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**Article title**

16S rRNA Gene Sequencing of Microbiota from the Preen Oil and Cloaca of Chipping Sparrows (*Spizella passerina*)

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**Running title**

*Chipping Sparrow Microbiome Analysis*

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**Abstract**

We present the results of 16S rRNA gene amplicon sequencing of the microbiota from preen oil and the cloaca of chipping sparrows (*Spizella passerina*) collected near Mountain Lake Biological Station (MLBS) in Pembroke, VA.

**Announcement**

New world sparrows (Passerillidae), specifically non-migratory dark-eyed juncos (*Junco hyemalis carolinensis*), are at the forefront of avian microbial ecology studies (1). These birds harbor symbiotic bacteria used for chemical communication through preen oil (2). In the same habitat is one overlooked species, the migratory chipping sparrow (*Spizella* *passerina*). Here, we describe microbial communities of the preen oil and cloaca of chipping sparrows to provide information for future interspecific comparative studies.

This study was conducted in compliance with Indiana University Bloomington Institutional Animal Care and Use Committee guidelines (15–026), US Fish and Wildlife Service (MB093279-1), and Virginia Department of Game and Inland Fisheries (058772). Birds were sampled as previously described (2). Microbial communities from the preen oil and cloaca were collected using a pre-moistened swab with sterile buffer (20 mM Tris pH 8; 2 mM EDTA; 1.2% Triton X-100). We extracted DNA with the QIAGEN DNeasy Powerlyzer PowerSoil DNA Isolation Kit with the following modifications: 1) Swabs were soaked in 500 µL bead solution and 200 µL phenol:chloroform:isoamyl alcohol for 10 min before using Biospec Products MiniBeadBeater-16 run 2X for 30 sec. 2) Samples received 100 µL each of solutions C2 and C3, plus 1 µL RNase A, and incubated at 4°C for 5 min before one-step centrifugation. 3) Lysates were mixed with 650 µL solution C4 and 650 µl 100% ethanol instead of using 1200 µL solution C4 alone. 4) DNA was eluted in 60 µL solution C6, reduced from 100 µL (1). We amplified bacterial DNA using nested PCR as described previously (2). The amplified V4 region of the 16S rRNA gene was prepared using the V2 500 cycle MiSeq Reagent Kit (Illumina MS102-2003) and sequenced on the Illumina MiSeq platform by Michigan State University Research Technology Support Facility’s Genomics Core generating 2 x 250 bp reads.

Analyses were performed using R Statistical Software v4.3.3 (3). We used DADA2 v1.30.0 (4) to process sequencing reads. Default parameters for DADA2 except reads were trimmed 10bp at the 5’ end and truncated at 240bp (F) and 200bp (R) at the 3’ end. Paired-end reads were merged and chimeric sequences were removed. Table 1 tracks reads through the DADA2 pipeline. We assigned taxonomy using the SILVA 138.1 data set with species information (5). Contaminating sequences from blank and water extractions were removed using decontam v1.22.0 (6). We used phyloseq v1.46.0 (7) to analyze alpha (Observed amplicon sequence variants (ASVs), Shannon diversity, and Simpson’s diversity index) and beta diversity (Bray-Curtis dissimilarity) metrics. We used vegan v2.6.6.1 (8) for statistical analyses and ggplot2 v3.5.1 (9) for generating figures.

A column chart comparing relative order abundance between preen oil and cloaca showed no noticeable differences (Fig. 1A). The Similarity Percentages function (simper) did not identify any statistically significantly different taxa in preen oil compared to cloaca. Alpha diversity analysis showed that the preen oil community was less diverse than that of the cloaca, though not significantly (Fig. 1B). We saw no significant difference in Bray-Curtis dissimilarity between the preen oil and cloaca communities (Fig. 1C).

**Data availability statement**

The 16S rRNA gene amplicon sequences have been deposited in the GenBank Sequence Read Archive (SRA) under the BioProject accession number [PRJNA1117373](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1117373) under the SRA accession numbers [SRR29202434](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202434)- [SRR29202455](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202455).

**Acknowledgments**

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**Figure 1. Microbial Diversity and Community Composition in Cloaca and Preen Gland Samples from chipping sparrows.** A) Relative abundance of orders obtained from 16S rRNA gene sequencing of preen oil and the cloaca. Orders with less than 5% abundance were grouped together as were orders that were unidentified. B) Alpha diversity of cloaca and preen oil communities. C) NMDS plot of Bray-Curtis dissimilarity.

**Table 1:** Sample information for sequencing reads.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Bird** | **Sample** | **Site** | **Input** | **Filtered** | **Denoised F** | **Denoised R** | **Merged** | **Non-Chimera** | **NCBI Accession** |
| CHSP02 | 262 | Cloaca | 23692 | 21166 | 20744 | 20850 | 19894 | 18729 | [SRR29202452](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202452) |
| CHSP03 | 8 | Cloaca | 53394 | 48310 | 46990 | 47125 | 43746 | 42312 | [SRR29202442](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202442) |
| CHSP04 | 39 | Cloaca | 11372 | 9649 | 9344 | 9322 | 8763 | 8707 | [SRR29202444](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202444) |
| CHSP05 | 2 | Cloaca | 40953 | 35685 | 34840 | 34926 | 33020 | 31999 | [SRR29202438](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202438) |
| CHSP06 | 377 | Cloaca | 58412 | 53359 | 52344 | 52381 | 50307 | 49818 | [SRR29202445](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202445) |
| CHSP07 | 372 | Cloaca | 45801 | 42088 | 41404 | 41524 | 40076 | 39548 | [SRR29202446](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202446) |
| CHSP08 | 20 | Cloaca | 54567 | 45840 | 44686 | 44668 | 40451 | 39019 | [SRR29202437](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202437) |
| CHSP09 | 184 | Cloaca | 55020 | 48926 | 48163 | 48153 | 46358 | 44916 | [SRR29202440](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202440) |
| CHSP10 | 214 | Cloaca | 19470 | 18049 | 17818 | 17853 | 17483 | 17483 | [SRR29202435](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202435) |
| CHSP11 | 180 | Cloaca | 112134 | 100851 | 100044 | 100117 | 89998 | 88560 | [SRR29202443](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202443) |
| CHSP12 | 186 | Cloaca | 29577 | 25491 | 25041 | 25060 | 23672 | 22923 | [SRR29202439](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202439) |
| CHSP02 | 123 | Preen | 3984 | 3585 | 3452 | 3488 | 3244 | 3202 | [SRR29202454](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202454) |
| CHSP03 | 298 | Preen | 9871 | 9269 | 9186 | 9208 | 9144 | 6304 | [SRR29202450](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202450) |
| CHSP04 | 93 | Preen | 11302 | 10028 | 9851 | 9844 | 9332 | 9012 | [SRR29202441](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202441) |
| CHSP05 | 103 | Preen | 43007 | 38430 | 37808 | 37882 | 36601 | 35407 | [SRR29202455](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202455) |
| CHSP06 | 237 | Preen | 38532 | 34316 | 33689 | 33706 | 32041 | 30803 | [SRR29202453](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202453) |
| CHSP07 | 207 | Preen | 8057 | 7037 | 6860 | 6878 | 6591 | 6542 | [SRR29202436](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202436) |
| CHSP08 | 283 | Preen | 1655 | 1461 | 1379 | 1374 | 1308 | 1308 | [SRR29202451](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202451) |
| CHSP09 | 319 | Preen | 20659 | 18642 | 18256 | 18305 | 17466 | 17133 | [SRR29202449](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202449) |
| CHSP10 | 326 | Preen | 44570 | 40618 | 39818 | 39740 | 37751 | 37589 | [SRR29202448](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202448) |
| CHSP11 | 22 | Preen | 59017 | 53469 | 52934 | 52982 | 48990 | 48558 | [SRR29202434](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202434) |
| CHSP12 | 329 | Preen | 40065 | 36088 | 35652 | 35645 | 34274 | 33466 | [SRR29202447](https://www.ncbi.nlm.nih.gov/sra/?term=SRR29202447) |