Analyzing Urban Coyote Scat through Shotgun Metagenomic Sequencing

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Abstract:

Coyotes (*Canis latrans*) are currently occupying urban areas where they were previously absent due to habitat loss and the high availability of anthropogenic food sources in cities. In the Twin Cities Metro Area, coyotes have successfully colonized the urban area. Due to the difficulty of capturing these urban coyotes, it's beneficial to study the coyotes non-invasively, such as by analyzing their readily available scat. This study aimed to examine the bacterial communities and endoparasites present in urban coyote scat samples, and potential differences between the inner and outer sections of samples. In this study, five coyote scat samples were randomly selected from five different areas around the Twin Cities Metro Area, and the inner core and outer crust sections were analyzed separately. DNA was extracted following a modified protocol for scat samples through the Qiagen DNeasy Blood and Tissue Kit, quantified via Qubit, and subjected to Quality Control (QC) tests at UMGC before being analyzed in CZID. Fluorometric quantification revealed variable DNA yields, with some samples failing due to low concentration. Eight samples met sequencing thresholds and were analyzed for bacterial composition in CZID. The results from sequencing indicate a mix of gut, diet-derived, and environmental bacteria, with a notable outlier being *Schistosoma japonicum*. A one-way ANOVA test was utilized to compare the bacterial diversity across the sample sections and showed no statistically significant differences ($F(7, 312) = 1.18$, $p = 0.315$), however, the t-test results indicate that there were statistically significant differences between the inner and outer sample values for only two of the scat samples. The findings from this study provide insights into urban coyote microbiomes and highlight the potential for using shotgun metagenomic

sequencing to analyze scat for ecological and health assessments. Future research could explore the potential microbiome differences between urban and rural coyotes.

Introduction:

Like many other wildlife species, coyotes (*Canis latrans)* are currently occupying cities and other urban areas where they were previously absent. These urban environments are often characterized by high availability of human food, habitat degradation, fragmentation, and prey assortments different than that of surrounding rural areas (Henger et al., 2022). Within the Twin Cities Metropolitan Area (TCMA), coyotes have successfully colonized the urban environment (Gehrt et al., 2009). The increasing urbanization worldwide will likely increase the number of predators and mesopredators that find themselves close to humans through living in urban environments (Wang et al., 2015). Because of this, understanding the basic biology of urban coyotes and other species is crucial to finding methods to better coexist with wildlife.

While capturing coyotes in urban areas to learn about their basic biology is difficult, their scat is readily available. The availability of urban coyote scat may allow for questions regarding the health and genetics of individual animals to be answered noninvasively. Diet composition can be determined by various scat dissection methods; however, these methods can be unsuccessful at identifying and detecting food processed for human consumption (Henger et al., 2022). Beyond diet composition detection, scat can be a source of DNA to identify individual coyotes. This is relevant because, in some studies, there is an indication that coyotes in urban areas experience more genetic isolation from surrounding populations (Henger et al., 2022). To detect diet composition and parasite presence, test for fecal pathogens, and identify individual coyotes simultaneously, we used shotgun metagenomic sequencing.

The goals for this research were fourfold. To specify the aims of this research, first, the efficacy of shotgun metagenomic sequencing for the identification of individual coyotes for population genetics was determined. Secondly, diet composition was analyzed by shotgun metagenomic sequencing, and then the results determined if it aligned with spatial trends in isotropic signatures from coyote hair samples previously analyzed across the Twin Cities Metro Area. Third, endoparasites were sequenced in individual scat samples to determine the diversity of intestinal parasites in the study animals. Lastly, the effect of subsampling certain regions of scat samples (i.e. outer and inner portions of the sample) on what diet, endoparasite, and host DNA can be detected was evaluated.

Methods:

Five scat samples collected between 2020-2024 were randomly selected for preliminary analysis via shotgun sequencing. The scat samples were only collected if they appeared fresh (i.e. still retained moisture and a tacky texture), and were frozen at -18 °C until DNA extraction and sequencing. Each scat sample was thawed and subsampled. The same subsampling process was completed for each scat sample. Three subsamples of the outer crust of the scat were taken such that 1x1 cm sections of crust were removed to a depth of 5mm, with sections taken at least 1 cm apart along the length of the scat to get a more representative sample of heterogeneous scat contents. In addition, three subsamples of the core of the scat were taken such that an approximate cubic centimeter was sectioned to exclude the outer crust of the scat. In cases where the scat sample was less than 2cm in diameter, the crust was removed at a depth of approximately 5mm, and the remaining scat was retained as the respective sample regardless of volume. Subsamples were taken at least 1 cm apart along the length of the scat. A separate

scalpel blade was used to take crust and core subsamples for each scat sample to avoid DNA contamination, and separate scalpel blades were used for each respective scat sample.

Once the respective subsamples were collected from a single scat sample, they were combined into a Whirl-pak. These samples were then manually homogenized for 1 minute, and re-frozen at −18°C prior to DNA extraction. DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit, with modifications following Henger et al. 2022. The modifications included having the Ethanol cooled in a freezer at −18°C for 1 hour. Also, the AE Buffer was heated to 70°C through immersion in a water bath. Some other important notes in the procedure included that Buffer ATL and AL may form a precipitate upon storage, and, if necessary, they should be warmed to 56°C until the precipitate is fully dissolved. Buffer AW1 and Buffer AW2 were supplied as concentrates. Before using it for the first time, the appropriate amount of ethanol (96–100%) was added as indicated on the bottle to obtain a working solution. Furthermore, it is important to preheat a thermomizer, shaking water bath, or rocking platform to 56°C for use in step 2 of the Qiagen DNeasy Blood and Tissue Kit. Since we were using frozen samples originally, they first needed to be equilibrated to room temperature (15–25°C) before subsampling.

After subsampling the scat samples, each subsample was sectioned into approximately 20 mg sections using a weigh boat and scale. The 20 mg sections were then placed into a 1.5 mL microcentrifuge tube. Next, 180 µl Buffer ATL was added to the microcentrifuge tubes followed by 20 µl Proteinase K. The tubes were then mixed thoroughly by vortexing (15 s pulse). The tubes were then incubated at 56°C on a rocking platform until the tissue was completely lysed. The lysis was completed in about 3 hours (could be 1-3 hours). After lysis, the samples were vortexed again for 15 seconds. Next 200 µl Buffer AL was added to the sample and mixed

thoroughly by vortexing. Then 200 µl ethanol (96–100%) frozen for 1 hr was added and mixed again thoroughly by vortexing. The sample, Buffer AL, and ethanol must be mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. While it was not used in this experiment, Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples. The mixture, including the precipitate, was then pipetted into the DNeasy Mini spin column and placed in a 2 ml collection tube (provided). The tube was centrifuged at 8000 x g for 1 min and the flow-through and collection tube were then discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl Buffer AW1 was added, and centrifuged for 1 min at 8000 x g. The flow-through and collection tube were again discarded. Again, the DNeasy Mini spin column was placed in a new 2 ml collection tube (provided), 500 µl Buffer AW2 was added, and centrifuged for 3 min at 18,213 x g to dry the DNeasy membrane. The flow-through and collection tubes were discarded. It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution. Following the centrifugation step, the DNeasy Mini spin column was removed carefully so that the column did not come into contact with the flow-through since this could result in a carryover of ethanol. The AE buffer was heated at 70 °C before eluting the DNA. We eluted with 100 µl AE buffer by performing two rounds of pipetting 50 µl of heated AE buffer to the DNeasy membrane, incubating at ambient temperature for 30 min, and centrifuging for 3 min at 8,000 rpm.

Following the DNA extraction, the DNA was quantified preliminarily with a Qubit before being sent to the University of Minnesota Genomic Center (UMGC) for further analysis. The University of Minnesota Genomic Center took the extracted DNA from the DNeasy Blood

and Tissue Kit and utilized a PicoGreen Assay kit and Nanodrop spectrophotometer to quantify the DNA and to show the purity. Next, they utilized an Illumina NextSeq for library preparation and to show the size distribution in the DNA samples. Lastly, they utilized Illumina sequencing to determine if the samples passed or failed the criteria to be further analyzed with downstream sequencing.

Results:

The five collected scat samples from the urban coyotes in the Twin Cities Metro Area were originally collected with different codes (see column 1 of Table 1 below) but were also given different extraction codes for this research (see column 2 of Table 1 below) which were used as the sample names. The site names from the original locations were also recorded.

Table 1: Summary of collection codes, extraction codes, site locations, and subsample status for each of the ten samples (from five fecal samples).

Collection Code	Extraction Code	Site Name	Subsample
83	S001O	Roselawn Cemetery	outer
83	S001I	Roselawn Cemetery	inner
78	S002O	Kenwood Bluff	outer
78	S002I	Kenwood Bluff	inner
49	S003O	Sunny Hollow	outer
49	S003I	Sunny Hollow	inner
44	S004O	Brickyard	outer
44	S004I	Brickyard	inner
58	S005O	Dodge Nature Center	outer
58	S005I	Dodge Nature Center	inner

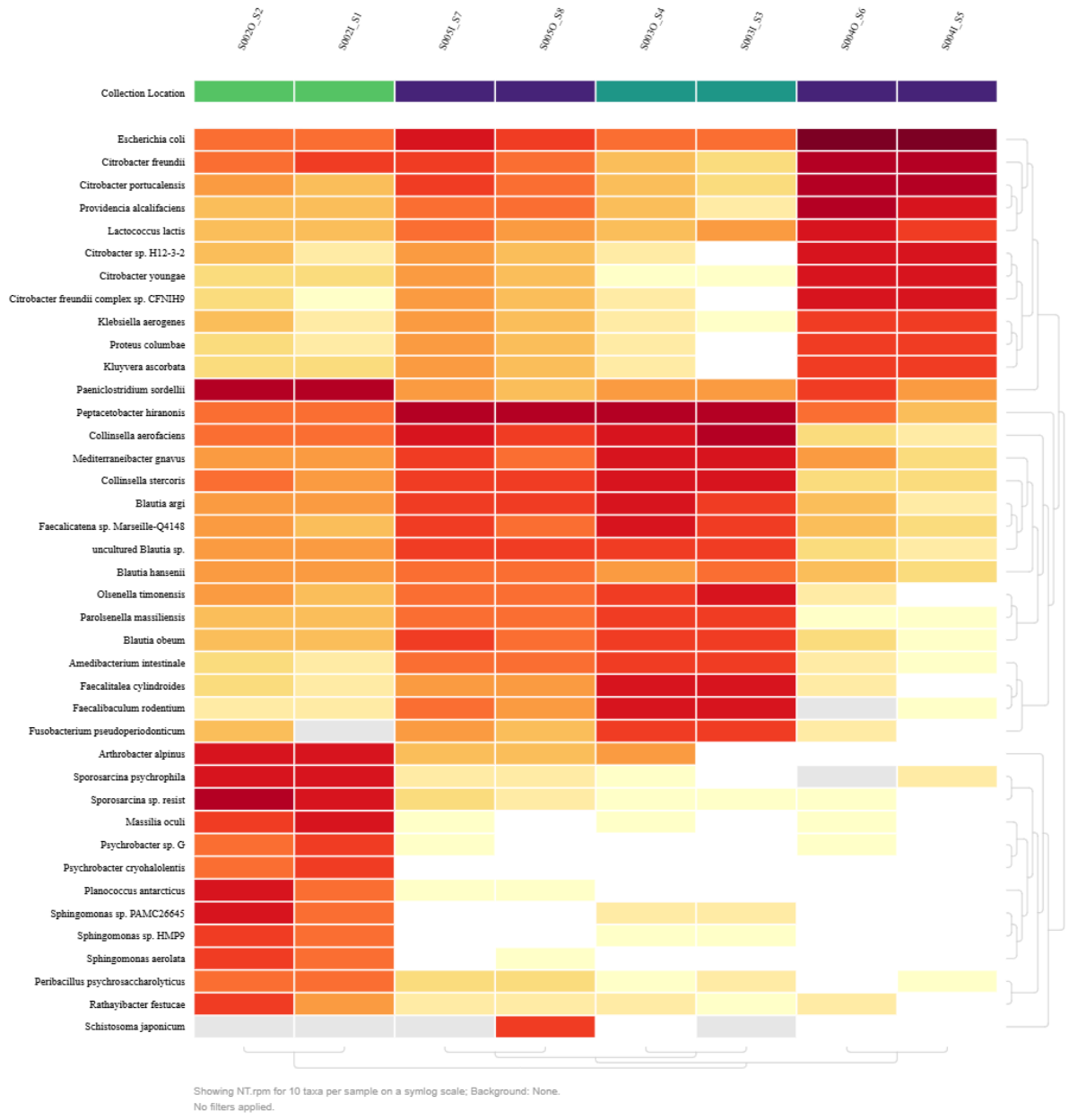
After subsampling and completing the DNeasy Blood and Tissue Kit protocol, the extracted DNA was sent to UMGC where it was analyzed for concentration, purity, and library size.

Table 2: Quality Control (QC) Metrics for DNA Extraction and Library Preparation from UMGC

Sample ID	PicoGreen Concentration (ng/µL)	NanoDrop Concentration (ng/µL)	260/280 Ratio	260/230 Ratio	Total Mass Based on PicoGreen (ng)	Library Size (bp)	Library Concentration (ng/µL)	Library Molarity (nM)	Pass/Fail (Molarity)	Notes
S001I	1.56	26.08	2.23	0.38	16.75	427	0.22	0.86	Fail	Shoulder at ~160-250 bp
S001O	1.68	31.37	2.1	0.28	18.72	444	0.03	0.12	Fail	Shoulder at ~160-250 bp
S002I	0.66	12.45	1.85	0.26	10.63	453	0.57	2.06	Marginal	Shoulder at ~160-250 bp
S002O	0.09	6.32	1.78	0.11	1.38	510	1.57	5.07	Pass	Shoulder at ~160-250 bp
S003I	0.5	43.66	1.72	0.25	7.97	540	1.17	3.57	Pass	No issues reported
S003O	0.3	30.77	1.74	0.22	4.75	556	1.63	4.82	Pass	No issues reported
S004I	10.62	47.89	1.82	0.73	169.86	550	6.68	20	Pass	No issues reported
S004O	17.17	156.5	1.62	0.56	274.74	505	2.03	6.63	Pass	Shoulder at ~160-250 bp
S005I	2.53	16.72	1.87	0.32	40.46	451	1.52	5.54	Pass	Shoulder at ~160-250 bp
S005O	0.95	12.12	1.75	0.22	15.22	418	0.77	3.04	Pass	Shoulder at ~160-250 bp

After UMGC analyzed the extracted DNA, it was put into the CZID online platform for DNA sequencing.

Figure 1: Bacteria taxons from the eight passing urban coyote scat samples quantified by reads per million (rPM) from an NT database.



The collection locations are classified by color in the top row where the dark purple color indicates samples from Dakota County in Minnesota, the green color indicates samples from Hennepin County, and the teal color indicates samples from Ramsey County, respectively. The colors making up the heatmap for the taxons that were found in the samples are classified from lighter to darker where the lighter colors indicate fewer reads per million (rPM) and the darker colors indicate more reads per million (rPM). The samples analyzed for the heatmap did not include samples S001O and S001I due to them not passing the QC metrics from UMGC.

Table 3. Results from two-way ANOVA analysis of the eight samples involved in sequencing.

Source of Variance	Values
Sum of the Squares (SS)	1.39E+10
Degrees of Freedom (df)	7
Mean Squares (MS)	1.98E+09
F-test	1.177888
P-value	0.315029

Table 4. Results from t-tests comparing the inner core results vs the outer crust results for each sample involved in sequencing.

Sample	Inner Core Mean rPM	Outer Core Mean rPM	Two-tailed p-value (between inner vs outer sections)
S002	4171.333	7714.329	0.040214
S003	7938.904	7692.797	0.718398
S004	20737.77	19932.57	0.728745
S005	4323.002	2611.306	0.038403

Discussion:

When originally subsampling the inner and outer sections of the scat, it was interesting to see the extreme visual and textural differences between scat samples. For example, one of the samples looked like it had a tooth in it from a small mammal, and another scat sample had a seed in it. Also, after DNA extraction, sample S004O still had a brownish tint while every other sample was clear. This color difference did not seem to play into the results.

The QC analysis of the DNA extracted from the coyote scat samples showed a large amount of variability in DNA concentration and quantity as shown in Table 2. PicoGreen concentrations can quantify double-stranded DNA (dsDNA) quantities (Ahn, 1996). The PicoGreen concentrations varied between samples, with some samples showing high DNA concentrations (e.g. S004I and S004O with 10.62 ng/μL and 17.17 ng/μL, respectively) and others having low concentrations (e.g. S002O and S003O). Furthermore, the NanoDrop Concentrations also varied significantly in the scat samples with the highest value being from sample S004O (156.5 ng/μL) and the lowest value being from S002O (6.32 ng/μL). The NanoDrop concentrations measure the DNA concentration using UV absorbance and can be affected by RNA, proteins, and other contaminations, often leading to overestimations (Biosearch Technologies). For this reason, the PicoGreen concentrations may be more accurate at quantifying dsDNA in degraded or impure samples (Biosearch Technologies). The 260/280 values can show potential protein contaminations and a value close to 2.0 is ideal (Reck et al., 2015). All of the 260/280 values from the samples were around 2.0 (ranging from 1.62 in sample S004O to 2.23 in sample S001I). The 260/230 values can show potential contaminations from salts, organic solvents, and inhibitory substances, with the ideal values being >2.0 (Reck et al.,

2015). These values also had some variability but were all less than 2.23, which shows that there are likely impurities in the samples.

The library preparation results also revealed variations in the library size, concentration, and molarity values, influencing the sequencing success (see Table 2). The library sizes in this study ranged from 418 bp in S005O to 556 bp in S003O, which mostly fits within the ideal range of 300-500 bp (Agilent) (see Table 2). While these are values mostly within the ideal range, a shoulder was found in seven of the samples at around 160 to 250 bp (Khetani et al.). According to Khetani et al., a shoulder can indicate a couple of things such as that a set of cells failed for some reason or that there are biologically different types or sizes of cells in the sample. Also, the library molarity values for the samples ranged from 0.12 nM in S001O to 6.63 nM in S004O (see Table 2). While it is possible to work with samples with a molarity concentration of 1nM, a concentration of 5 nM or more is ideal (Froenicke, 2013). For this reason, samples S001I and S001O failed and were not used for further analysis.

The CZID sequencing showed a mix of gut, diet-derived, and environmental bacteria. One interesting discovery from the CZID sequencing was that *Schistosoma japonicum*, which is a parasitic flatworm, specifically a blood fluke, found in China, the Philippines, and Sulawesi (CDC, 2024). The CDC does not currently recognize that this parasitic flatworm exists in the United States. Also, the causative agents for botulism *(Paeniclostridium sordelli)* and dysentery (*Providencia alcalifaciens)* were found (Nyaoke et al., 2020). The other bacteria found were typical for that to be found in soil, water, or the intestines of coyotes. A one-way ANOVA test was conducted to compare the bacterial diversity across sample sections as shown in Table 3. The p-value was found to be $p=0.315029$ which was above the statistical significance amount of $p<0.05$. Also, the F statistic value of 1.18 was smaller than the F critical value of 2.04, again

indicating that the results between samples did not show statistical significance. T-tests were conducted to further analyze the results between the inner core and outer crusts of the same samples as shown in Table 4. The two-tailed p-value for S002 comparing the inner vs outer samples was $p=0.040214$ which is less than the $p<0.05$ cutoff to count the results as statistically significant (see Table 4). There was also a higher mean read per million (rPM) value for the outer section compared to the inner section of S002. For S003, the inner sample had slightly more average reads per million (rPM) than the outer sample, and the two-tailed p-value was $p=0.718398$ which was not statistically significant. For S004, the inner sample also had slightly more reads per million (rPM) compared to the outer sample and the p-value was $p=0.728745$, again indicating that the results from S004 were not statistically significant from each other. Lastly, for sample S005, the inner sample had more reads per million (rPM) compared to the outer sample and the p-value was $p=0.038403$, indicating that the results were statistically significant from one another.

Conclusion:

With the increasing number of wildlife finding homes in urban areas, understanding more about the urban wildlife and their potential interactions with humans or pets is crucial. This study aimed at examining the diets and microbiomes of urban coyotes noninvasively. To do so, it examined the differences between inner and outer subsamples of urban coyote scat samples collected in the Twin Cities Metro Area. Furthermore, the study looked at different bacteria present in the scat samples. The University of Minnesota Genomic Center conducted a QC report which showed the DNA concentrations, library sizes, and molarities of the samples. Of the ten samples submitted to UMGC, only eight of the samples passed and were able to be evaluated

further in the CZID online platform. The differences between inner and outer subsamples were further examined via a two-way ANOVA test for all results and t-tests comparing each inner vs outer sample. S001I and S001O were not examined further past the QC report conducted by UMGC due to their values not meeting the passing criteria. The QC report also showed that the observed differences in DNA between samples may be due to environmental or biological factors rather than sample degradation or processing errors. S002I and S002O as well as S005I and S005O both showed significantly different results when comparing the inner vs outer crust samples ($p=0.0402$ and p-0.0384 respectively). The inner and outer subsamples from S003 and S004 did not show statistically significant differences.

The differences observed between different samples, as well as the inner and outer crust of the same samples, may be attributed to biological composition, environmental exposure, structural variation, or other unknown influences. The QC report from UMGC also supports the integrity of the DNA samples, ruling out technical artifacts, contamination, or degradation as primary causes of variation. This validation strengthens confidence in the observed patterns and suggests that the differences may stem from biological or environmental factors rather than errors in sample processing.

To expand on these findings, future research could be conducted with a larger sample size and comparing rural vs urban coyotes to understand if there are differences in microbiomes from geographic locations. Environmental factors such as diet, pollution exposure, and habitat variation could play a significant role in shaping genetic and structural differences between populations. The same research could also be conducted with other canid populations to understand differences in microbiomes and diet between species and locations. By utilizing genomic sequencing and statistical analyses, this research lays the foundation for a more

comprehensive understanding of the spatial and structural variation in canid samples, or biological samples as a whole. Future studies should aim to build upon these findings, contributing to a broader understanding of how the environment and genetics interact in shaping wildlife populations.

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