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Evaluating the efficacy of sample collection approaches and DNA metabarcoding for identifying the diversity of plants utilized by nectivorous bats

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17 ABSTRACT

18 In this study, we evaluated the efficacy of sample collection approaches and DNA
19 metabarcoding to identify plants utilized by nectivorous bats. Samples included guano collected
20 from beneath bat roosts and pollen-swabs from bat fur, both of which were subjected to DNA
21 metabarcoding and visual identification of pollen (microscopy) to measure plant diversity. Our
22 objectives were to determine whether DNA metabarcoding could detect likely food plants of
23 nectivorous bats, whether sample types would produce different estimates of plant diversity, and
24 to compare results of DNA metabarcoding to visual identification. Visual identification found
25 that 99% of pollen was from *Agave*, which is thought to be the bats' main food source. The
26 dominant taxon found by metabarcoding was also Agavoideae, but a broader diversity of plant
27 species was also detected, many of which are likely "by-catch" from the broader environment.
28 Metabarcoding outcomes differed between sample types, likely because pollen-swabs measured
29 the plant species visited by bats and guano samples measured all items consumed in the bat's
30 diet, even those that were not pollen or nectar. Overall, metabarcoding is a powerful, high-
31 throughput tool to understand bat ecology and species interactions, but careful analysis of results
32 is necessary to derive accurate ecological conclusions.

33

34 Key Words

35 Diet, diversity, DNA metabarcoding, guano, nectivorous bats, plant barcodes, pollen, *Agave*

37 **Introduction**

38 Identifying the diversity of food resources used by animals is critical for understanding their
39 behavior, physiological requirements, and habitat needs, all of which are important for effective
40 conservation. Traditionally, techniques to understand the composition and diversity of animal
41 diets have relied on direct behavioral observations and visual identification of feces or gut
42 contents (Bjugstad et al. 1970; Holechek et al. 1982). While providing valuable contributions
43 toward deciphering complex food webs, these techniques are not without limitations (Norbury
44 and Sanson 1992); the feeding behavior of many animal species, particularly those that are
45 nocturnal, is difficult to observe visually, while the visual identification of diet items dissected
46 from fecal samples is painstaking (Ward 1970; Holechek and Gross 1982) and may provide low
47 resolution for identifying the items consumed by an animal. A newer approach to understand
48 animal diet is DNA metabarcoding, which combines high-throughput DNA sequencing, DNA
49 barcodes, and bioinformatic analysis to identify the diversity of species within a sample, even for
50 those diet items that have low representation in fecal droppings (Deagle et al. 2009; Boyer et al.
51 2015).

52 Bats are a group in which visual feeding observations and the identification of the
53 contents of guano (i.e., bat fecal samples) using dissections are particularly limited both in scale
54 and ability to identify the full suite of diet items consumed (Belwood and Fenton 1976; Kunz and
55 Whitaker 1983), particularly for diet items that lack hard remains (e.g., soft-bodied invertebrates;
56 Rabinowitz and Tuttle 1982). For insectivorous bats, knowledge of their diet has been greatly
57 expanded by DNA metabarcoding (Zeale et al. 2011; Vesterinen et al. 2013; Burgar et al. 2014),
58 but the use of metabarcoding has thus far not been well explored for nectivorous bat diet (Voigt
59 et al. 2009). Traditionally, diet analysis of nectivorous bats has relied on microscopic

identification of pollen grains in guano, which requires specialized knowledge of pollen morphology of the regional flora (Voigt et al. 2009). With its speed and potential for greater taxonomic resolution, DNA metabarcoding may be a useful approach to determine the diet of nectivorous bats; however, little is known about the most effective way to conduct DNA metabarcoding studies in nectivorous bats.

In this study, we explored the use of DNA metabarcoding to determine the diet of two species of nectivorous bats native to North America, *Choeronycteris mexicana* (Mexican long-tongued bat; Arroyo-cabrales et al. 1987) and *Leptonycteris yerbabuenae* (lesser long-nosed bat; Cole and Wilson 2006) in Arizona, USA. Both species are migratory; for example, migratory populations of *Leptonycteris yerbabuenae* overwinter in Mexico, migrate northward along the coast of Mexico in spring as they follow the blooming of columnar cacti, spend the summer in the southwestern United States where they form maternity colonies, then migrate southward and inland in the fall as they follow the blooming of *Agave* species (Fleming et al. 1993; Wilkinson and Fleming 1996; Rojas-Martínez et al. 1999; Molina-Freaner and Eguiarte 2003). In the southern part of their range in Mexico, a previous study that conducted visual analysis of guano showed that populations of *Leptonycteris yerbabuenae* consume pollen and nectar of plants from a diverse range of plants, including species in the families Cactaceae, Bombacaceae, and Agavaceae, among others (Stoner et al. 2003). In addition to pollen and nectar, both species have also been observed to consume cactus fruit as well as insects found on fruit and flowers (Stoner et al. 2003). In Arizona, both species are thought to forage primarily on *Agave* during the maternity season, but to our knowledge, no comprehensive investigation of nectivorous bat food resources has been conducted on these two species in Arizona.

We collected guano samples and pollen-swabs of the fur of two species of nectivorous bats and subjected them to both visual analysis of pollen and DNA metabarcoding. Our objectives were to determine whether DNA metabarcoding could detect likely food plant species of nectivorous bats, whether the two types of samples would produce different estimates of plant diversity, and how estimates of plant diversity using DNA metabarcoding would compare to visual identification of pollen isolated from the same samples. Ultimately, this research was designed to understand whether DNA metabarcoding would provide more rapid, refined, and information-rich capabilities for determining trophic interactions, foraging sites, and other related ecological dynamics of bats, whose behavior and diet are challenging to study using conventional techniques.

Materials and Methods

Sample Collection

We obtained all necessary state and federal permits and ethics approvals prior to conducting research. We collected two sample types, guano and pollen-swabs, from *C. mexicana* and *L. yerbabuenae* throughout southeastern Arizona. Sample collection focused on the region of southeastern Arizona where there are large *L. yerbabuenae* maternity roosts and many plant species with bat pollination syndromes (including *Agave* -- *A. chrysantha*, *A. palmeri*, and *A. parryi*) that flower during and following the mid-summer monsoons, making it a prime foraging location for nectivorous bats. Guano was collected by placing new plastic sheeting attached to PVC pipe frames beneath the primary flight corridor of a large *L. yerbabuenae* maternity roost, Pyeatt Cave, and beneath a night roost, Wren Bridge, used by both *C. mexicana* and *L. yerbabuenae* (Fig 1A & B). Small numbers of *C. mexicana* may also occur in Pyeatt Cave. The

guano of nectivorous bats is dropped as a “splat”. We obtained 130 "splat" samples from these sites during four collection periods, totaling ten days, in August and September of 2009 and 2010. We collected the splat samples using sterile wooden sticks to scrape the material from the sheeting surface into sterile 2.0 mL screw-cap tubes filled with Invitrogen™ RNAlater™ Stabilization Solution (ThermoFisher Scientific; Fig 1C & D). The wooden sticks were cut off into the tube before capping to retain as much guano as possible prior to DNA extraction. Because roosts contained both species of nectivorous bats, the identity of bat species that produced a guano sample was unknown.

Pollen-swab samples were collected from 152 *C. mexicana* and *L. yerbabuenae* captured by bat experts at sites throughout southeastern Arizona between August and November of 2010 and 2011. Capture and handling protocol followed the guidelines of the American Society of Mammalogists (Sikes et al. 2011). Collections were made individually using a new, sterile Catch-All™ Sample Collection Swab (Epicentre) moistened with RNAlater™ Stabilization Solution and swiped across each bat’s muzzle and shoulders (whether pollen was visible or not; Fig 1E & F). Swab tips were then clipped off into a sterile, DNA-free 2.0 mL screw-cap tube filled with RNAlater™.

Guano and Pollen-Swab Sample Grouping and Visual Identification of Pollen

Because many samples contained only a small amount of pollen, we pooled samples that were of the same type (guano or pollen-swab), location (nearest town or bridge/cave), and date of sampling. For the pollen-swab samples, we also divided the samples by species (*L. yerbabuenae* or *C. mexicana*), which was unknown for guano samples. Although we collected a similar number of total samples using the two approaches, because the number of samples that could be collected each night varied, the number of pooled sample groups and number of

128 samples within each group varied. For guano samples, a larger number of samples could often be
129 collected in a single night, but the bat species corresponding to each fecal sample was unknown,
130 resulting in a total of 5 pooled samples, each containing between 2 and 40 individual guano
131 samples. For pollen-swab samples, because fewer samples could be collected per night and the
132 bat species was known, the samples were divided into a greater number of pooled samples,
133 resulting in 43 pooled pollen-swab samples, each containing samples from between 1 and 14
134 individual bats (Table 1).

135 We identified four pooled guano samples and four pooled pollen-swab samples for
136 quantification of plant diversity by direct visual identification of pollen using light microscopy.
137 For these pooled samples, half was removed for visual analysis and the other half was reserved
138 for DNA extraction and metabarcoding (see below). The pooled guano samples analyzed by
139 visual analysis were samples 1, 2, 4, and 5 and the pooled pollen-swab samples were 6, 12, 13,
140 and 23. Visual analysis of pollen composition was conducted at the University of Wyoming, with
141 all samples undergoing acetolysis following the standard protocol in Faegri et al. 1989. Samples
142 were initially centrifuged to concentrate pollen in the *RNAlater*TM Stabilization Solution to
143 estimate total pollen volume (to the nearest fraction of a milliliter). A single *Lycopodium* tablet
144 with known concentration (average = 12,542; University of Lund batch number 124961) was
145 added to each sample (N = 10) as a standard of known concentration against which pollen in the
146 samples can be compared to determine total pollen concentration per sample. *Lycopodium* spores
147 were encased in a bicarbonate matrix, dissolved with the addition of 1 mL of 10% hydrochloric
148 acid (which also broke down the matrix of *RNAlater*TM), allowing for the continuation of
149 standard pollen acetolization (Faegri et al. 1989). No further treatment of these samples was
150 required for proper identification of the pollen grains. Samples were counted to a standard sum

of 300 pollen grains or 1000 *Lycopodium* spores. These count values balance the need for a statistically complete evaluation of pollen diversity in each sample (Maher 1963) and provide a reasonable assessment of samples with little to no pollen.

DNA Extraction, Metabarcoding, and Sequencing

DNA was extracted from each guano (N = 130) and pollen-swab (N = 152) sample individually using a modified CTAB extraction protocol (Doyle and Doyle 1987; Lalmangaihi et al. 2015). Genomic DNA concentrations (ng/μl) were determined with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies), then equimolar concentrations were pooled as listed in Table 1.

Library preparation followed the 454 Sequencing System Guidelines for Amplicon Experimental Design (Roche 2011). We employed three plant chloroplast DNA barcode regions, *rbcL*, *psbA-trnH*, and *trnL-F* (Table S1). To enable pooling of the libraries for all samples in a single 454 run, we ordered a unique set of the three plant chloroplast primers for each sample that were tagged with a sample-specific multiple identifier (MID); the MID (1-47; Roche 2009) assigned to each were the same as the pooled sample names as shown in Table 1. To enable sequencing on a Roche 454 GS FLX DNA sequencer, the 5' end of each forward and reverse PCR primer was tagged with the A and B 454 adapter (A: CGTATCGCCTCCCTCGCGCCATCAG, B: CTATGCGCCTTGCCAGCCCGCTCAG), respectively. PCR reactions were conducted in 25 μL volumes consisting of 2 U of Platinum Taq DNA polymerase (Invitrogen), 1× Taq DNA Polymerase PCR Buffer without magnesium [containing Tris HCl (pH 8.4), and KCl], 2.0 mM MgCl₂, 0.2 mM of each dNTP, 1 μM of both forward and reverse primers, and nuclease-free water. PCR temperature cycling conditions were as follows: (I) 3 min at 95 °C, (II) denaturation for 30 s at 95 °C, (III) annealing for 30 s at 55

174 °C, (IV) extension for 30 s at 72 °C, (V) eight repetitions of steps 2–4, and (VI) a final
175 elongation at 72 °C for 5 min. PCR products were quantified, pooled in equimolar ratios, and
176 submitted for next-generation DNA sequencing using a ¼-plate run with on a Roche 454 GS
177 FLX DNA sequencer using titanium chemistry at the University of Florida’s Interdisciplinary
178 Center for Biotechnology Research.

179 *Bioinformatic Analysis*

180 The resulting reads were first processed using the 454 amplicon processing pipeline. The
181 resulting file was demultiplexed by MID and quality filtered using split_libraries.py (QIIME
182 V1.91; Caporaso et al., 2010) with the following settings; minimum sequence length of 170
183 nucleotides, maximum homopolymer size of 8, mean quality score above 25 and allowing for up
184 to 2 primer mismatches. Chimeric sequence filtering was performed on each primer separately
185 using identify_chimeric_seqs.py and filter_fasta.py (QIIME) with the implemented USEARCH
186 de novo algorithm using default settings (Edgar 2010). Taxonomic assignment of sequences was
187 conducted using BLASTN (blastn-2.2.31+; Camacho et al. 2009), with local alignments of query
188 sequences to the NCBI nt database (downloaded 08/25/2017; NCBI Resource Coordinators
189 2017). BLAST searches were run in parallel using GNU parallel (Tange 2011), with a word size
190 of 11 and an *E-value* cutoff of 1E-10. Taxonomic binning was performed on the top 10% of
191 BLAST hits to a query sequence by bit score using Megan6 CE v6.11.4 with the naive lowest
192 common ancestor algorithm (Huson et al. 2016) with default settings, except we used a percent
193 identity cutoff of 92%. Megan filters hits by e-values and percent identity and summarizes the
194 taxonomic hits to a query, finding the “lowest common ancestor,” or lowest taxonomic rank
195 common to the hits, such that each read has only one assignment at the most appropriate
196 taxonomic rank. Because we obtained relatively few annotated sequences for some samples, to

maximize the data and number of samples analyzed, we did not filter out singletons. Furthermore, because we obtained relatively few sequences for two of the three markers, we summarized the results using a “total evidence” approach, combining all of the annotations across all markers, such that each annotation, regardless of marker, was treated as a separate identification. To determine whether the results generated using the three types of analysis differed significantly, we used a chi-squared test to analyze whether the relative proportions of Agavoideae vs. other types of taxa were similar between each pair of approaches.

Results

Visual Identification of Pollen

For the visual identification, the mean proportion of *Agave* in the observed pollen from both the guano and pollen-swab samples was 99.6%. In half of the samples, *Agave* pollen was the only type identified. The second most common pollen type identified was the *Yucca* type according to the keys used. However, the keys require mature pollen, and it is possible that some percentage of *Agave* pollen may have been immature; it is therefore possible that pollen grains identified to *Yucca* may have been immature Agavaceae pollen, as the morphologies of these types are fairly similar. Only three other pollen types were identified, including an Anacardiaceae grain (Group 1), *Ambrosia*-type (Group 2), and Malvaceae (Group 6).

DNA Metabarcoding of Pollen

We obtained 60,662 total DNA sequences with amplicon length >170bp, of which 42,950 were *trnL-F*, 14,265 were *psbA-trnH*, and 3,447 were *rbcL*. The mean and median length of these sequences was 349 bp and 322 bp, respectively, which corresponds closely with the expected sizes of *trnL-F* and *psbA-trnH*. Because we recovered relatively small numbers of sequences for

220 both *rbcL* and *psbA-trnH*, the three primers from each sample were combined and analyzed
221 together. When all markers were combined, 52,409 sequences (86%) were from the guano
222 samples and 8,253 (14%) were from the pollen-swab samples. After annotating
223 and filtering the annotations, across all samples, a total of 15,074 sequences were annotated to
224 85 plant taxa. Plant DNA sequences were recovered from four of the five pooled guano samples
225 and 19 of the 43 pooled pollen-swab samples (Table 1). A total of 8,662 sequences were
226 annotated to plants from the guano samples and 6,412 were annotated to plants from the pollen-
227 swab samples. The number of sequences annotated to plants per group ranged from 620 to 3,354
228 for the guano samples and from 1 to 1,912 for the pollen-swab samples. Across all samples, the
229 majority of sequences (8,889) were assigned to *Agave*, *Yucca*, and other taxa in Agavoideae,
230 which were all combined in figures under “Agavoideae” (Fig. 2).

231 When the metabarcoding results for all guano samples were combined into one analysis
232 (Fig 2a), 32.6% of the sequences were from Agavoideae, 40.8% were from the genus *Wolffia*,
233 7% were from the order Zingiberales, 5.7% was from the genus *Musa*, 3.2% were from the
234 subclass Petrosaviidae (which contains the order Asparagales), 2.9% were from the
235 Cupressaceae family, 1.8% were from Lemnoideae, 1% were from the genus *Pisium*, and a
236 number of other taxa were recovered at lower sequence depths (i.e., <1% of the total sequences
237 recovered from guano), which are combined in the category “other angiosperms” in Fig. 2. The
238 chi-square test comparing the results of visual identification of pollen to those of metabarcoding
239 of fecal samples showed that the relative proportions of Agavaoideae vs. other taxa differed
240 significantly ($\chi^2 = 97.0588$, $p < .00001$). When the guano samples were each analyzed
241 individually, Agavoideae was the main DNA sequence type for two of four guano samples (Fig

2c). The third guano sample was made up primarily of DNA sequences of *Wolffia*, while the fourth guano group was composed of roughly equal proportions of sequences from *Musa* and Zingiberales (Fig 2c).

For the metabarcoding of pollen-swab samples, when the read counts were summed across all samples (Fig. 2b), we obtained a total of 6,412 sequences annotated to plants, 93.6% of which were Agavoideae (Fig. 2b), with all other taxa recovered at frequencies lower than 1%. Agavoideae was the main DNA sequence type found in 17 of the 19 pollen-swab samples (Fig. 2c). The second most common group was the “other angiosperms” group, which is comprised of all of the taxa found at frequencies <1%. Two samples were dominated by “other angiosperms”, but both of these samples produced very few sequences (i.e., <10 sequences per sample). The relative proportions of Agavaoideae vs. other taxa found by metabarcoding of pollen-swab samples did not differ significantly from those found through visual identification of pollen ($\chi^2=3.701$, $p=0.054$), but they differed significantly with those obtained from metabarcoding of fecal samples ($\chi^2=80.2718$, $p < .00001$).

Discussion

In this study, the first objective was to determine whether DNA metabarcoding could detect likely foods utilized by nectivorous bats. DNA metabarcoding successfully identified plant DNA sequences from both guano and pollen-swab samples, and the majority of sequences recovered were from Agavoideae, which corresponds to the most common food source utilized by nectivorous bats in this region based on behavioral observations. These dominant species recovered by metabarcoding also corresponds to that found using morphological identification of the pollen in the samples, which found that 99.6% of pollen from *C. mexicana* and *L. yerbabuena* guano and pollen-swab samples was *Agave*. These results suggest that

265 metabarcoding analysis of both pollen-swab and guano samples is able to detect the main food
266 source utilized by nectivorous bats.

267 Contrary to the results of visual identification of pollen, metabarcoding also found
268 sequences from a diverse range of plant species in the samples, indicating that metabarcoding is
269 a very sensitive approach to determine the plants with which nectivorous bats interact. Some of
270 these taxa may be utilized as food sources by bats; for example, we detected sequences of taxa in
271 the genus *Musa* and the order Zingiberales, many of which have been shown to be visited by
272 nectar bats in other regions (Liu et al. 2002; Fleming et al. 2009; Pedrozo et al. 2018). Although
273 neither of these groups is native to the study area, both are commonly cultivated for food or as
274 ornamental plants and could be utilized occasionally by bats as a food source.

275 Many of the additional plant species found in the metabarcoding results are not bat-
276 pollinated and are unlikely to be utilized by bats as food resources; instead, they may occur in the
277 sample from incidental transfer or pollen by-catch from the broader network of species
278 interactions within the community. For example, metabarcoding of guano samples found that
279 many sequences in two guano samples matched with high similarity to the small, freshwater,
280 aquatic taxa *Wolffia* and Lemnoideae (Araceae). Although *Wolffia* are not present in the region,
281 multiple species of two closely related genera in the Lemnoideae subfamily, *Lemna* and
282 *Spirodela*, are present. These could have arisen in the sample through incidental transfer from
283 bats drinking water from freshwater ponds, and indeed, these plants are thought to be dispersed
284 by animals transporting vegetative tissue and seeds (Keddy 1976). In addition, metabarcoding of
285 one guano sample showed a large proportion of sequences from the Cupressaceae, which are
286 wind-pollinated and unlikely to be a food source for bats. Given that Pinyon-juniper forests are
287 common in the region, *Juniperus* pollen may have been deposited by the wind onto collecting

surfaces, into the nectary wells or the stigma of the flowers visited by the bats, or onto the bats themselves and then consumed through grooming. Other small numbers of sequences may have arisen in the sample by incidental transfer from the broader network of species interactions within the plant-pollinator community.

The other goals of the study were to determine whether the two types of samples would produce different estimates of plant diversity, and how estimates of plant diversity using DNA metabarcoding would compare to visual identification of pollen isolated from the same samples. Although the results of visual analysis of both types of samples and metabarcoding of pollen-swab samples were highly similar, the results of metabarcoding of guano samples differed dramatically. Visual identification of pollen in both sample types found that 99% was Agavoideae, and results of metabarcoding of pollen-swab samples showed a comparable proportion of sequences from Agavoideae (93.6%). In contrast, results of metabarcoding of guano samples were significantly different from visual analysis, showing fewer sequences from the Agavoideae (32.6%), and a greater number from taxa such *Wolffia* (40.8%), Zingiberales (7%), *Musa* (5.7%), etc. However, despite the proportions of reads differing across the different types of analysis, it should be noted that Agavoideae sequences were consistently recovered in almost all samples for each of the three types of analysis, indicating that it is an important component of the diet of these bats.

These differences in the estimates of diversity among the types of samples and analyses are likely because they are measuring slightly different things. Visual identification of pollen in both types of samples measured only pollen, whereas metabarcoding measures any type of DNA present in the sample. Results of metabarcoding of the pollen-swab samples were more similar to visual analysis, likely because the sample was composed primarily of pollen and contained very

little other plant DNA. Metabarcoding of guano samples demonstrated larger differences relative to the results of visual analysis, likely because the samples may contain anything consumed by the bats, including pollen, nectar, or any other items such as fruit, leaf tissue, or insects. Because we used chloroplast genes for metabarcoding, if the bats consumed foods such as fruit (i.e., *Musa*, or bananas) or leaf tissue (i.e., the small freshwater aquatic plants such as *Wolffia* or Lemnoideae) that have higher densities of chloroplasts than pollen/nectar, these items may be disproportionately represented in metabarcoding results. Because relative proportions of sequences per diet item in our study may be biased according to the type of tissue consumed, data from guano samples should therefore not be used to determine the relative abundance of food items in the diet. Overall, these results indicate that sample collection techniques could be tailored depending on the question, with pollen-swab samples used to understand what plant species the bats are visiting and guano samples used to understand other potential aspects of bat diet and ecology.

It is also worth noting that the efficiency and ease of collecting and working with the two types of samples differed. Collecting pollen-swab samples involved capturing the bats to remove the pollen from their muzzles, requiring significant animal-handling expertise and potentially causing stress to the bats; however, this also enabled visual identification of the species of bat from which the sample originated, thereby increasing resolution. Pollen-swab samples were also generally very small in volume, contained very little DNA, and produced relatively few sequences per sample; however, this may partially have been an artifact of the smaller average number of individual samples pooled together for pollen-swab samples (Table 1). The data produced by pollen-swab samples were generally of higher quality, as most of the sequences produced from pollen-swab samples were annotated to plants. In contrast, guano samples were

much easier to collect, as it simply required collecting guano samples from plastic sheeting placed under roots or flyways, which caused little to no disturbance or stress to the bats. One drawback of guano samples is that we were unable to visually determine the species of bat associated with each sample; however, if each fecal sample is analyzed individually, then DNA sequencing of loci to distinguish bat species could be employed to obtain this information (Swift et al. 2018). Guano samples generally were also of larger volume, produced a greater quantity of DNA, and provided more sequences per sample than pollen-swab samples; however, only a small proportion (17%) of the 52,409 DNA sequences were annotated to plants, with the remaining sequences annotated to bacteria, fungi, and a large proportion of sequences not able to be annotated. The guano samples also may have contained a variety of tissue types, which may have biased the relative proportion of sequences recovered for the diet items. These relative strengths and drawbacks should be considered carefully when choosing sampling strategies for future studies.

Strengths of DNA metabarcoding include the ability to process more samples, faster, than visual identification of pollen. It is more sensitive than visual identification of pollen and able to recover information about diversity from unidentifiable samples (i.e., digested fruit pulp or leaf tissue in fecal samples). Although metabarcoding detected a wider range of taxa in the present study than visual analysis, one limitation of metabarcoding is that its power to detect taxa may be limited somewhat by low taxonomic resolution; for example, many taxa were identified only to the level of subfamily or family and this low resolution could have led to some false positives and could explain some of the unexpected taxa detected in this study. The low taxonomic resolution observed in the metabarcoding data may be the result of issues with plant DNA barcodes, which frequently have low levels of variability and often lack the power to resolve

DNA sequences to the species level (Fahner et al. 2016; Hollingsworth et al. 2016). Low taxonomic resolution in metabarcoding may also arise from problems related to DNA sequence databases, such as low accuracy of species identifications or poor representation of the taxa of a region; however, this was less likely to be a factor affecting our study, as the plant species in this region are generally well represented in NCBI. Low taxonomic resolution, however, is not unique to metabarcoding, as visual analysis of pollen is similarly limited in its ability to provide taxonomic resolution for many taxa.

In contrast to visual analysis, which provides a quantitative estimate of the relative proportion of different types of pollen in a sample, another weakness of DNA metabarcoding is that it may not provide an accurate estimate of relative abundance of items in the diet. Previous studies have shown that the proportion of sequence reads corresponded only weakly to the relative abundance of items in samples of known composition (Bell et al. 2018). Similarly, we found a great deal of variability in the relative proportions of diet items recovered among the different approaches used in the study, suggesting that at least some of them do not accurately reflect the relative abundance of diet items.

For future studies, we would recommend employing individual-level samples in analyses and making use of DNA sequencing technologies that produce a greater number of sequences (i.e., Illumina MiSeq), as this will provide even greater resolution of nectivorous bat diet. To detect potential contamination that may be introduced during sample collection (e.g., wind-borne pollen or plant debris that may contaminate samples when collecting guano from plastic sheeting) or during laboratory processing, we recommend that future studies follow the sample processing protocols and negative controls outlined in detail in Swift et al. (2018). To ensure good identification and coverage of regional taxa in DNA sequence databases, we also

recommend that researchers simply develop DNA barcoding databases of the potential taxa in their samples. Finally, given that many of the plant species identified by DNA metabarcoding in this study were not likely utilized as food resources by bats and instead may result from incidental transfer or pollen by-catch, we emphasize that careful analysis and interpretation of results is necessary to derive accurate and robust ecological conclusions. Overall, the results of this study indicate that metabarcoding, particularly of fecal samples, is a rapid, high-throughput, and efficient approach to understand nectivorous the diet and species interactions of nectivorous bats.

Data availability

Raw sequencing data can be downloaded from both https://github.com/Kenizzer/Nectar_bat_metabarcoding and the NCBI Sequence Read Archive under BioProject ID PRJNA492812. Instructions and code for sequence processing and data analysis is provided at https://github.com/Kenizzer/Nectar_bat_metabarcoding.

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References

Arroyo-cabral, J., Hollander, R.R., and Jones, J.K. 1987. *Choeronycteris mexicana*. Mamm. Biol. (291): 1–5. doi:10.2307/3503823.

Bell, K.L., Burgess, K.S., Botsch, J.C., Dobbs, E.K., Read, T.D., and Brosi, B.J. 2018. Quantitative and qualitative assessment of pollen DNA metabarcoding using constructed species mixtures. Mol. Ecol. doi:10.1111/mec.14840.

Belwood, J.J., and Fenton, M.B. 1976. Variation in the diet of *Myotis lucifugus* (Chiroptera: Vespertilionidae). Can. J. Zool. **54**(10): 1674–1678. doi:10.1139/z76-194.

Bjugstad, A.J., Crawford, H.S., and Neal, D.L. 1970. Determining forage consumption by direct observation of domestic grazing animals. In Range and Wildlife Habitat Evaluation. US Forest Service Miscellaneous Publications, Washington, D.C. pp. 101–104.

Boyer, S., Cruickshank, R.H., and Wratten, S.D. 2015. Faeces of generalist predators as “biodiversity capsules”: A new tool for biodiversity assessment in remote and inaccessible habitats. Food Webs **3**: 1–6. doi:10.1016/j.fooweb.2015.02.001.

Burgar, J.M., Murray, D.C., Craig, M.D., Haile, J., Houston, J., Stokes, V., and Bunce, M. 2014. Who’s for dinner? High-throughput sequencing reveals bat dietary differentiation in a biodiversity hotspot where prey taxonomy is largely undescribed. Mol. Ecol. **23**(15): 3605–

- 426 3617. doi:10.1111/mec.12531.
- 427 Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., and Madden,
428 T.L. 2009. BLAST+: architecture and applications. *BMC Bioinformatics* **10**(1): 421.
429 doi:10.1186/1471-2105-10-421.
- 430 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K.,
431 Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights,
432 D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M.,
433 Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T.,
434 Zaneveld, J., and Knight, R. 2010. QIIME allows analysis of high- throughput community
435 sequencing data Intensity normalization improves color calling in SOLiD sequencing. *Nat.*
436 *Methods* **7**(5): 335–336. doi:10.1038/nmeth0510-335.
- 437 Cole, F.R., and Wilson, D.E. 2006. *Leptoncyteris yerbabuenae*. *Mamm. Species* (797): 1–7.
438 doi:10.1644/797.1.
- 439 Coordinators, N.R. 2017. Database Resources of the National Center for Biotechnology
440 Information. *Nucleic Acids Res.* **45**(D1): D12–D17. doi:10.1093/nar/gkw1071.
- 441 Deagle, B.E., Kirkwood, R., and Jarman, S.N. 2009. Analysis of Australian fur seal diet by
442 pyrosequencing prey DNA in faeces. *Mol. Ecol.* **18**(9): 2022–2038. doi:10.1111/j.1365-
443 294X.2009.04158.x.
- 444 Doyle, J., and Doyle, J. 1987. A rapid isolation procedure for small quantities of fresh tissue.
445 *Phytochem. Bull.* **19**: 11–15.
- 446 Edgar, R.C. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*
447 **26**(19): 2460–2461. doi:10.1093/bioinformatics/btq461.
- 448 Faegri, K., Kaland, P.E., and Kzywinski, K. 1989. *Textbook of Pollen Analysis*. 4th edition.

- 449 Wiley, New York.
- 450 Fahner, N.A., Shokralla, S., Baird, D.J., and Hajibabaei, M. 2016. Large-Scale Monitoring of
451 Plants through Environmental DNA Metabarcoding of Soil: Recovery, Resolution, and
452 Annotation of Four DNA Markers. PLoS One **11**(6): e0157505.
453 doi:10.1371/journal.pone.0157505.
- 454 Fay, M.F., Swensen, S.M., and Chase, M.W. 1997. Taxonomic Affinities of *Medusagyne*
455 *oppositifolia* (Medusagynaceae). Kew Bull. **52**(1): 111–120. doi:10.2307/4117844.
- 456 Fleming, T.H., Geiselman, C., and Kress, W.J. 2009. The evolution of bat pollination: A
457 phylogenetic perspective. Ann. Bot. **104**(6): 1017–1043. doi:10.1093/aob/mcp197.
- 458 Fleming, T.H., Nuñez, R.A., and Sternberg, L. da S.L. 1993. Seasonal changes in the diets of
459 migrant and non-migrant nectarivorous bats as revealed by carbon stable isotope analysis.
460 Oecologia **94**(1): 72–75. doi:10.1007/BF00317304.
- 461 Holechek, J.L., and Gross, B. 1982. Training Needed for Quantifying Simulated Diets from
462 Fragmented Range Plants. J. Range Manag. **35**(5): 644–647. doi:10.2307/3898655.
- 463 Holechek, J.L., Vavra, M., Pieper, R.D., Journal, S., May, N., Holechek, J.L., Vavra, M., and
464 Pieper, R.E.X.D. 1982. Botanical Composition Determination of Range Herbivore Diets : A
465 Review. J. Range Manag. **35**(3): 309–315.
- 466 Hollingsworth, P.M., Li, D.-Z., van der Bank, M., and Twyford, A.D. 2016. Telling plant species
467 apart with DNA: from barcodes to genomes. Philos. Trans. R. Soc. B Biol. Sci. **371**(1702):
468 20150338. doi:10.1098/rstb.2015.0338.
- 469 Huson, D.H., Beier, S., Flade, I., Górská, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.-J., and
470 Tappu, R. 2016. MEGAN Community Edition - Interactive Exploration and Analysis of
471 Large-Scale Microbiome Sequencing Data. PLOS Comput. Biol. **12**(6): e1004957.

- doi:10.1371/journal.pcbi.1004957.
- Keddy, P.L. 1976. Lakes as islands: the distributional ecology of two aquatic plants, *Lemna minor* L. and *L. trisulca* L. *Ecology* **57**(2): 353–359.
- Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A., and Janzen, D.H. 2005. Use of DNA barcodes to identify flowering plants. *Proc. Natl. Acad. Sci.* **102**(23): 8369–8374.
doi:10.1073/pnas.0503123102.
- Kunz, T.H., and Whitaker, J.O.J. 1983. An evaluation of fecal analysis for determining food habits of insectivorous bats. *Can. J. Zool.* **61**(6): 1317–1321.
- Lalmangaihi, R., Ghatak, S., Laha, R., Gurusubramanian, G., and Kumar, N.S. 2015. Protocol for Optimal Quality and Quantity Pollen DNA Isolation from Honey Samples. *J. Biomol. Tech.* **25**(4): 92–95. doi:10.7171/jbt.14-2504-001.
- Liu, A., Li, D., Wang, H., and Kress, W.J. 2002. Ornithophilous and Chiropterophilous Pollination in *Musa itinerans* (Musaceae), a Pioneer Species in Tropical Rain Forests of Yunnan , Southwestern China. *Biotropica* **34**(2): 254–260.
- Maher, L.J. 1963. Pollen Analyses of Surface Materials from the Southern San Juan Mountains, Colorado. *Geol. Soc. Am. Bull.* **74**(12): 1485–1503.
- Molina-Freaner, F., and Eguiarte, L.E. 2003. The pollination biology of two paniculate agaves (Agavaceae) from Northwestern Mexico: contrasting roles of bats as pollinators. *Am. J. Bot.* **90**(7): 1016–1024.
- Norbury, G.L., and Sanson, G.D. 1992. Problems with measuring diet selection of terrestrial, mammalian herbivores. *Aust. J. Ecol.* **17**(1): 1–7. doi:10.1111/j.1442-9993.1992.tb00774.x.
- Pedrozo, A.R., Gomes, L.A.C., and Uieda, W. 2018. Feeding behavior and activity period of three Neotropical bat species (Chiroptera: Phyllostomidae) on *Musa paradisiaca*

- 495 inflorescences (Zingiberales: Musaceae). *Iheringia. Série Zool.* **108**: 1–8. doi:10.1590/1678-
496 4766e2018022.
- 497 Rabinowitz, A., and Tuttle, M.D. 1982. A test of the validity of two currently used methods of
498 determining bat prey preferences. *Acta Theriol.* **27**(21): 13–24.
- 499 Rojas-Martínez, A., Valiente-Banuet, A., del Coro Arizmendi, M., Alcántara-Eguren, A., and
500 Arita, H.T. 1999. Seasonal distribution of the long-nosed bat (*Leptonycteris curasoae*) in
501 North America: does a generalized migration pattern really exist? *J. Biogeogr.* **26**(5): 1065–
502 1077. doi:10.1046/j.1365-2699.1999.00354.x.
- 503 Sang, T., Crawford, D.J., and Stuessy, T.F. 1997. Chloroplast DNA phylogeny, reticulate
504 evolution, and biogeography of *Paeonia* (Paeoniaceae). *Am. J. Bot.* **84**(8): 1120–1136.
505 doi:10.2307/2446155.
- 506 Sikes, R.S., Gannon, W.L., and The Animal Care and Use Committee of the American Society
507 Mammalogists 2011. Guidelines of the American Society of Mammalogists for the use of
508 wild mammals in research and education: *J. Mammal.* **92**(1): 235–253.
509 doi:10.1093/jmammal/gyw078.
- 510 Stoner, K.E., O.-Salazar, K.A., R.-Ferdandez, R.C., and Quesada, M. 2003. Population
511 dynamics, reproduction, and diet of the lesser long-nosed bat (*Leptonycteris curasoae*) in
512 Jalisco, Mexico: implications for conservation. *Biodivers. Conserv.* **12**: 357–373.
513 doi:10.1023/A:1021963819751.
- 514 Swift, J.F., Lance, R.F., Guan, X., Britzke, E.R., Lindsay, D.L., and Edwards, C.E. 2018.
515 Multifaceted DNA metabarcoding: Validation of a noninvasive, next-generation approach
516 to studying bat populations. *Evol. Appl.* **11**(7): 1120–1138. doi:10.1111/eva.12644.
- 517 Taberlet, P., Coissac, E., Pompanon, F., Gielly, L., Miquel, C., Valentini, A., Vermat, T.,

- 518 Corthier, G., Brochmann, C., and Willerslev, E. 2007. Power and limitations of the
519 chloroplast trnL (UAA) intron for plant DNA barcoding. *Nucleic Acids Res.* **35**(3).
520 doi:10.1093/nar/gkl938.
- 521 Tange, O. 2011. GNU Parallel: the command-line power tool. ;login USENIX Mag. **36**(1): 42–
522 47. doi:10.5281/zenodo.16303.
- 523 Vesterinen, E.J., Lilley, T., Laine, V.N., and Wahlberg, N. 2013. Next Generation Sequencing of
524 Fecal DNA Reveals the Dietary Diversity of the Widespread Insectivorous Predator
525 Daubenton's Bat (*Myotis daubentonii*) in Southwestern Finland. *PLoS One* **8**(11): e82168.
526 doi:10.1371/journal.pone.0082168.
- 527 Voigt, C.C., Kelm, D.H., Bradley, B.J., and Ortmann, S. 2009. Dietary Analysis of Plant-visiting
528 Bats. *In* *Ecological and Behavioral Methods for the Study of Bats*, 2nd ed. *Edited by* T.H.
529 Kunz and S. Parsons. John Hopkins University Press, Baltimore. pp. 593–609.
- 530 Ward, A.L. 1970. Stomach content and fecal analysis. *In* *Range and Wildlife Habitat Evaluation*.
531 US Forest Service Miscellaneous Publications, Washington, D.C. pp. 146–158.
- 532 Wilkinson, G.S., and Fleming, T.H. 1996. Migration and evolution of lesser long-nosed bats
533 *Leptonycteris curasoae*, inferred from mitochondrial DNA. *Mol. Ecol.* **5**(3): 329–339.
534 doi:10.1111/j.1365-294X.1996.tb00324.x.
- 535 Zeale, M.R.K., Butlin, R.K., Barker, G.L.A., Lees, D.C., and Jones, G. 2011. Taxon-specific
536 PCR for DNA barcoding arthropod prey in bat faeces. *Mol. Ecol. Resour.* **11**(2): 236–244.
537 doi:10.1111/j.1755-0998.2010.02920.x.
- 538
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Supplemental materials legends

Figure S1. The diversity of plant taxa found for the three DNA regions used to conduct metabarcoding analysis of guano (G) and pollen-swab samples (S). Stacked bar graphs represent the proportion of reads by plant taxon in each sample group.

Table S1. Information on primers used in the study. To enable sequencing on a Roche 454 GX FLX, Forward primers were tagged on the 5' end with the 454 Adaptor A (CGTATCGCCTCCCTCGCGCCATCAG). Reverse primers were tagged on the 5' end with the 454 Adaptor B CTATGCGCCTTGCCAGCCCGCTCAG. For each primer, we ordered the same primer but plus a group-specific MID.

Table S2. Number of sequencing reads and annotated sequences per sample and marker, including the total, and mean in parentheses.

Table 1. Sample collection information, including the type of sample, collection date, number of samples collected per group, and the species represented.

Pooled sample Group and MID	Sampling Location	Sampling Date(s)	Sample Type	N	Species	Visual pollen analysis?	Produced Plant Sequences?
1	Pyeatt Cave	SEP 10-11 2009	Guano	30	both		
2	Wren Bridge	SEP 09-12 2009	Guano	26	both	Yes	Yes
3	Wren Bridge	AUG 30 2010 - SEP 03 2010	Guano	2	both	Yes	Yes
4	Wren Bridge	SEP 03 2010	Guano	32	both	Yes	Yes
5	Pyeatt Cave	SEP 04 2010	Guano	40	both	Yes	Yes
6	Arivaca	AUG 09-16 2010	Swab	6	<i>L. yerbabuenae</i>	Yes	Yes
7	Arivaca	AUG 10-16 2010	Swab	3	<i>C. mexicana</i>		Yes
8	Bisbee	AUG 16 2010	Swab	3	<i>L. yerbabuenae</i>		Yes
9	Wren Bridge	AUG 30 2010	Swab	11	<i>L. yerbabuenae</i>		Yes
10	Ramsey Canyon	AUG 31 2010	Swab	2	<i>C. mexicana</i>		Yes
11	Ramsey Canyon	AUG 31 2010	Swab	7	<i>L. yerbabuenae</i>		Yes
12	Nogales	SEP 01 2010	Swab	12	<i>L. yerbabuenae</i>	Yes	Yes
13	Nogales	SEP 01 2010	Swab	4	<i>C. mexicana</i>	Yes	Yes
14	Arivaca	SEP 21 2010	Swab	5	<i>L. yerbabuenae</i>		Yes
15	Arivaca	SEP 21 2010	Swab	1	<i>C. mexicana</i>		
16	Hubbard Residence	SEP 24 2010	Swab	1	<i>L. yerbabuenae</i>		
17	Manderscheid Residence	SEP 29 2010	Swab	1	<i>L. yerbabuenae</i>		
18	Osbrink Residence	OCT 01 2010	Swab	1	<i>L. yerbabuenae</i>		
19	Pedrogrosa Mountains	OCT 04 2010	Swab	1	<i>L. yerbabuenae</i>		
20	Nogales	OCT 06 2010	Swab	13	<i>L. yerbabuenae</i>		Yes
21	Nogales	OCT 06 2010	Swab	5	<i>C. mexicana</i>		Yes
22	Arivaca	OCT 11-18 2010	Swab	14	<i>C. mexicana</i>		Yes
23	Arivaca	OCT 12 2010	Swab	3	<i>L. yerbabuenae</i>	Yes	Yes
24	Tucson	AUG 25 2011	Swab	6	<i>L. yerbabuenae</i>		Yes
25	Green Valley	AUG 29 2011	Swab	2	<i>C. mexicana</i>		Yes
26	Green Valley	AUG 29 2011	Swab	7	<i>L. yerbabuenae</i>		Yes
27	Vail	SEP 01 2011	Swab	2	<i>C. mexicana</i>		Yes
28	Vail	SEP 01 2011	Swab	3	<i>L. yerbabuenae</i>		Yes
29	Tucson	SEP 06 2011	Swab	6	<i>L. yerbabuenae</i>		Yes
30	Oro Valley	SEP 15 2011	Swab	6	<i>L. yerbabuenae</i>		Yes

31	Elfrida	AUG 23 2011	Swab	1	<i>L. yerbabuenae</i>	
32	Wilcox	AUG 31 2011	Swab	1	<i>L. yerbabuenae</i>	
33	Nogales	AUG 22 2011	Swab	1	<i>C. mexicana</i>	
34	Wilcox	AUG 08 2011	Swab	1	<i>L. yerbabuenae</i>	
35	Rio Rico	AUG 15 2011	Swab	1	<i>L. yerbabuenae</i>	
36	Arivaca	AUG 14 2011	Swab	2	<i>C. mexicana</i>	
37	Arivaca	AUG 14 2011	Swab	6	<i>L. yerbabuenae</i>	
						Yes
38	Arivaca	SEP 05 2011	Swab	3	<i>C. mexicana</i>	
						Yes
39	Arivaca	SEP 05 2011	Swab	3	<i>L. yerbabuenae</i>	Yes
40	Arivaca	OCT 03 2011	Swab	1	<i>C. mexicana</i>	
41	Arivaca	OCT 03 2011	Swab	4	<i>L. yerbabuenae</i>	
42	Arivaca	OCT 04 2011	Swab	2	<i>L. yerbabuenae</i>	
43	Pyeatt Cave	NOV 08 2011	Swab	1	<i>L. yerbabuenae</i>	

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Figure 1. Images showing sample collection techniques. A-D) Approach to collect guano samples, including: A) sheeting placed in a flyway in Pyeatt cave, B) Sheeting placed under wren bridge, C) collection of guano sample from sheeting, D) collection tube containing RNAlater, sample, and swab. E-F) Approach to collect pollen-swab samples, where E) *Leptonycteris yerbabuenae*, and F) *Choeronycteris mexicana* were captured and swabbed to remove pollen from their muzzles. Photo credits: A and B, Ronnie Sidner; C, E, and F, Eric Britzke; D, Joel Swift.

Figure 2. The diversity of plant taxa found through metabarcoding analysis of guano (A) and pollen-swab samples (B). Pie charts depict the number of total reads recovered for each plant taxon, with all samples of a type (i.e., guano or swab) combined (information for slices >1% combined into the category “other angiosperms”). (C) Stacked bar graphs represent the proportion of reads by plant taxon in each pooled sample.

Fig. 1

254x338mm (300 x 300 DPI)

Figure 2.

177x162mm (300 x 300 DPI)