have been driven by different functions of the microbial communities in plumage and soil. Despite the ubiquity of most bacterial groups isolated from the plumage, differences in plumage bacterial composition were primarily driven by the abundance of *Pseudomonas* spp. The greatest differentiation was found between the plumage microbial communities of Black-throated Blue Warblers and other avian species. However, these differences were largely driven by individual variation. Habitat type at the Neotropical nonbreeding site plays a greater role in bacterial community composition of American Redstart plumage than geographic location. Birds occupying moist habitats harbored a greater abundance of plant-specific bacteria than those sampled in dry habitats. Our results further show that soil may play a less important role in bacterial acquisition by migratory birds than has been thought. Repeated sampling of the same individuals throughout the annual cycle will provide a more detailed look at the changes in the bacterial community of the plumage. Future research should also focus on the mechanism by which birds acquire their plumage bacteria. In sum, microbial community in the plumage of migratory birds appears to differ in composition and dynamics from that of the soil and surrounding habitat. Understanding the differences may help us better understand the evolution of avian plumage and the dispersal of microorganisms by birds.

Acknowledgment

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Appendix 1

Table 4. Identification of cloned bacterial sequences and LH-PCR fingerprinting (LH operational taxonomic unit (OTU) length in base pairs) results from representative sequences sampled from the feathers of one American Redstart captured in Jamaica (JA39) and one captured in Maryland (MD34)

Cloned sample	*RDP description*[a]	*Closest BLAST match*[b] *(accession number)*	*LH OTU size (bp)*
JA39	PSEUDOMONAS	Ps.stutz11 *Pseudomonas stutzeri* str. LS401 (DQ856170)	309
JA39	RHIZOBIUM–AGROBACTERIUM	str. TM41 (X97087)	309
JA39	CAULOBACTER	*Brevundimonas* MCS17 str. MCS17 (AJ227799)	309
MD34	RHIZOBIUM–AGROBACTERIUM	clone 49519 (AF097813)	311
JA39	CY.AURANTIACA	Sps. lingua *Spirosoma linguale* str. Mc1 ATCC 23276 (DQ856168)	321
MD34	PSEUDOMONAS	Ps.pavonac IAM 1155 (DQ856229)	325
MD34	PSEUDOMONAS	*Moraxella osloensis* str. Ben 58 (X95304)	332
MD34	PSEUDOMONAS	*Moraxella osloensis* str. Ben 58 (X95304)	332
JA39	PSEUDOMONAS	Mrx.oslons *Moraxella osloensis* NCTC 10465 (T) (DQ856180)	333
MD34	FRANCISELLA	Fnc.phmira *Francisella philomiragia* ATCC 25017 (DQ856244)	333
JA39	SACCHAROPOLYSPORA	str. LAA1 (AJ006999)	334
JA39	ACIDOVORAX	*Comamonas* sp. str. R7 (AJ002810)	334
MD34	ACIDOVORAX	*Comamonas* D22 str. D22 (AF188304)	334
MD34	PSEUDOMONAS	*Pseudomonas mendocina* ATCC 25413 (AJ006109)	334
MD34	PSEUDOMONAS	Ps.veronii *Pseudomonas veronii* str. CFML 92–134 CIP 104663 (T) (DQ856224)	335
MD34	PSEUDOMONAS	F.lutescen *Flavobacterium* "lutescens" ATCC 27951 (DQ856221)	336
MD34	PSEUDOMONAS	*Pseudomonas* DhA-91 str. DhA-91 (AF177916)	336
MD34	PSEUDOMONAS	*Pseudomonas* PsK str. PsK (AF105389)	336
MD34	PSEUDOMONAS	*Pseudomonas plecoglossicida* str. FPC951 (AB009457)	336
MD34	PSEUDOMONAS	*Pseudomonas* SF1 str. SF1 (AF135269)	336
MD34	CY.AURANTIACA	IFAM 3359 (X90702)	336
JA39	PSEUDOMONAS	Acn.junii2 *Acinetobacter junii* DSM 1532 (DQ856146)	336
JA39	PSEUDOMONAS	Ps.pavonac IAM 1155 (DQ856145)	336
JA39	PSEUDOMONAS	str. S2 (AJ002813)	336
JA39	PSEUDOMONAS	Ps.veronii *Pseudomonas veronii* str. CFML 92–134 CIP 104663 (T) (DQ856187)	336
JA39	PSEUDOMONAS	*Pseudomonas* PsK str. PsK (AF105389)	336
JA39	PSEUDOMONAS	Ps.aspleni *Pseudomonas asplenii* LMG 2137 (T) (DQ856167)	336
JA39	PSEUDOMONAS	*Pseudomonas jessenii* CIP 105274 (AF068259)	336
JA39	PSEUDOMONAS	*Pseudomonas plecoglossicida* str. FPC951 (AB009457)	336
JA39	PSEUDOMONAS	clone SJA-129 (AJ009491)	336
MD34	CYTOPHAGA_GROUP_I	str.1044 str. (SBR1044)	337
JA39	CYTOPHAGA_GROUP_I	str.1044 str. (SBR1044)	337
JA39	CYTOPHAGA_GROUP_I	str.2091 str. (SBR2091)	338
MD34	CYTOPHAGA_GROUP_I	*Capnocytophaga sputigena* str. 897 CIP 100 (AF133536)	338
MD34	AZOARCUS	*Hydrogenophilus thermoluteolus* str. TH-4 (AB009829)	338
MD34	ALTM.MACLEODII	*Alteromonas macleodii* (Y18230)	339
MD34	CY.AURANTIACA	IFAM 3359 (X90702)	340
JA39	XANTHOMONAS	*Stenotrophomonas maltophilia* LMG 10888 (AJ131915)	341
MD34	PROPIONIBACTERIUM	Clone BPC009 (AF154099)	342
Ja39	PROPIONIBACTERIUM	Clone BPC009 (AF154099)	343
Md34	XANTHOMONAS	*Stenotrophomonas maltophilia* LMG 10857 (AJ131117)	344
MD34	XANTHOMONAS	*Stenotrophomonas* sp. str. S3 (AJ002814)	344
JA39	XANTHOMONAS	*Stenotrophomonas maltophilia* LMG 11104 (AJ131912)	345
MD34	B.MEGATERIUM	*Bacillus thermoleovorans* str. B23 (AB034836)	345
JA39	ARTHROBACTER	*Kocuria rhizophila* str. TA68(type strain) DSM 11926 (Y16264)	346
JA39	STAPHYLOCOCCUS	Stp.warner *Staphylococcus warneri* (DQ856134)	349
MD34	PPS.TETRADIUS	Pps.octavi *Peptostreptococcus octavius* str. Davey 1 NCTC 9810 (T) (DQ856287)	356
MD34	B.SUBTILIS	Exg.acety2 *Exiguobacterium acetylicum* NCIMB 9889 (T) (DQ856233)	357
JA39	B.SUBTILIS	Exg.acety2 *Exiguobacterium acetylicum* NCIMB 9889 (T) (DQ856192)	382

[a]Ribosomal Database Project (RDP) class level 4.
[b]The species in GenBank with the closest sequence match as determined with the BLAST algorithm.

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