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APPLICATION ARTICLE

DNA metabarcoding reveals greater plant diversity than morphological seed analysis of bird feces

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Abstract

Premise: Fruit-eating birds drive seed dispersal in recovering tropical ecosystems, shaping forest regeneration. Molecular techniques, such as DNA metabarcoding, enable diet analysis from feces and can provide complementary frugivory data where dispersal is infrequent, as well as aid in seed identification in hyper-diverse regions lacking morphological reference collections.

Methods: We compared the plant taxa detected in bird droppings using DNA metabarcoding and morphological seed classification. Plant DNA extracted from 52 seed-containing droppings was amplified using a universal plant primer for an *rbcL* mini-barcode. Morphologically detected seeds were cross-referenced with metabarcoding-detected genera.

Results: DNA metabarcoding and morphological classification of seeds are shown to be complementary methods, with the same plant taxa detected in only 38% of dropping samples. Although metabarcoding did not recover DNA of some seeds present in the dropping samples, metabarcoding revealed an overall higher diversity of plant genera compared to morphological seed analysis.

Discussion: Our results suggest that the use of a metabarcoding approach can be complemented by established seed analysis methods. Diet information obtained from metabarcoding feces may shed light on cryptic bird–plant interactions and potential seed dispersal events, especially in high biodiversity areas.

KEYWORDS

bird droppings, diet analysis, *rbcL* mini-barcode, seed dispersal, seed identification

Frugivory, the consumption of fruits by animals, is a keystone mutualism that shapes both animal and plant communities, especially in the tropics (Bascompte and Jordano, 2007; Escribano-Avila et al., 2018). Fruits are a critical resource for many tropical vertebrates, which consume fruit at proportionally higher rates than vertebrates in other regions (Fleming et al., 1987; Jordano, 2000). In the Neotropics, nearly 40% of bird species are at least partially frugivorous, consuming fruits from hundreds of plant species (Kissling et al., 2009; Almeida and Mikich, 2018; Carlo et al., 2022). Simultaneously, most tropical woody plants depend on frugivores for seed dispersal (Herrera, 2002). Animal-mediated seed dispersal shapes plant population dynamics, ecosystem functioning, and forest regeneration (Jordano, 2000; Tabarelli and Peres, 2001; Carlo et al., 2011). Neotropical birds are particularly important seed dispersers, exhibiting a high potential to transport seeds away from the parent plant, often contributing to the establishment of new forests (Godínez-Alvarez et al., 2020; Nevo et al., 2023). Among the most biodiverse regions on the planet, Neotropical ecosystems face significant threats from deforestation and habitat loss (Bogoni et al., 2024; Fuzessy et al., 2024). Gaining deeper insights into seed dispersal networks is therefore essential for designing effective strategies for conservation and forest restoration (e.g., Torre et al., 2015), especially in degraded habitats

where natural recruitment depends heavily on effective seed dispersal (Ribeiro da Silva et al., 2015). Accurate quantification of plant–frugivore interactions is key to unraveling these ecological processes and informing efforts to preserve and restore tropical biodiversity.

Plant–frugivore interactions between plants and birds are most often studied using focal observations of bird visits to a single fruiting plant (Pizo and Camargo, 2018), as well as the morphological identification of seeds found in bird feces (Hernández-Dávila et al., 2015; Stone et al., 2017), allowing for the quantification of dispersal events and evaluation of seed disperser effectiveness (Schupp, 1993; Schupp et al., 2010). While these methods are well established, both focal observations and the morphological identification of seeds from bird feces can be labor intensive and time consuming (Jordano, 2016; Schlautmann et al., 2021; Quintero et al., 2022). Also, morphological identification is further hindered by the lack of comprehensive reference collections and the immense plant richness and diversity, particularly in biodiverse regions like the Neotropics (Nesbitt et al., 2003; Flemming and Grassi, 2024). Many plant species in tropical ecosystems produce seeds with similar morphologies, and the high species diversity often surpasses the capacity of available taxonomic keys, posing significant challenges for accurate identification (Zappi et al., 2015). Additionally, the degradation of the seeds consumed, especially of smaller seeds, can further complicate the identification process (Rompf et al., 2024). Bird species traits can also influence the frequency with which seeds are found in feces, as many small Neotropical frugivores have short gut passage times (less than 20 minutes) and some seeds are regurgitated rather than excreted (Gasperin and Pizo, 2012). As a result, these seeds may not be available for morphological analysis in the fecal samples, restricting the dietary and seed dispersal information that could be obtained. Therefore, both bird and plant traits can limit the seed diversity that can be found and identified in feces, ultimately constraining the scope and accuracy of diet and seed dispersal assessments based solely on morphological analysis (Bracho-Estévanez et al., 2024). These limitations highlight the need for complementary approaches that can overcome the taxonomic and logistical challenges inherent in traditional methods.

Advances in molecular techniques have offered a promising alternative for studying seed dispersal by identifying DNA from seeds and plant material present in fecal samples (Galimberti et al., 2016). DNA barcoding has been used in conjunction with morphological identification when seeds could not be identified using reference collections, or to create a short-list of possible taxa (Viana et al., 2016; Timóteo et al., 2018; González-Varo et al., 2021). More recent advances in environmental DNA (eDNA) techniques have allowed for the application of DNA metabarcoding approaches to identify plant material in fecal samples (Ando et al., 2020; García et al., 2024). DNA metabarcoding enables the identification of multiple plant taxa from a single fecal sample, which can provide more comprehensive and accurate analysis of bird diets, improving understanding of seed dispersal networks (Cuff et al., 2022; Tang et al., 2023). Additionally, DNA metabarcoding can reveal cryptic interactions that are not detectable through field observation methods, such as species that consume small seeds or those involved in sporadic interactions, providing more detailed information on plant-derived diets (Tang et al., 2023; García et al., 2024). Rare or sporadic interactions may happen when fruit yield is low or competition is high, resulting in infrequent interactions (Bender et al., 2017; Dehling et al., 2022). These uncommon events are unlikely to be captured by focal observations focused on an individual plant and may cumulatively contribute to bird diets and seed dispersal (Carlo and Morales, 2016). Furthermore, DNA metabarcoding has the potential to detect fruit consumption even in cases where seeds are excreted or regurgitated before fecal sample collection for morphological analyses, allowing us to infer seed dispersal from frugivory (Simmons et al., 2018). Applying DNA metabarcoding in seed dispersal studies can therefore potentially increase the temporal detection window, as well as provide frugivory data on a plant community level.

In this study, we compared the efficacy of DNA metabarcoding with morphological detection of seeds present in bird feces. By evaluating the strengths and limitations of each method, we aimed to assess the potential of metabarcoding as a tool for identifying plants from seeds that are present in bird feces and detecting fruit consumption. We worked in naturally recovering secondary forest fragments in a mixed urban, industrial, and agricultural matrix within an ecotone region of Atlantic Forest and Cerrado biomes in Brazil. Understory birds were captured using mist netting to collect droppings once per month in 10 study sites. Bird droppings containing seeds were analyzed using metabarcoding of a universal plant mini-barcode to identify plant diet items. We then compared the plant genera detected using DNA metabarcoding to the seeds morphologically detected in bird feces to quantify the efficacy of each method. Due to the rapid gut passage time of small Neotropical birds, we predicted that metabarcoding would detect a higher number of plant taxa than seed morphology, given its ability to identify other plant materials (e.g., pulp) and its potentially broader temporal detection window. This research contributes to advancing methodological frameworks for ecological studies and informs conservation strategies focused on plant–animal interactions, particularly in diverse and complex ecosystems.

METHODS

Study system

Our study area is located in the Corumbataí River basin (22°13′22.1″S, 47°37′24.2″W) in the state of São Paulo, Brazil (Figure 1). This region is an ecotone of the Atlantic Forest and Cerrado biomes, characterized by seasonal, semi-deciduous

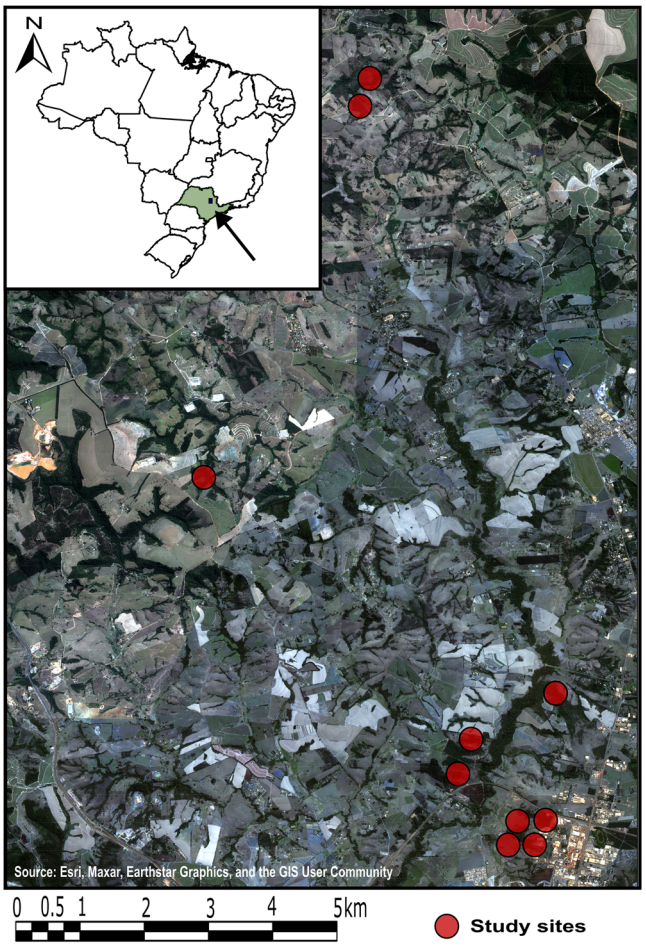

FIGURE 1 Map of study area and the 10 study sites where birds were captured and bird dropping samples were collected, located in the Corumbataí River basin (22°13′22.1″S, 47°37′24.2″W) in the state of São Paulo, Brazil.

forests (César et al., 2021). To better capture seed dispersal interactions in the region, we worked in 10 sites of naturally recovering secondary forest fragments located on private property. The surrounding landscape matrix includes industrial and urban areas, as well as pasture lands where cattle are raised (Borda-Niño et al., 2021). Detailed survey data at each site include identifications and abundance of woody tree species individuals (César et al., 2021). Approximately half of the woody tree species present are dispersed by animals, including species from the families Euphorbiaceae, Meliaceae, Myrtaceae, Rubiaceae, and Salicaceae (Simioni, 2024). Fruit-eating, understory birds in the region include both forest-dependent and habitat-generalist species from the families Passerellidae, Pipridae, Thraupidae, Turdidae, and Tyrannidae (Maluf Napoleão, 2022).

Bird dropping sample collection

Birds were captured once per month at each of the 10 sites to collect droppings from August 2022 to August 2023, totaling 2763 net hours. We used five mist nets (12 × 3 m, 15-mm mesh) arranged in an "L" formation, with plastic sheets placed beneath the nets to collect any droppings from birds captured in the nets. The mist nets were opened at sunrise and checked every 30 minutes until they were closed at 11:00 hours. A total of 58 bird species were captured across all sites, with 38 of these being considered fruit-eating. Birds were classified as "fruit-eating" if seeds were found in feces, or if percent use of fruit in their diet is greater than 0 (Wilman et al., 2014). A total of 443 individuals were captured, with 483 captures (40 individual recaptures) of all species and 273 captures (10 recaptures) of fruit-eating species. The capture rate per net hour averaged 0.175 ± 0.05 for all species and 0.099 ± 0.03 for fruit-eating species across all 10 sites. Droppings were collected from 417 out of 483 bird captures. Of these 417 dropping samples, 252 dropping samples came from the 273 captures of fruit-eating species.

Droppings were collected in two ways: using the plastic sheets beneath the mist nets and by placing birds in paper bags lined with plastic (Figure 2). Upon each capture, we inspected the plastic sheets for droppings, collecting any samples found (Figure 2A). Regardless of the presence of droppings on the sheet, captured birds were held in paper bags and observed every 10 minutes for up to 30 minutes to allow for additional collection through defecation or regurgitation (Figure 2B, C). All collected droppings were transferred to 2-mL autoclaved polypropylene microtubes containing 99.5% alcohol and stored at −20°C the same day upon returning from the field (Figure 2D). Following dropping collection, each bird was identified and released. Bird capture and sample collection were authorized by the Comissão de Ética no Uso de Animais (Decisão CEUA No. 05/2022) and the Sistema de Autorização e Informação em Biodiversidade (Sisbio; No. 81210-1).

Morphological detection and identification of seeds in bird droppings

In order to preserve DNA integrity, a small amount of fecal material (50–100 mg; 200 µL alcohol from sample) was reserved for DNA extraction prior to the manual removal of intact seeds. The remaining fecal sample was used to search for seeds. Seeds were counted and identified by manually disassembling droppings in Petri dishes under a dissection microscope. We found seeds in 64 (15%) of the 417 collected droppings (Table 1). All seeds found were classified to morphotype. Metabarcoding results were used to create a list of possible taxa in each dropping sample. Photos of seeds of genera detected using metabarcoding were found using online seed identification keys (e.g., https://www.frutosatrativosdocerrado.bio.br/chave-interativa) or other identification resources (e.g., Lorenzi, 1992) and used to match each morphotype to taxa detected in the same sample using metabarcoding. When possible, physical seeds in seed

FIGURE 2 Bird dropping collection after mist netting at study sites in the state of São Paulo, Brazil. (A) Bird droppings on the plastic sheet placed below the mist net. (B) Paper bag used to hold birds for up to 30 minutes, or until defecation. (C) Bird droppings in a paper bag with plastic liner. (D) Bird droppings containing pulp and seeds collected from captured *Turdus leucomelas* (Turdidae) individuals conserved in 99.5% alcohol in autoclaved 2-mL tubes.

TABLE 1 Number of fecal samples sampled and analyzed by metabarcoding and seed morphology.

		No. of droppings	
Bird family	**Bird species**	**Sampled**	**Analyzed**
Passerellidae	*Arremon flavirostris*	2	2
Pipridae	*Antilophia galeata*	17	13
	Manacus manacus	5	5
Thraupidae	*Nemosia pileata*	1	1
	Ramphocelus carbo	6	5
	Tachyphonus coronatus	6	5
	Thlypopsis sordida	1	1
	Thraupis sayaca	1	0
	Eucometis penicillata	4	3
Turdidae	*Turdus amaurochalinus*	3	3
	Turdus leucomelas	7	7
Tyrannidae	*Cnemotriccus fuscatus*	1	1
	Lathrotriccus euleri	1	0
	Leptopogon amaurocephalus	1	0
	Myiarchus ferox	3	3
	Myiodynastes maculatus	1	0
	Pitangus sulphuratus	4	3
	TOTAL	64	52

reference collections maintained by collaborators were also consulted. When seeds did not match any taxa detected using metabarcoding, we identified seed morphotypes to family and, when possible, genus, using all possible seed identification resources.

Metabarcoding of plant material in bird droppings

While the presence of seeds in droppings provides direct evidence of fruit consumption and seed dispersal, DNA metabarcoding enables us to determine if droppings contain additional plant material, offering a more comprehensive view of plant-based diet and potential seed dispersal. For that, we selected all 64 dropping samples that contained seeds, collected from 17 frugivorous bird species (Table 1). Following sequencing and quality control filtering (described below), 52 samples from 13 bird species remained and are presented in our results. DNA extraction and library preparation were conducted at the Laboratory of Molecular Ecology, Department of Biodiversity, Universidade Estadual Paulista (UNESP) in Rio Claro, São Paulo, Brazil.

DNA extraction

A small portion of the dropping samples (50–100 mg; 200 µL alcohol from sample) was dried in 2-mL microtubes for two hours in a dry bath (65°C) under a fume hood before the DNA extraction. We used a modified cetyltrimethylammonium bromide (CTAB) protocol with increased lysis time and the use of sodium acetate and NaCl in the wash step (https://dx.doi.org/10.17504/protocols.io.8epv527x5v1b/v1; Motta et al., 2024). DNA extractions were conducted under a fume hood sterilized with 10% bleach solution and a nucleic acid inhibitor solution (Cleaner, Nova Biotecnologia, São Paulo, Brazil), wearing a mask and gloves.

Mini-barcode amplification, library preparation, and metabarcoding sequencing

Universal plant primers allow for the amplification and detection of specific regions in the plant DNA, independent of

the tissue source (i.e., leaves, fruit), present in a sample using DNA barcode markers, usually from *rbcL*, *matK*, *trnH-psbA*, and *ITS2* gene regions (Hollingsworth et al., 2011; Kress, 2017). In our study, we selected a universal plant mini-barcode within the *rbcLa* subregion of the *rbcL* (ribulose-1,5-bisphosphate carboxylase) chloroplast gene due to a higher availability of reference sequences of plants from our study area for this mini-barcode and because of its taxonomic resolution as demonstrated in previous studies (Espinosa Prieto et al., 2024). For the mini-barcode amplification, the primers rbcL1 Forward: 5′-TTGGCAGCATTYCGAGTAACTCC-3′ and rbcLB 5′-AACCYTCTTCAAAAAGGTC-3′ were used (Palmieri et al., 2009; Little, 2014). When used together, the forward primer rbcL1 and reverse primer rbcLB result in a mini-barcode region amplification of a 184-bp fragment, which is therefore ideal for degraded plant DNA found in droppings (Shutt et al., 2021).

For metabarcoding sequencing, six tagged versions of the primer pair were synthesized to include an Illumina sequencing overhang adapter and one of six 5-bp tags obtained from Axtner et al. (2019) (Figure 3). Utilizing tags, we reduced the metabarcoding costs by indexing and pooling samples in-house, while still being able to separate samples according to their tag in our bioinformatics pipeline and conduct analyses at an individual level (Saranholi et al., 2023).

PCR conditions were set as follows: 1.25 μL of 10× reaction buffer (Bioline-Meridian Bioscience, Memphis, Tennessee, USA), 1.25 μL of $MgCl_2$ (25 mM; Bioline-Meridian Bioscience), 0.8 μL of the dNTP mix (10 mM; Sinapse Biotecnologia, São Paulo, Brazil), 0.5 μL of each the forward and reverse primers (10 pmol), 0.2 μL of Taq polymerase (5 U/μL, Taq Platinum; Invitrogen, São Paulo, Brazil), 0.2 μL of bovine serum albumin (BSA) (20 μg/mL; New England Biolabs, Ipswich, Massachusetts, USA), and 3 μL of template DNA for a final volume of 12.5 μL. Cycling conditions consisted of an initial denaturation at 94°C for 10 min, followed by 30 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 40 s of extension at 72°C, and a final extension at 72°C for 5 min. Following amplification, PCR products were visualized by electrophoresis in a 1.5% agarose gel. To increase yield and guarantee successful amplification, each sample was amplified three times and 6 μL of each PCR product was combined into a single sample of 18 μL. PCRs were conducted for one tagged primer at a time, using 5–6 DNA samples from droppings and one blank sample (negative control), in which extracted DNA was replaced with purified water during PCR. The PCR mix was prepared in a designated flux chamber sterilized with a 10% bleach solution and a nucleic acid inhibitor solution (Cleaner, Nova Biotecnologia) in addition to ultraviolet light, along with pipettes, pipette tips, and tubes prior to each PCR. Library preparation was conducted wearing a mask and gloves, and the environment was sterilized between each PCR.

Samples were pooled according to similar product concentration (ng/μL) estimated by visual inspection of band strength in the electrophoresis gel. We avoided pooling samples that had strong bands with weakly amplified samples to avoid a bias on the number of reads of the metabarcoding. When possible, PCR products from birds of the same species were grouped together. A volume of 5 μL was taken from each sample obtained from the combination of the 18 μL of the three replicates, differentially tagged with one of the six synthesized primers, and combined in a single tube, resulting in a final volume of 30 μL. A grouped sample of blanks was also combined using 5 μL of each tag-associated blank, resulting in a final volume of 30 μL. Pools were sent for sequencing in two batches. The first batch consisted of six pools, with five of the pools each containing six pooled samples ($n = 30$), and one blank pool. The second batch consisted of seven pools, with six of the pools each containing six samples ($n = 36$), and one blank pool. A total of 13 pools were sent for sequencing, with 11 pools of 66 samples (64 dropping samples with seeds and an additional two dropping samples that did not contain seeds), and two blank pools. The results of the two samples that did not contain seeds are not presented here.

Metabarcoding sequencing was performed by EcoMol Consultoria in Piracicaba, São Paulo, Brazil. Pooled samples were purified using magnetic beads (Agencourt AMPure XP; Beckman Coulter, Brea, California, USA) and quantified using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Standardized samples (50 ng/μL) were indexed using a Nextera Index kit (Illumina, San Diego, California, USA), and the pooled library was quantified using real-time PCR (Kapa Biosystems, Wilmington, Massachusetts, USA). Paired-end metabarcoding sequencing was performed on an Illumina iSeq platform, using an iSeq 100

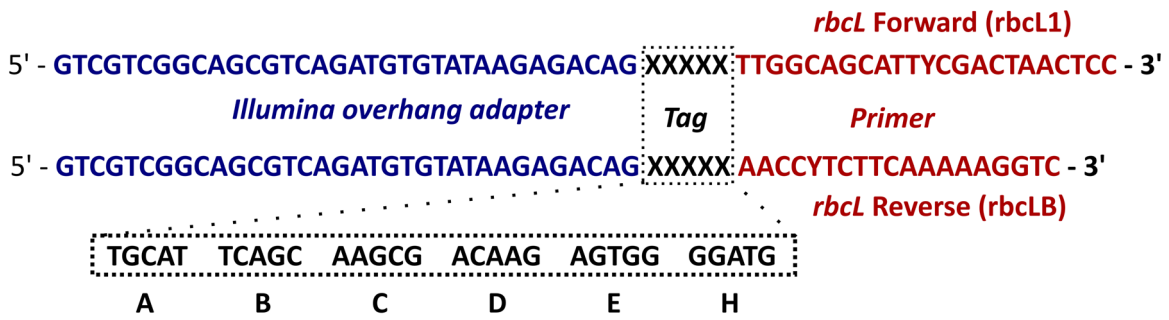

FIGURE 3 Schematic demonstrating how multiple versions of the forward primer rbcL1 and reverse primer rbcLB were synthesized with the tags A, B, C, D, E, and H (Axtner et al., 2019) used to differentiate each of the six versions of the rbcL primer pair (Palmieri et al., 2009; Little, 2014).

v2 300 Cycle Reagent kit (2 × 150 bp), for a total of up to 70,000–100,000 reads per each of the 13 pools, expecting approximately 12,000–16,000 reads per tagged sample or blank.

Bioinformatics pipeline and identification of operational taxonomic units

Bioinformatics processing was conducted following Saranholi et al. (2023, 2024). Obtained sequences were inspected using FastQC software (Andrews, 2010) to confirm sequence quality (>33 Phred score). Sequences were then demultiplexed with the process_radtags program in Stacks v2.59 (Catchen et al., 2013) in order to separate sequences by tag and retrieve individual-level sample information (Figure 3). Using PEAR (Zhang et al., 2014), corresponding forward and reverse sequences were merged and trimmed using the following conditions: a minimum overlap (−v) of 60 bp, a minimum length (−n) of 100 bp, and minimum quality score threshold (−q) of 15 (Zhang et al., 2007; Rodgers et al., 2017). Singletons were discarded and operational taxonomic units (OTUs) were obtained by clustering unique sequences with at least 97% similarity using USEARCH v.11.0.667 (Edgar, 2010). Nine of the 64 droppings sampled did not generate OTUs due to PCR failure. Quality control filtering was conducted with the remaining samples ($n = 55$), using a read threshold (>20 reads) to retain the OTUs. Read thresholds are often implemented to avoid including OTUs that result from sequencing artifacts (Sales et al., 2020; Broadhurst et al., 2021; Mojica and Caballero, 2021). Three samples were excluded due to all associated OTUs having less than 20 reads. After these filtering steps, 52 samples were retained for further analyses.

The Basic Local Alignment Search Tool (BLAST) using the Nucleotide collection (nr/nt) database was used to compare the obtained OTUs to reference sequences available from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/; accessed October 2024) and the Barcode of Life Data Systems (BOLD version 4; https://www.boldsystems.org/index.php) databases. Further filtering of each OTU was conducted by evaluating its percentage of identity match to reference sequences. OTUs with poor identity match (<97.99%) with any plant taxa were removed. If an OTU from a blank control appeared in a tagged sample and had the same or fewer reads than in the blank, it was excluded from the tagged sample (Drake et al., 2021).

Because all OTUs matched (>97.99%) with multiple species within the same genus, we therefore conducted the analyses at genus level. Finally, to improve OTU taxonomic curation, we confirmed the geographic distribution of the genera detected and, in cases where a >97.99% match was obtained with multiple genera, geographic distribution and presence in our study area was used to determine the most likely genus. Geographic distribution was evaluated using the Global Biodiversity Information Facility (https://www.gbif.org/) and lists of woody tree species (individuals with >10 diameter at breast height [DBH]) present in our study plots throughout the region (César et al., 2021). In two cases, a 100% sequence match occurred with two very closely related genera that are sister clades from the same family, both of which are present in our study area. We therefore treated each pair of genera, *Serjania/Paullinia* (Sapindaceae) and *Lippia/Lantana* (Verbenaceae), as a single identification in our analyses (Marx et al., 2010; Joyce et al., 2023). When comparing these ambiguous OTU assignments to genera identified through seed morphology, we considered them a match if either of the two possible genera corresponded. For example, if a seed was identified as *Lantana* and the metabarcoding result was *Lippia/Lantana*, we counted it as a match.

Because DNA metabarcoding detects plant DNA regardless of the source tissue, detections of plant genera may not necessarily reflect fruit consumption. To assess the likelihood that the detected DNA originated from fruit rather than other plant material, we investigated the type of fruit produced by each genus (Cuff et al., 2022; García et al., 2024). We therefore determined whether a majority of the species in each associated genus produce fleshy or dry fruits, and if any associated species are known to be consumed by birds (https://reflora.jbrj.gov.br/reflora/herbarioVirtual/).

Similarity and overlap between plants detected using metabarcoding and seeds

For each dropping, we calculated the number of taxa detected by each method individually, mutually by both methods (i.e., shared taxa), and in total. We then evaluated whether taxa detected using metabarcoding matched with seeds found in feces, classifying the resulting overlap as total (100%), partial (100% > overlap > 0%), or absent (0%). When overlap was partial or absent due to the detection of different taxa, we assessed whether both methods detected the same number of taxa or whether one method identified more than the other. The Jaccard index (Real and Vargas, 1996; Verma and Kumar, 2020) was used to evaluate the similarity of taxa detected using metabarcoding analyses and seed morphology. We divided the intersection of taxa detected using metabarcoding and seed morphology by the union of taxa detected using the following formula:

$$\text{Jaccard index (metabarcoding and seed morphology)} = \frac{|\textit{Metabarcoding taxa} \cap \textit{Seed morphology taxa}|}{|\textit{Metabarcoding taxa} \cup \textit{Seed morphology taxa}|}$$

The intersection and union of the taxa detected were calculated in R (version 4.1.1; R Core Team, 2021).

To assess how comprehensively each method captured the plant taxa present in each fecal sample, we calculated the recall metric from both the morphological and metabarcoding perspectives (Verma and Kumar, 2020). Recall is defined here as

the proportion of taxa identified by a given method relative to the total number of taxa mutually detected by both methods in a sample. Specifically, we evaluated how well the metabarcoding method recovered the taxa identified by seed morphology by dividing the number of taxa detected by both methods (i.e., the overlap) by the total number of taxa detected through morphology, as shown in the following formula:

$$\text{Recall (Metabarcoding)} = \frac{\text{No. taxa mutually detected by both methods in dropping sample}}{\text{No. taxa detected using seed morphology in dropping sample}}$$

Conversely, we evaluated how well seeds present in droppings detected plants relative to metabarcoding by dividing the number of taxa detected using both methods (i.e., overlap) by the total number of taxa detected by metabarcoding, as shown in the following formula:

$$\text{Recall (Seed morphology)} = \frac{\text{No. taxa mutually detected by both methods in dropping sample}}{\text{No. taxa detected using metabarcoding in dropping sample}}$$

An average recall value for both metabarcoding and seed morphology was calculated across all samples.

The difference between the number of taxa detected by each method was visualized using a lollipop graph from ggplot2 in R (Wickham, 2016). We also created interaction webs connecting dropping samples to plants detected for both methods using the ggplot2 extension ggalluvial in R (Brunson, 2020). The interaction webs and lollipop graph were combined and edited using Inkscape (version 1.4; https://inkscape.org/).

Plants detected using both metabarcoding and morphological methods, as well as plant taxa uniquely identified by each approach, were visualized using the VennDiagram package in R (version 1.6.20; Chen and Boutros, 2011). We therefore compared the 39 OTU classifications identified using metabarcoding to the 29 taxa of seed morphotypes identified using morphology.

RESULTS

Morphological detection and identification of seeds in bird droppings

We classified the 1741 seeds found in 52 dropping samples as 36 morphotypes. Using metabarcoding data and seed reference collections for comparison, we identified 33 morphotypes to 22 families and 30 morphotypes to 23 genera, with three morphotypes remaining at family level and another three morphotypes remaining unidentified (Appendix S1; see Supporting Information with this article). We detected between one and three plant taxa per dropping sample (mean ± SD: 1.36 ± 0.65 plant genera). Each plant genus was detected in one to 19 droppings, with most genera occurring in approximately two samples on average (1.79 ± 1.72 samples). The three most detected plant genera using morphological classification include *Miconia* (Melastomataceae; $n = 19$), *Casearia* (Salicaceae; $n = 9$), and *Piper* (Piperaceae; $n = 4$) (Figure 4).

Metabarcoding of plant material in bird droppings

Before quality filtering, the combined read count of all pooled sequences (excluding control pools) resulted in a total of 223,124 paired reads, with a mean of 772 ± 3854 paired reads per OTU. All OTUs produced belonged to plant species, with a total of 290 OTUs obtained. Following quality filtering, a total of 215,319 paired reads from 52 samples were maintained, with an average of 2446 ± 6708 reads per OTU and 5.25 ± 8.89 OTUs per sample. The number of reads retrieved per dropping sample varied between 22 and 26,745, with an average of 4140 ± 9029 reads per sample. We discarded 163 OTUs due to having an insufficient number of reads (<20 total paired reads). An additional 36 OTUs were discarded due to a <97.99% match with any plant taxa, and two OTUs were discarded due to suspected contamination. Following quality filtering, we maintained 88 OTUs for further analyses.

For each dropping sample sequenced, we retrieved between one and seven OTUs (1.69 ± 1.2 OTUs). We identified all 88 OTUs retained as being from 28 plant families (Appendix S2). We were able to assign 83 OTUs to a unique genus, representing 38 different genera. The remaining five OTUs were classified as being of two different taxa, with four classified as *Serjania/Paullinia* and one as *Lippia/Lantana*. We detected between one and seven plant genera for each sample sequenced, averaging approximately two plant genera per sample (1.92 ± 1.4 plant genera). Each plant genus was detected in between one and 16 dropping samples, with a majority of plant genera being detected in approximately two dropping samples (2.05 ± 2.66 dropping samples). The three most detected plant genera using metabarcoding include *Miconia* (Melastomataceae; $n = 19$), *Casearia* (Saliaceae; $n = 9$), and *Serjania/Paullinia* (Sapindaceae; $n = 4$) (Figure 4). When evaluating the fruit type of each taxa, we classified nine of the genera detected as containing more species that produce dry, non-fleshy fruits than those that produce fleshy fruits (Figure 4).

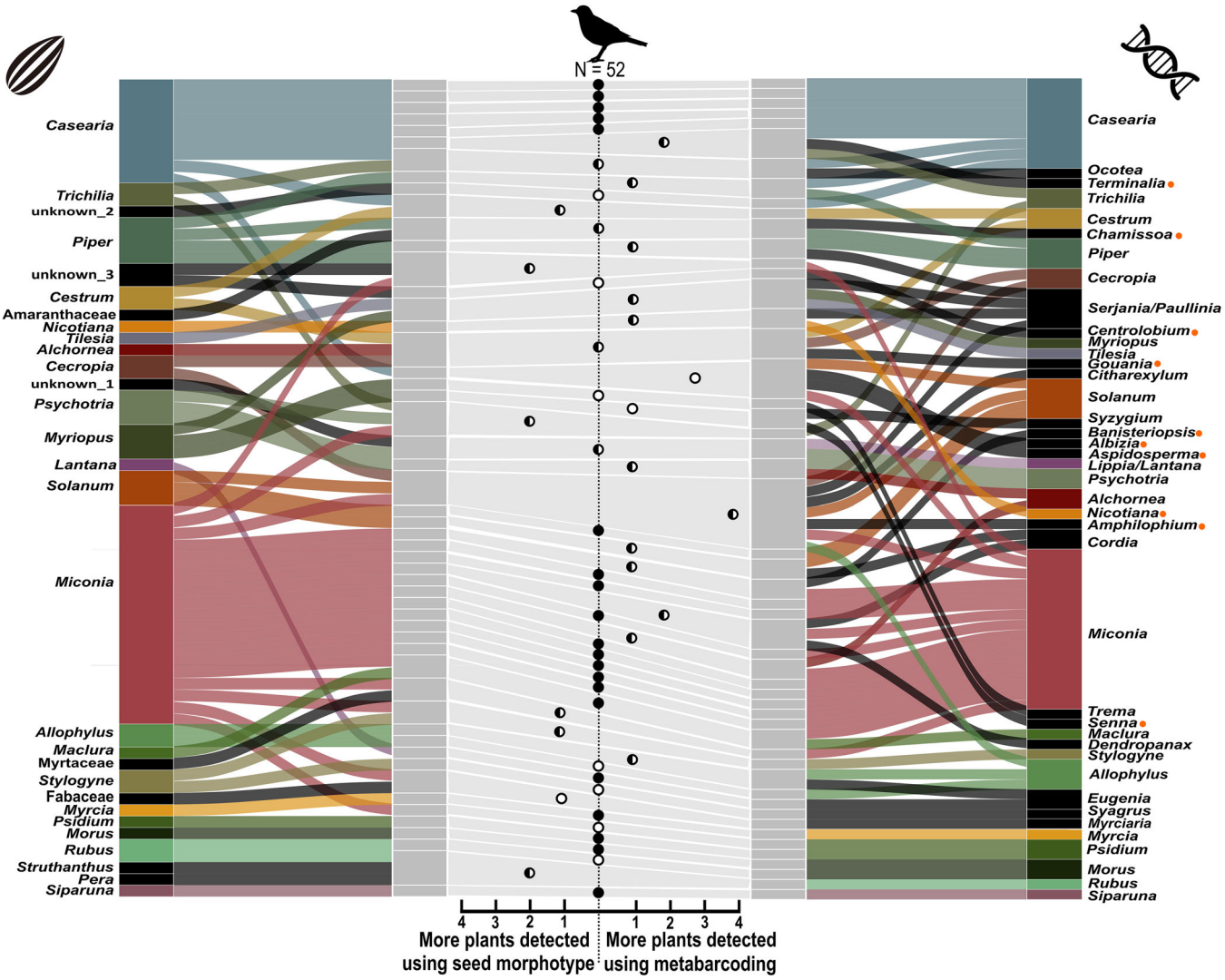

FIGURE 4 Interaction web of bird dropping samples (center; $N = 52$) with plants detected using morphological identification of seeds present in droppings (left) and metabarcoding of plant material (right). The seed morphology interaction web includes seeds identified to family and genus level, as well as unknown morphospecies. The metabarcoding interaction web includes the genera identified, as well as the two operational taxonomic units (OTUs) classified as one of two plant genera. Plants detected in both datasets, independent of the sample in which they were detected, have the same color in both interaction webs, while plants detected using only one method are depicted in black. Points representing bird dropping samples are filled solid if metabarcoding and seed datasets detected the same plants (100% overlap), half-filled if the two methods detected at least one of the same genera (100% > overlap > 0%), and empty if the two methods detected completely different genera (no overlap, 0%). Points are centered if morphology and metabarcoding methods identified the same number of plants. Points located to the right of the center had a higher plant diversity detected by metabarcoding and are positioned according to the difference between the number of taxa obtained from metabarcoding vs. morphology. Points located to the left of the center had a higher plant diversity detected by using seeds and are positioned according to the difference between the number of taxa obtained from morphology vs. metabarcoding. Orange points next to the plant names indicate the production, by a majority of the species in that genus or family, of dry, non-fleshy fruits that are not known to be consumed by birds.

Overlap and similarity between metabarcoding results and seeds found in droppings

Of the 52 dropping samples, 38% ($n = 20$) showed total overlap between plants detected using metabarcoding and seeds, while 42% ($n = 22$) showed partial overlap, and 19% ($n = 10$) of samples did not have any overlap (Appendix S3). In the case of partial overlap, metabarcoding more frequently detected more plants than the morphological analysis of seeds. When overlap was absent, the same number of plants was most frequently detected by both methods. Similarity represented by the Jaccard index resulted in a mean of 0.56 ± 0.39. Mean values of recall metrics calculated per sample show that the metabarcoding data captured on average 68% of plants detected using seed morphology (Appendix S3). Meanwhile, the seed morphology data captured on average 65% of plants detected using metabarcoding.

We detected a total of 49 plant taxa using both seed morphology ($n = 29$) and metabarcoding ($n = 40$), with 21

taxa (43%) detected by both methods, 19 (38%) uniquely detected by metabarcoding, and eight (16%) uniquely detected by seed morphology (Figure 5). Metabarcoding also recovered a higher proportion of genus-level identifications, with 38 out of 40 taxa (95%) assigned to genus, compared to 23 out of 29 (79%) identified using morphology. Focusing only on genus-level detections, a total of 41 genera were identified: 38 using metabarcoding and 23 using seed morphology. Of these, 21 genera (51%) were shared between methods, 18 (44%) were unique to metabarcoding, and two (5%) were unique to morphology (Figure 5).

DISCUSSION

Our study highlights the efficacy of using DNA metabarcoding and seed morphological methods in conjunction when analyzing bird diet and seed dispersal. Both methods detected, on average, one to two plant taxa per fecal sample, but complete overlap of detected taxa was found in only 38% of samples, highlighting their complementarity (Appendix S3). As predicted, DNA metabarcoding revealed a greater diversity of plant taxa and ultimately identified a greater proportion of detected taxa to genus level (Figure 5). While metabarcoding captured taxa detected by both methods at a slightly higher rate than seed morphology (68% and 65%, respectively), metabarcoding did not detect some of the taxa of seeds present in droppings (Figure 5). These results highlight the potential of metabarcoding not only to help identify seeds in feces but also to uncover additional diet items and potentially dispersed plant taxa.

Although metabarcoding detected up to seven plant taxa in a single dropping—compared to a maximum of three identified by seed morphology—both methods detected, on average, between one and two plant taxa per sample. A relatively low number of seed morphotypes per sample is expected due to rapid gut passage time of small Neotropical birds and their foraging habits (Gasperin and Pizo, 2012). The number of fruits consumed and trees visited per day depends on bird species' degree of frugivory (Quitián et al., 2019; González-Varo et al., 2022) and the availability of fruiting plants in the landscape (Saracco et al., 2004; Guerra et al., 2017). The degree of frugivory varies greatly among the nearly 40% of Neotropical birds that consume fruit, with many complementing fruits with the consumption of invertebrates and other food resources (Wilman et al., 2014; Gomes et al., 2022). The low recovery rate of OTUs per dropping sample is very likely an accurate reflection of the low fruit availability in our study area. Phenological data collected in our study area show a low number of fruits available in the landscape (Moreira, 2024), as is reflected by only 15% of the 417 droppings collected containing seeds. Our metabarcoding protocol prioritized sample number over sequencing depth, likely reducing OTU recovery. OTUs per sample decreased from five to two after filtering, mostly due to low read counts. Increasing reads per sample could reduce data loss, although most samples with >3 OTUs still corresponded to only two plant taxa after assignment. These findings highlight the importance of accounting for landscape-scale fruit availability when interpreting metabarcoding results in ecological studies of frugivory.

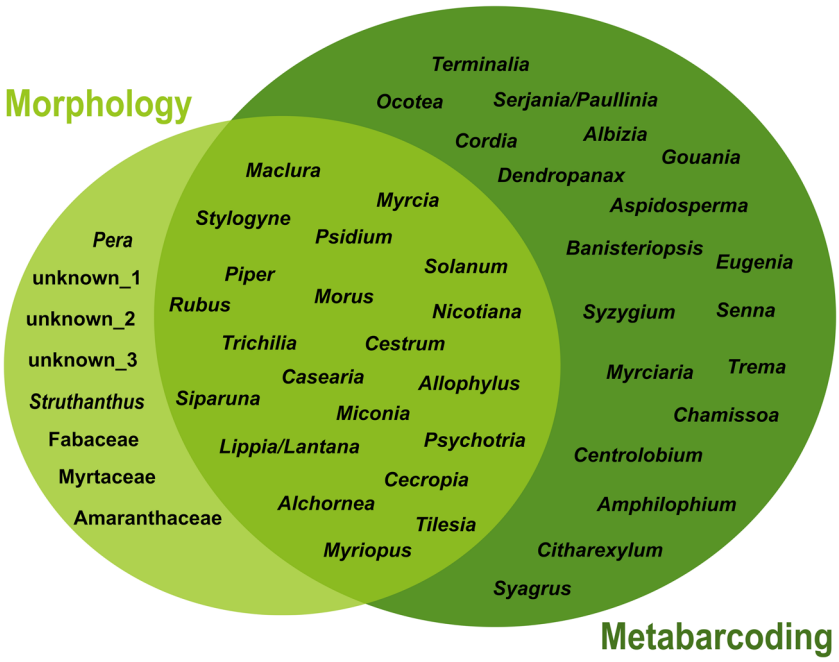

FIGURE 5 Venn diagram depicting unique and shared genera and/or morphotypes identified using metabarcoding-based and seed-based detections.

The incomplete detection of plant taxa by metabarcoding, compared to those identified from seeds present in feces, may be attributed to the relative abundance of diet items and temporal factors. Different plant tissues (i.e., pulp and seeds) likely travel through the digestive system at distinct rates, potentially causing a discrepancy in plant DNA detected using metabarcoding and seeds found in feces (Snider et al., 2022; Vallin, 2024). Differences in detection may be due to variation in the relative abundance of DNA from consumed items or the time elapsed since ingestion. DNA associated with seeds may have been less abundant than more recently consumed plant material from other taxa at the time of sampling, leading to fewer copies being amplified during PCR and ultimately resulting in non-detection of seed-associated taxa (Pawluczyk et al., 2015; Krehenwinkel et al., 2017). Once again, it is possible that taxa detected using seed morphology were retrieved using metabarcoding but ultimately excluded due to low read count. Therefore, the probability of detecting a more complete suite of plants present in bird droppings may be increased by reducing the number of samples pooled during in-house PCR multiplexing, or by increasing the overall number of sequence reads per pool, although it is not guaranteed (Alberdi et al., 2018; Shirazi et al., 2021). Alternatively, secondary plant metabolites can act as PCR inhibitors (Demeke and Jenkins, 2010; Trombley Hall et al., 2013), potentially resulting in a taxon-specific reduction in amplification and detection (Rucińska et al., 2021). A better understanding of how the relative abundance of diet items affects detection and potential taxon-specific biases is crucial in order to improve metabarcoding techniques applied in community-level studies.

The greater diversity of plants identified through metabarcoding highlights the potential of this method to detect fruit consumption and infer potential seed dispersal. Even when seeds are absent from bird droppings, this method can capture DNA traces of seeds that were regurgitated or defecated before they could be detected in the fecal samples (Quintero et al., 2022). Pulp and other fruit structures consumed by birds may stay in the digestive system longer than seeds, allowing for detection using metabarcoding for a longer period (Simmons et al., 2018; García et al., 2024). A recent study suggests that the detection of *Psidium guajava* L. (Myrtaceae) DNA in *Turdus leucomelas* (Turdidae) droppings is influenced more by time since collection than by the quantity of fruit consumed, although further research using other food items and bird species is needed (De Godoy et al., unpublished manuscript). The use of metabarcoding to detect fruit consumption could therefore increase the sampling period window from approximately 20 minutes, the average time required for seeds to pass through a bird's digestive system (Gasperin and Pizo, 2012), to up to 48 hours following consumption (De Godoy et al., unpublished manuscript). While DNA metabarcoding may extend the detection period for fruit consumption, further research is needed to better understand how the time since ingestion and the type of plant tissue consumed affect DNA abundance and detection success.

Several plant genera detected through metabarcoding do not produce fleshy fruits typically consumed by birds (Figure 4), suggesting that some of the detected DNA may originate from the consumption of non-fruit plant parts. These cases likely reflect instances where birds ingested material other than seeds and fruits (e.g., flowers, leaves) not identifiable through seed morphology alone. For example, it is unlikely that birds are consuming dry fruits, such as the legumes produced by *Albizia* Durazz. spp. and *Senna* Mill. spp. (Fabaceae) (Figure 5). Because metabarcoding does not allow the identification of the specific plant part from which the DNA originated, the detected DNA may therefore come from various plant materials, such as leaves or flowers, rather than exclusively from consumed fruits (Ando et al., 2018, 2020; Pappalardo et al., 2021). The detection of DNA from plant genera not typically consumed by birds may be a consequence of accidental ingestion of flowers while foraging for insects or from secondary detection of plants ingested by insect prey (Tercel et al., 2021; García et al., 2024). The accidental consumption of uncommon diet items by birds was also pointed out by Schumm et al. (2023) using next-generation sequencing. These findings underscore the need for caution when interpreting metabarcoding data in dietary studies, particularly in distinguishing between intentional fruit consumption and incidental ingestion.

Metabarcoding is a powerful tool for studying bird diets and seed dispersal, capable of detecting a broader diversity of plant taxa, including cryptic interactions and morphologically unidentifiable species, even when seeds are absent from feces. However, its application in seed dispersal studies requires careful interpretation and a thorough understanding of local natural history (Simmons et al., 2018). In our study, the limited resolution of the mini-barcode used, along with gaps in reference databases, made it essential to incorporate complementary information, such as species distribution data, to refine taxonomic assignments. This need for contextual data has been echoed across metabarcoding studies (Pappalardo et al., 2021; Van Leeuwen and Michaux, 2023; Saranholi et al., 2024). Due to the potential detection of non-fruit plant materials and secondary DNA sources, we encourage complementing metabarcoding with traditional methods like morphological seed identification or direct observations to improve ecological interpretation. To maximize its utility, continued efforts to expand reference libraries and adopt more informative barcodes are essential, especially in hyper-diverse systems like the Neotropics.

AUTHOR CONTRIBUTIONS

C.I.M. and M.C.C. conceived the analysis and designed the field collection methods. B.H.S., C.I.M., and V.M.G. designed laboratory and metabarcoding methods. C.I.M. collected field data. C.I.M. and V.M.G. conducted laboratory work. C.I.M. led data presentation with input from B.H.S., V.M.G., and M.C.C. C.I.M. led the writing of the paper, with contributions from B.H.S. and feedback from M.C.C. All authors discussed

the results, commented on the manuscript, and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The full DNA extraction protocol can be found at https://dx.doi.org/10.17504/protocols.io.8epv527x5v1b/v1 (Motta et al., 2024). Raw sequence data are available in the NCBI BioProject and in the Sequence Read Archive repository under accession number PRJNA1203590. Raw data sets and associated code can be found at https://github.com/carinamotta/metabarcoding-neotropical-bird-feces.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Summary table of seed morphotypes classified based on the morphology of seeds found in feces.

Appendix S2. Summary of plant families and genera detected using DNA metabarcoding of bird feces.

Appendix S3. Overlap of genera detected using metabarcoding and seeds found in droppings.

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