

Assessing Diet and Genotyping Success of Goat Pellet Surveys from 2019 in Glacier National Park

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Recommended citation: Scoresby, S, LM Dose, J Belt, TA Graves. 2025. Assessing Diet and Genotyping Success of Goat Pellet Surveys from 2019 in Glacier National Park. National Park Service, Glacier National Park. 23 pp.

<https://irma.nps.gov/DataStore/Reference/Profile/2311387>

Introduction

Fecal pellets contain genetic information and can be used to identify individuals, their diet, and more. Individual identification can be useful in understanding movements of individuals, developing population estimates, assessing vital rates, genetic diversity and structure, and evaluating trends over time (e.g., Epps et al 2024). Successful genotyping depends on the quality of the sample, which can be influenced by many things including the initial state of the sample, environmental conditions that the pellets were exposed to, and the method of storage.

The diet of mountain goats (*Oreamnos americanus*) is hard to study as they are habitat specialists in some of the most remote and rugged alpine environments (Festa-Bianchet & Côté 2012). These iconic ungulates epitomize the renowned wilderness character of Glacier National Park (GNP), yet the population has declined by 45% since 2008 likely due at least in part to changing climate (Graves et al. 2025). This is well above the International Union for the Conservation of Nature's population decline criterion used to designate a species as vulnerable (IUCN 2012).

Mountain goats are known as dietary generalists employing highly variable diets across systems, but diets have not been identified for GNP. Identifying goat diet composition in GNP can provide the information needed to determine whether this population decline could be related to climate-change induced shifts in forage phenology, diversity, productivity, and plant community structure. Other anthropogenic changes in GNP, such as increases in exotic and invasive plant species, will continue to affect plant community composition (Lesica et al. 1993).

These alterations may reduce optimal forage availability and quality, which could have a deleterious effect on mountain goat survival. To examine these possibilities, we must first ask- What precisely are the mountain goats of Glacier National Park eating?

DNA metabarcoding of fecal pellets provides a cutting-edge, non-invasive method for assessing herbivore diet. Previous studies of mountain goat diet have used methods such as fecal crude protein analysis (Festa-Bianchet & Côté 2012), rumen analysis (Saunders 1955), fecal microhistology (Cobb et al. 2012), and bite-for-bite feeding observations (Daily et al. 1984). Many of these studies have only been able to identify diet components to functional plant type and the methods used are biased towards less digestible diet components such as grasses (McInnis et al. 1983). Metabarcoding of fecal pellets can provide a higher taxonomic resolution than previous methods (Scasta et al. 2019, Stapleton et al. 2022) and open the field for increased participation of volunteers, also called community or citizen scientists.

Here, we use mountain goat fecal pellets to conduct 1) an evaluation of which conditions might influence genotyping success and 2) a preliminary assessment of diet using metabarcoding. This pilot study allowed us to evaluate which pellets to focus on collecting, identify the best storage methods, explore approaches for analyzing metabarcoding data, examine the utility of metabarcoding for acquiring taxonomically specific diet information, and to identify genetic sequences that could not be resolved to genus or species levels using these methods. We examined the frequency of occurrence and relative diet composition of identified forbs, shrubs, graminoids, mosses, and trees in mountain goat diets.

Methods

Sample collection

We conducted a pilot study to test the best methods for collecting fecal pellets in Glacier National Park. Goals for our study included developing and testing approaches for training citizen scientists, comparing success rates of genotyping individual goats from pellets using dried, frozen, or ethanol-based preservation methods, and improving our understanding of the feasibility and logistics required to obtain pellets. We address a subset of those goals here. Scientists and trained citizen scientists collected pellets using protocols in Appendix A. After conducting a few initial trainings, Glacier National Park's Citizen Science program developed a training video to improve accessibility and flexibility for training.

Samples were collected by 21 sets of searchers from June 5th through October 15th, 2019 ($n=168$). Of these, 110 were sent to Oregon State University for genotyping, and a subsequent subset of 52 pellet samples that had been successfully genotyped as goats (versus bighorn sheep, deer, or elk) were sent to Jonah Ventures laboratory in Boulder, Colorado, for diet metabarcoding. The pellets sent for metabarcoding were collected from 38 locations in the park and preserved dried ($n=22$), frozen ($n=27$), or in ethanol ($n=3$). Searchers were instructed to collect pellets that were as fresh as possible and estimates of sample freshness ranged <1 day

old ($n=24$), >1 day ($n=28$). Collectors recorded information on pellet age, pellet color, pellet texture, recent weather, and mountain goat presence and activity.

Individual Goat Genotyping Success

We sent 110 of 168 pellet samples to Oregon State University. We did not send pellets that did not have an age estimation ($n=4$), had been recorded as > 1 week old ($n=50$), or were marked as low priority by the collector ($n=4$). Low priority could be indicated when there was high species uncertainty or pellet observations indicated they might be duplicative of another sample or might be unlikely to run. The 110 pellet samples that were sent for analysis were preserved dried ($n=55$), frozen ($n=43$), or in ethanol ($n=13$), and the success of each of these preservation methods was evaluated using logistic regression. One pellet sample was not processed due to mold and is not included in the regression. Though we targeted mountain goats for our sample collection, when examining genotyping success of pellet samples collected, we marked as “Successful” all samples which generated microsatellite genotypes even if they were from a non-target species i.e., mule deer or bighorn sheep because we thought it likely that characteristics of successful genotyping should be consistent across species.

After we fit univariate logistic regression models for all binary variables recorded, we simplified variables within the weather and color categories based on those results. Black and brown pellets had similar effect sizes, which led to us to compare green pellets with black/brown pellets as a binary categorical variable. Recent weather was only intermittently recorded. The incomplete recording of weather observations by collectors led to a very low sample size for any of the weather categories. We therefore combined weather conditions of recent rain or snow into a single covariate for wet weather.

We combined the weather conditions of wet and dry with pellet conditions of moist and squishy representing interactions of the binary conditions to create four new variables: Pellet Moist/Weather Wet, Pellet Squishy/Weather Wet, Pellet Squishy/Weather Dry, and Pellet Moist/Weather Dry.

We ran all possible combinations of the covariates with univariate model p values < 0.15 and used the corrected Akaike information criterion (AICc) for small sample sizes to identify the most parsimonious models and chose models with $AICc < 2$ for interpretation (Hurvich and Tsai 1989).

Diet Analysis

We analyzed 52 fecal pellet samples from 40 genotyped individual mountain goats for plant content using DNA metabarcoding. Metabarcoding analyses were conducted by the Jonah Ventures laboratory using the *c* and *h* trnL primers that target chloroplast in plants (Appendix B; Taberlet et al. 2007). Because limited sequencing and database referents exist currently, we did not use primers targeting the presence of lichen or fungi in the diet (Gueidan & Li 2022, Mitchell et al. 2022).

Each sample was checked for read depth of $\geq 1,000$ reads per sample. To minimize sequencing errors in our analysis, we eliminated exact sequence variants (ESVs) with $<98\%$ match to a library reference sequence (Appendix B). The reads for each remaining ESV were relativized to percentages of each sample to obtain relative read abundance (RRA) (Deagle et al. 2019). Because mountain goats are known to be generalist feeders, we used a moderately low cumulative RRA threshold of .05% for ESVs to include the most likely diet components while excluding possible contaminants such as wind-blown pollen (Fig. 1) (Littleford-Colquhoun et al. 2022, Deagle et al. 2019).

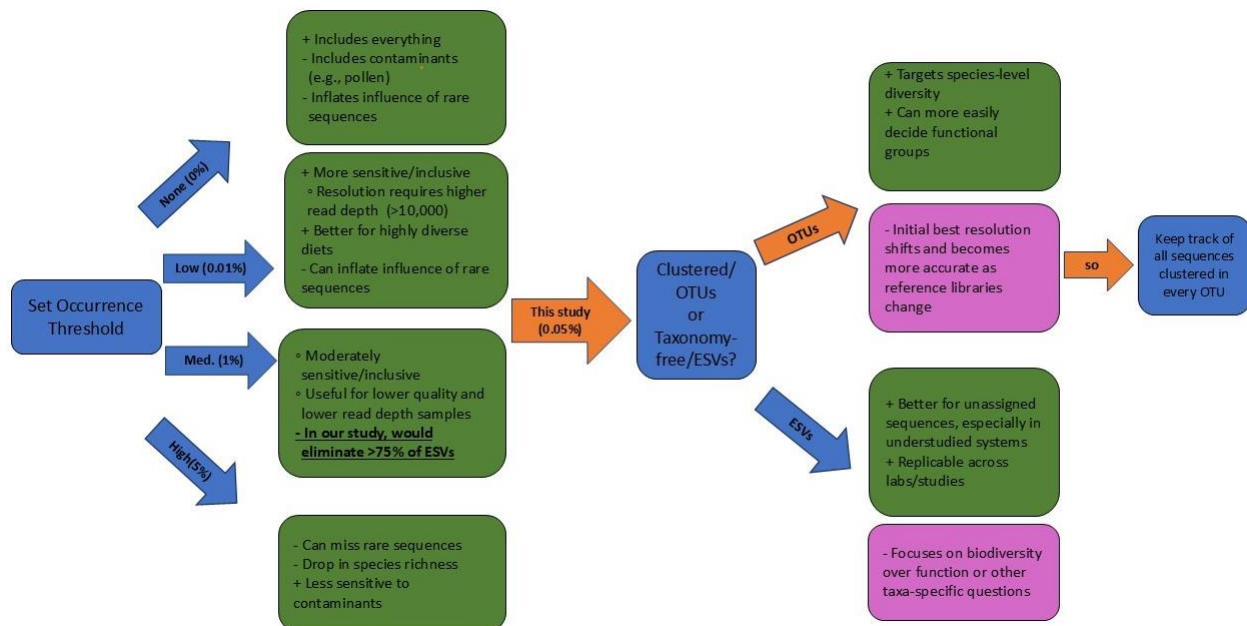


Figure 1. Flowchart of decisions for filtering metabarcode reads. Operational taxonomic units (OTUs) are the finest resolution taxonomic unit for the given data and analysis. Orange arrows reflect the choices for this report.

We compared the list of possible taxa by matching each ESV with a regional flora (Lesica 2002). For species in the reference library not found in the regional flora (which included all bryophytes), we looked in the Montana Natural Heritage Program databases for mosses found in Glacier or Flathead Counties (available at:

https://mtnhp.org/Reports/BOT_Montana_Moss_Checklist_2018_ver20200103.pdf, accessed 12/13/2022) and vascular plants occurring in the state (available at: <http://mtnhp.org/plants/>, accessed 12/13/2022). We combined multiple ESVs into the finest taxonomic resolution

possible—often genera, but occasionally only as specific as subfamily or, when multiple ESVs matched repeated groupings of species in the same family or family groups. The resultant taxonomic resolution showed 98 operational taxonomic units (OTUs) identified to the level of at least family. Once identified to this level, we assigned each OTU to a functional type of Forb, Shrub, Sub-shrub, Moss, Graminoid, Tree, Conifer, or Unknown if not identifiable to a taxonomic resolution that would lend itself to this classification.

Frequency of occurrence (FOO) is the percentage of samples that contain a sequence, or in the case of some OTUs, multiple sequences. Using both RRA and FOO data allows us to obtain an understanding of the relative proportions of diet items while also ensuring that important diet species which may be consumed frequently in small quantities are not overlooked (Deagle et al. 2019, Ando et al. 2020).

Results

Genotyping Success

Of the 110 processed samples, the lab successfully identified 83 samples to species using microsatellite loci. Sixty samples yielded complete or near-complete genotypes of mountain goats, comprising 46 individual goats. The other 23 successful samples yielded microsatellite genotypes from bighorn sheep ($n=22$) or mule deer ($n=1$). The remaining 27 unsuccessful samples included five samples that did not generate enough loci to be included in individual-matching analyses and 22 samples that failed to generate clear microsatellite genotypes.

Samples which were stored in ethanol or frozen had higher success rates than those stored dried (85% ethanol, 84% frozen, 67% dried successful, Table 1, Figure 2).

The model including Age < 1 day + Pellet Moist/Weather Wet + Pellet Cond: Green had the lowest AICc, carrying 15% of the model weight (Table 3). The eight other top models within two AICc contained subsets of these variables or subsets plus covariates for the pellet conditions of squishy and weathered.

Table 1 Logistic regression of preservation methods

(vs. Dried)	Estimate	SE	p
Ethanol	1.012	0.82	0.218
Frozen	0.945	0.50	0.061

Samples less than one day old or green had higher genotyping success rates than those estimated to be over one day old, brown, or black. Samples of pellets which were moist, squishy, weathered, or moist and recently exposed to wet weather had lower success rates. Although samples Age < 1 day is in seven of the eight top models, it has relatively high variance associated with it. This indicates it is less useful for distinguishing between samples likely to have successful genotypes than the other pellet characteristics in top models (pellet moist/wet weather, green color, weathered, and squishy).

Table 2 Estimates from logistic regressions of univariate models based on collector observations

Model	Estimate	SE	p
Age <1 day	0.783	0.49	0.111
Bedding Area	-0.904	0.80	0.258
Defecation Observed	0.511	1.12	0.648
Goat Observed	0.265	0.50	0.595
Pellet Cond: Black	-0.431	0.55	0.436
Pellet Cond: Brown	-0.495	0.45	0.270
Pellet Cond: Dry	-0.362	0.55	0.511
Pellet Cond: Green	0.787	0.51	0.127
Pellet Cond: Hard	-0.131	0.58	0.821
Pellet Cond: Moist	-1.012	0.49	0.041
Pellet Cond: Pliable	-0.595	0.46	0.196
Pellet Cond: Shiny	-0.496	0.46	0.285
Pellet Cond: Smooth	-0.176	0.49	0.722
Pellet Cond: Squishy	-1.019	0.51	0.046
Pellet Cond: Weathered	-1.286	0.68	0.058
Pellet Moist/Weather Dry	0.666	0.81	0.409
Pellet Moist/Weather Wet	-2.438	0.73	0.001
Pellet Squishy/Weather Dry	1.038	1.09	0.340
Pellet Squishy/Weather Wet	-1.709	0.64	0.008
Weather: Dry	0.000	0.46	1.000
Weather: Wet	0.000	0.46	1.000

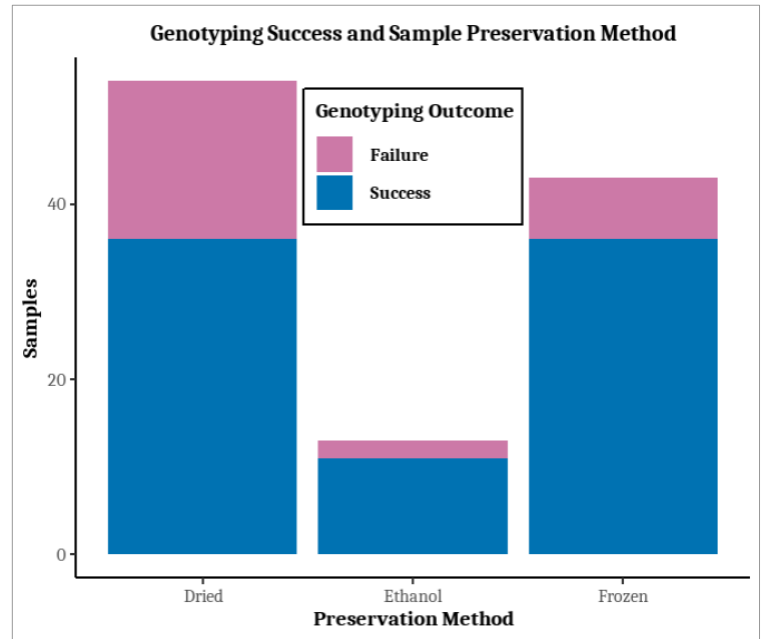


Figure 2 Genotyping success across three methods of sample preservation methods

Table 3. Model comparison for top models considering all combinations of covariates with univariate model p values < 0.15 using the corrected Akaike information criterion (AICc) for small sample sizes

Model	Delta_AICc	AICcWeight
Age < 1 Day + Pellet Moist/Weather Wet + Green	0.00	0.15
Age < 1 Day + Pellet Moist/Weather Wet	0.42	0.13
Age < 1 Day + Pellet Moist/Weather Wet + Weathered	0.43	0.12
Age < 1 Day + Pellet Moist/Weather Wet + Green + Weathered	1.10	0.09
Age < 1 Day + Pellet Moist/Weather Wet + Green + Squishy	1.35	0.08
Pellet Moist/Weather Wet + Green	1.65	0.07
Age < 1 Day + Pellet Moist/Weather Wet + Squishy + Weathered	1.66	0.07
Age < 1 Day + Pellet Moist/Weather Wet + Squishy	1.73	0.06
Age < 1 Day + Pellet Moist/Weather Wet + Green + Squishy + Weathered	2.46	0.05
Pellet Moist/Weather Wet + Green + Weathered	2.63	0.04
Pellet Moist/Weather Wet + Green + Squishy	3.57	0.03
Pellet Moist/Weather Wet + Weathered	3.72	0.02
Pellet Moist/Weather Wet	4.16	0.02
Pellet Moist/Weather Wet + Green + Squishy + Weathered	4.61	0.02
Pellet Moist/Weather Wet + Squishy + Weathered	5.60	0.01
Age < 1 Day + Pellet Squishy/Weather Wet + Moist + Weathered	5.91	0.01
Pellet Moist/Weather Wet + Squishy	6.03	0.01
Age < 1 Day + Pellet Squishy/Weather Wet + Moist	6.94	0.00
Age < 1 Day + Pellet Squishy/Weather Wet + Moist + Green	7.47	0.00
Age < 1 Day + Pellet Squishy/Weather Wet + Moist + Green + Weathered	7.48	0.00
Age < 1 Day + Pellet Squishy/Weather Wet + Weathered	8.34	0.00
Pellet Squishy/Weather Wet + Moist + Green	8.50	0.00
Pellet Squishy/Weather Wet + Moist + Weathered	8.58	0.00
Age < 1 Day + Pellet Squishy/Weather Wet	8.73	0.00
Pellet Squishy/Weather Wet + Moist + Green + Weathered	8.87	0.00
Pellet Squishy/Weather Wet + Weathered	9.30	0.00
Pellet Squishy/Weather Wet + Moist	9.58	0.00
Pellet Squishy/Weather Wet + Green	9.69	0.00
Age < 1 Day + Pellet Squishy/Weather Wet + Green	9.73	0.00
Pellet Squishy/Weather Wet	9.97	0.00
Age < 1 Day + Pellet Squishy/Weather Wet + Green + Weathered	10.07	0.00
Pellet Squishy/Weather Wet + Green + Weathered	10.12	0.00
Age < 1 Day + Squishy + Moist + Weathered	13.61	0.00
Age < 1 Day + Moist + Weathered	14.14	0.00
Age < 1 Day + Squishy + Weathered	14.68	0.00
Age < 1 Day + Squishy + Moist	15.39	0.00
Age < 1 Day + Moist + Green + Squishy + Weathered	15.49	0.00
Age < 1 Day + Moist	15.81	0.00
Age < 1 Day + Squishy	15.92	0.00
Age < 1 Day + Green + Moist + Weathered	15.94	0.00
Age < 1 Day + Moist + Green + Squishy	16.43	0.00

Diet Analysis

Within the 52 metabarcoded samples we found a total of 1,704,710 reads, with all samples having a read depth of >10,000 (mean read depth = 32783, SE=1453). We identified a total of 930 plant exact sequence variants (ESVs) with a mean of 66 (SE=2.3) ESVs per sample. We matched $n=880$ sequences to vascular plants, $n=46$ bryophytes, $n=1$ alga; $n=3$ sequences had no matches. After filtering, 177 ESVs remained with a mean of 41 (SE=1.3) ESVs per sample.

After grouping ESVs with matching taxa we had 98 OTUs, all but one of which was identified to at least the family level. The mean number of OTUs found per sample was 29.7 (SE=0.87), indicating a highly diverse daily diet. Sequences belonging to *Penstemon* spp. occurred in 100% of samples and were found in larger amounts than any other OTU with a mean of 13.8% (SE=1.6) of reads. These 98 OTUs belong to 41 different plant families, with sequences of Plantaginaceae making up 13.9% (SE=1.6), Rosaceae 10.5% (SE=1.9), Pinaceae 10.1% (SE=1.6), Saxifragaceae 9.2% (SE=1.3), Onagraceae 8.7% (SE=1.4), Hypericaceae and Polygonaceae each 8.2% (SE=1.9 and 1.1), and all other families <5% of OTUs. Frequency of occurrence (FOO) differed from the relative abundance of sequences (Figure 4). We found Plantaginaceae (*Penstemon* spp.) in every sample (100% FOO); Polygonaceae and Asteraceae 98%; Rosaceae, Saxifragaceae, and Onagraceae 96%; Pinaceae 92%; and all other families at <90% FOO.

Table 4 Operational Taxonomic Units comprising top 90% of relativized reads

Operational Taxonomic Units by Form and Family					
Form	Family	OTU	Mean	SE	POO
Conifer	Pinaceae		10.1%	1.6	92.3%
			10.1%	1.6	92.3%
		<i>Abies</i> spp.	7.2%	1.5	76.9%
		<i>Pinus contorta</i>	1.4%	0.5	61.5%
		<i>Pinus</i> spp.	1.1%	0.5	50%
Forb	Asteraceae		70.3%	2.9	100%
			5%	1.2	98.1%
		<i>Arnica</i> spp.	1.5%	0.3	86.5%
		<i>Asteraceae 2</i>	1.7%	0.9	78.8%
			8.2%	2.0	86.5%
	Hypericaceae	<i>Hypericum</i> spp.	8.2%	2.0	86.5%
			8.7%	1.4	96.2%
	Onagraceae	<i>Chamerion/Epilobium</i> spp.	8.7%	1.4	96.2%
			1.3%	0.5	69.2%
	Orobanchaceae	<i>Castilleja</i> spp.	1.1%	0.4	69.2%
			13.9%	1.6	100%
	Plantaginaceae	<i>Penstemon</i> spp.	13.8%	1.6	100%
			8.2%	1.1	98.1%
	Polygonaceae	<i>Eriogonum ovalifolium</i>	2.4%	0.4	71.2%
		<i>Eriogonum</i> spp.	2.4%	0.9	69.2%
		<i>Oxyria digyna</i>	3.2%	0.7	76.9%
	Ranunculaceae		2.7%	1.4	53.8%
		<i>Aquilegia coerulea</i>	2.6%	1.4	53.8%
	Rosaceae	<i>Potentilla</i> spp.	7.8%	1.4	94.2%
		<i>Rosoideae</i>	2.7%	0.7	53.8%
	Saxifragaceae		9.2%	1.3	96.2%
		<i>Heuchera</i> spp.	3.4%	0.7	90.4%
		<i>Micranthes</i>	2.9%	0.9	46.2%
		<i>Saxifraga 1</i>	1.1%	0.4	36.5%
	Violaceae		1%	0.7	9.6%
		<i>Viola</i> spp.	1%	0.7	9.6%
Graminoid	Juncaceae		5.8%	1.3	96.2%
			3.9%	1.2	82.7%
		<i>Juncus drummondii</i>	1.4%	1.1	23.1%
	Poaceae	<i>Luzula</i> spp.	2.4%	0.6	76.9%
			1.8%	0.6	84.6%
Shrub	Ericaceae	<i>Poaceae 1</i>	1.3%	0.5	71.2%
			11.8%	1.7	92.3%
			4.8%	0.7	88.5%
	Grossulariaceae	<i>Vaccinium myrtillus</i>	1.1%	0.5	28.8%
		<i>Vaccinium</i> spp.	2.9%	0.6	76.9%
	Rosaceae		2.7%	0.9	57.7%
		<i>Ribes</i> spp.	2.7%	0.9	57.7%
	Salicaceae	<i>Sorbus aucuparia</i>	1.4%	0.9	28.8%
		<i>Salix</i> spp.	2.1%	0.7	51.9%

Note:

Mean = Relativized mean of sequence reads

SE = Standard error

POO = Percentage of occurrence

Within the functional groups into which we classified each OTU, forbs were by far the dominant group, found at a relative abundance of 70.2% (SE=2.9), followed by shrubs 11.8% (SE=1.7), conifers 10.1% (1.6), graminoids 5.8% (SE=1.3), and bryophytes 1.3% (SE=0.7), with deciduous trees, ferns, and unknown comprising <1% each. The FOO of each group showed forbs in 100% of samples, graminoids slightly less frequently (96%), followed by shrubs and conifers in 92% of samples, bryophytes in 34.6%, trees in 7.7%, and ferns/allies in 3.8% of samples.

Figure 4 The most abundantly found operational taxonomic units comprising 90% of reads found, shown by percent frequency of occurrence.

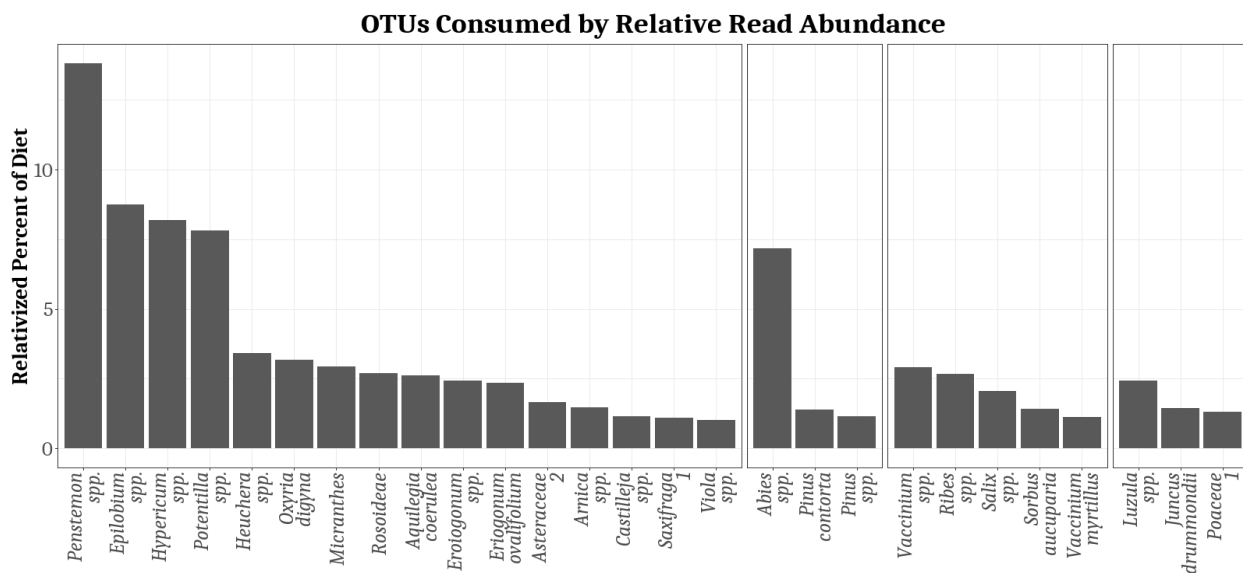
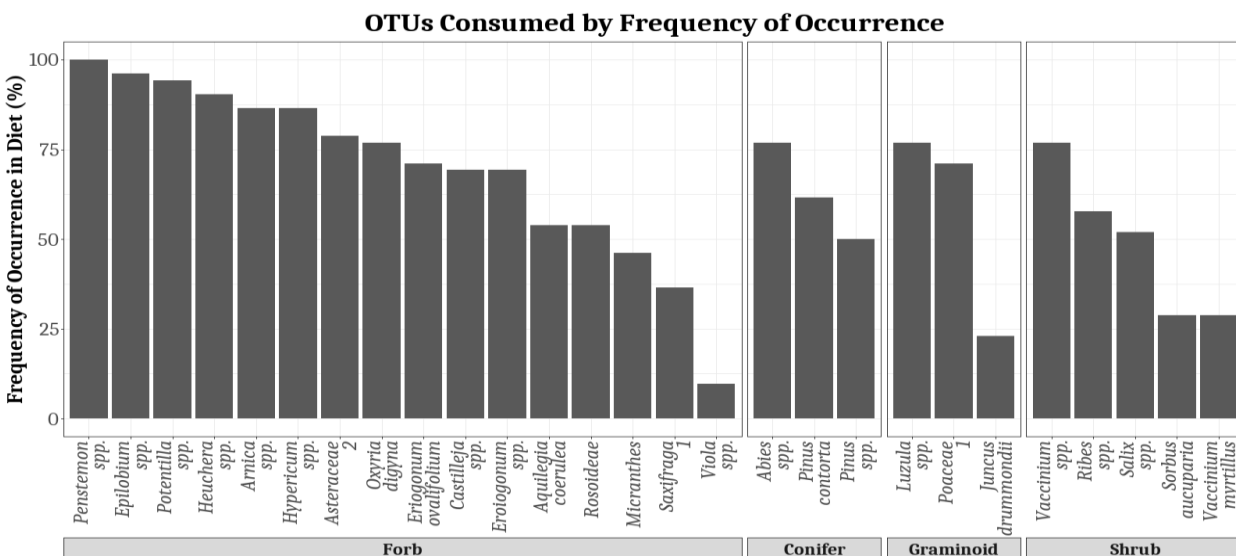


Figure 3 The most abundantly found operational taxonomic units comprising 90% of reads found, shown by relative read abundance



Discussion

Genotyping Success

Green pellets generated more complete microsatellite loci, whereas dark, hard, weathered pellets and moist, squishy, washed-out pellets were less successful. Green, unweathered pellets were often aged as <1 day old, but high variance made sample age less informative.

Information that we recorded about mountain goat behavioral observations (bedding area, goat observed, defecation observed), and pellet conditions of smooth, shiny, or pliable did not provide substantial information for separating the successfully genotyped samples. Low sample size and incomplete responses prevented us from being able to reliably assess the effect of weather on genotyping success. Future sample collection focusing on age of pellets, color, and moist from weather could be useful if subsampling is required, for example due to budget constraints.

Ethanol and freezing had similar success rates (ethanol 85%, freezing 84%). Ethanol is an easily accessible preservation method in the field, and our results agree with other studies that have shown a higher success rate preserving fecal pellets in ethanol than drying using silica desiccant (67% success rate; Ando et al. 2020). Dry sample success rates were lower (67%), but can be more easily stored, weigh less, are more portable, and are more straightforward to ship. We did not evaluate whether samples that were dry at collection had higher genotyping success with the ethanol approach, but it is possible that collecting dry samples using a dry protocol might result in similar success rates because of one less transition from wet to dry (the lab dries and then scrapes the ethanol samples). Tradeoffs exist that might determine which method to use.

With a larger sample size, more complete information on weather, or in other study areas, underperforming and additional covariates may provide more information on which pellet and collection conditions lead to higher genotyping success.

Diet Analysis

In this pilot study, we found summer mountain goat diet to be predominantly composed of forbs (in 100% of samples, with RRA of 70.2%), especially those within the genera *Penstemon*, *Potentilla*, *Chamerion/Epilobium* and *Hypericum*. Graminoids were in most samples as well (92%). Conifers were only 9.7% of the overall sequences found, although *Abies spp.* accounted for 7.2% of the overall sequences, on par with the most common forbs found. These findings contrast with the bulk of previous work on mountain goat diet. A 1994 review of 11 studies conducted through a variety of methods indicated an average summer diet of 52% grass, 30% forb, and 16% browse (Laundré 1994); in a more recent microhistology study, which equated pellet biomass with diet proportions, graminoids (sedges, rushes, and grasses) constituted 63.9% of the diet, forbs 22.2%, ferns 8.1% and mosses, lichens, and shrubs 9% (Cobb et al. 2012). Similarly, a study in the Cascade Range in Washington at 2 sites using a microhistology approach found graminoids comprised ~63-81% of diets by volume in early to late summer,

with forbs comprising 3.3-11% of diets by volume across the same sites and times (Harris et al. 2017). Only one experimental study using grazing trials and recording the number of bites found forbs to be 65-100% of the dry matter eaten, but these captive animals had been fed alfalfa hay and grain ad libitum until 1 day before the trials (Dailey et al. 1984).

Our study identified more forb use than previous studies, but the possible biases in all methods mean that caution should be used in inferring exact proportions of taxa consumed. Sequence recovery from fecal sampling with metabarcoded DNA can vary (Nakahara 2015). Previous methods such as microhistology have shown biases towards preferential physical recognition of taxa containing higher amounts of less digestible elements including graminoids (McInnis et al. 1983) and underestimates of dicot forbs (Vavra and Holecheck 1980, Shrestha and Wegge 2006), which may indicate that our study methods more accurately portray mountain goat diets compared to the historical understanding of a graminoid-dominant diet. The identification of *Hypericum sp.* raises a potential concern about the expansion of the noxious weed *H. perforatum*, which is now present in the park, is phototoxic, and causes hyperthermia and acts as a stimulant in domestic sheep and to a lesser extent goats (Bourke et al. 1997, Collett 2019).

Our methods identified a higher taxonomic resolution of vascular plants than previous studies. Using both the relative read abundance and the frequency of occurrence to examine sequences acts as a safeguard against either overemphasizing or entirely missing possibly important high-occurrence low-volume diet items (Deagle et al. 2019). When examining metabarcoding sequence data there are several different decision points to carefully consider, such as sequence match filtering, choosing occurrence thresholds, species matching, and OTU clustering (Ando et al. 2020). The possible effects of different analysis pipelines could produce different estimates of which diet items are considered important, as well as different estimates of the number of species within the diet. In our modest filtering, we removed 535 sequences which had a match of >98%, but which were <0.05% of the reads in any samples.

Unlike most other diet study methods, DNA metabarcoding techniques are continuing to rapidly improve. Taxonomical precision of sequence identification is increasing as reference libraries grow and primer specificity increases (Espinosa Prieto 2024). To contribute to further studies of herbivore diet in Glacier National Park we can identify plant species which have low sequence matches or are absent from reference libraries and send in voucher samples to be added. It has recently been shown that utilizing DNA metabarcoding primers targeting multiple gene regions increases both the accuracy and precision in assessing the diet of generalist herbivores (Goldberg et al. 2020), a consideration in further studies. Additional primers may also be useful in assessing lichen in the diet as this technology progresses (Gueidan & Li 2022).

All pellet samples submitted for barcoding had previously been positively genotyped as mountain goat, an important element considering that mountain goat and bighorn sheep (*Ovis canadensis*) have substantial habitat overlap and fecal pellets which are indistinguishable in the

field and likely have different diets (Dailey et al. 1984). This ability to genotype scats to sex and individual can be combined with DNA metabarcoding to examine issues such as possible differential resource use by sex and breeding status, and whether survival is related to specific diet items. In light of the recent decline of mountain goats in Glacier NP (Graves et al. 2025, identifying possible relationships between mountain goat diet and changes in alpine plant communities will inform future studies and conservation efforts.

Acknowledgements

Funding was provided by the U.S. Geological Survey Species Management Research Program and the National Park Service Focused Condition Assessment. We also thank Rachel Crowhurst and Clint Epps from Oregon State University for genotyping. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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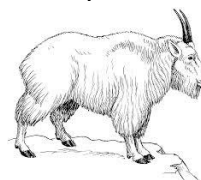
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Appendix A: Protocol provided to citizen scientists participating in the fecal pellet study.



**Glacier National Park High Country Citizen Science
Mountain Goat Fecal Pellet Collection Protocol**
Dry Envelope and Ethanol Solution



Introduction:

Ethanol vials are our first choice as they preserve the DNA more effectively. Citizen Scientists should carry envelopes in their packs at all times so that they can collect dry samples if they are out and don't have ethanol vials.

Equipment:

- ☐ Pencil (pen ink can run if writing gets wet)
- ☐ Smartphone with Survey 123 app (QR code), navigation app
- ☐ Barcode datasheet



Ethanol kit:

- ☐ Ziploc bag to limit leakage
- ☐ Vials with 10mL ethanol solution with label and barcodes

Dry envelope kit:

- ☐ Ziploc bag with *Desiccant*- Desiccant must be kept in sealed bag to work properly
- ☐ Coin envelopes with label and barcode

Major Principles to Observe

1. **Pellets must be relatively fresh**- usually less than a few weeks old and not rained on. Avoid collecting pellets with visible mold (whitish) or cracks.
2. **Avoid contaminating samples with DNA from other Mountain Goat individuals** (or potential bighorn individuals). Don't reuse collection materials.
3. **Avoid contaminating samples with human DNA**. Don't touch the inside of the envelope, the ends of the sticks you use to collect pellets, or the pellets with bare hands/ your skin.
4. **Pellets must be dried immediately and completely when using the envelopes.**

Tips for Scat Collection

- If you see a goat defecate, wait until the animal has **moved away at least 25 yards** before collecting sample.
- It is better to collect a questionable sample than no sample at all, especially if you're in a remote area. Where goat and sheep populations overlap, we will use genetics to identify species.
- It is better to collect from discrete piles as much as possible. This will allow us to calculate a minimum number of goats and possibly to estimate population size.

For All Fecal Pellet Sample Collection

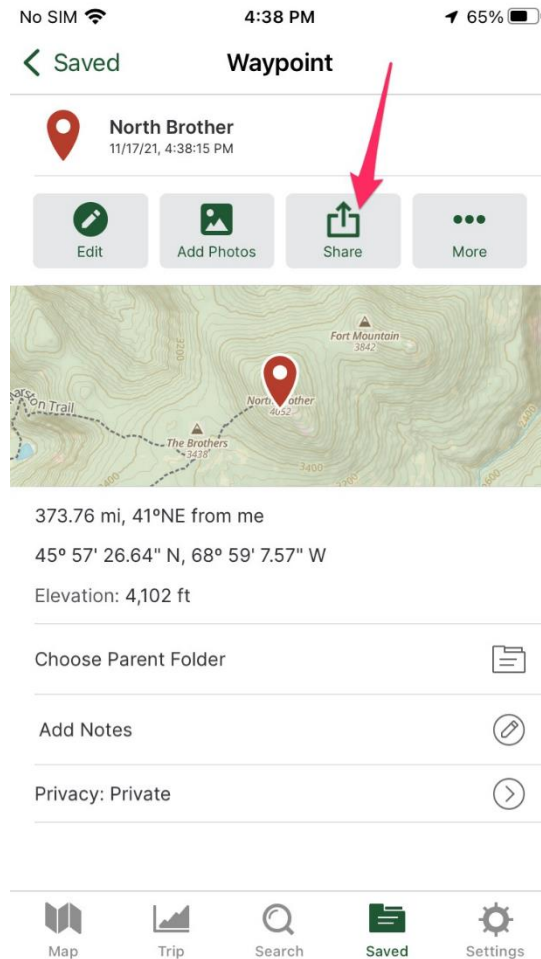
- 1.) If possible, initiate track on your navigation app (once entering alpine area/ goat habitat)
 - a. If uncertain, it is better to record a longer track or start earlier than for track to be shorter than your search area

- 2.) Initiate Survey 123 on your smartphone, initiate a new "survey" in the app, and prepare to complete relevant fields in the app. If possible, use the ethanol vial sample collection directly below, if not available, use the dry sample collection protocol (see Dry Protocol)
- 3.) Observe the pellets before collection and record in the app
 - a. **Time:** Please use military time (e.g., 1 p.m. = 13:00)
 - b. **Site Description:** describe trail or ridge with as much detail as you can provide
 - c. **GPS Location:** Latitude/longitude coordinates of where the pellets were collected
 - d. **Weather:** Can affect the pellet condition. Record/Circle whether it has rained or snowed in the last 24 hours
 - e. **Age of scat:** <1day=pliable and/or squishy >1day=can still be moist inside, can still be dark in color >1week: light in color, possibly weathered
 - f. **Observation of Scat:** DO=defecation observed, BA=pellets found in bedding area, GO= goats observed in pellet area before pellet collection, NG=pellets found in area with no goats observed that day
 - g. **Comments:** note things such as "fecal pellets found in water," "fecal pellets probably rained on," "only one pellet collected, not a discrete pile," or "bighorn present"

Texture	Fresh pellets will be squishy or pliable, older pellets will be hard and/or weathered. Use a stick or rock to break a pellet apart to check the pliability (do not touch pellets with bare skin, discard any pellets that might have your DNA on them).
Color	Fresh pellets will be dark green, dark brown, or black. Sunlight bleaches pellets a lighter color over the course of a few weeks and degrades DNA.
Moisture	Fresh pellets are moist on the outside and slightly damp inside (note that moisture should not be due to rain/snow). Do not collect moldy pellets.
Surface Appearance	Fresh pellets will often have tiny rocks or sand adhered to the surface or appear smooth and shiny. If pellets appear weathered (cracked, worn by long exposure to the air; weather-beaten) they are too old.

- 4.) Collect pellets from a single defecation event. Usually there is a single pile of pellets. Collect up to 6 pellets.
 - a. Use dead twigs or rocks as chopsticks to put pellets into the vial or envelope. Don't handle the ends of twigs or rocks that will touch the pellets. **DISPOSE OF TWIGS/ROCKS AFTER EACH SAMPLE** to prevent contamination of genetic material.
 - b. If pellet groups are mixed on the ground, (this can occur in high-use areas), collect **ONLY** pellets in discrete piles. **If you cannot distinguish an individual pile than take only 1 pellet from that group of scattered scat.**
- 5.) Return all samples and email route track file to:

Glac_Citizen_Science@nps.gov
 Citizen Science Office
 Crown of the Continent Research Learning Center
 Glacier National Park
 194 Mather Drive
 P.O. Box 128, West Glacier, MT 59936
 (406) 888 7986
- 6.) You can export "tracks" and "routes" from the Gaia app as a GPX or a KML file. To export all tracks and routes, tap "Share", then tap "Export File." Select "export file," select "KML" as the file type, and select email as the preferred export method. Please send to: Glac_Citizen_Science@nps.gov (Glac_Citizen_Science@nps.gov)



Ethanol Vial Fecal Sample Collection

- 1.) Vial will have ~10 mL 95% ethanol. Pellets should be submerged in ethanol.
- 2.) Close vial securely. Place in a ziplock bag to protect other gear from leakage. Store in a cool, dry place. Store in a cooler if available.
- 3.) Once you have returned from the field, **DO NOT OPEN SAMPLES**. Return to RLC staff to place all samples in freezer immediately, or store in your freezer until you can return samples to staff. Do not allow to freeze/thaw as this destroys DNA.

If you find yourself in the field without adequate ethanol vials, proceed to:

Dry Fecal Sample Collection

1. Initiate Survey 123 on your smartphone, initiate a new "survey" in the app, and prepare to complete relevant fields in the app.

- Fill out label on envelope used to collect fecal pellets with **Date, Observer ID (Obs. Id) at minimum**. If you do not have the app for some reason, fill out the envelope completely.
Example label:

Date: ___/___/___ Obs. ID: _____ Sample ID _____
Please fill out section below if not able to enter using SURVEY123 Form
Time: ___:___ Lat _____
Long _____
Weather (last 24 hrs): Rain Snow No Precip
Age of Scat: <1day >1day >1week
Scat Observation: Defecation Seen; Bedding Area; Goats Observed; No Goats
Condition (circle all that apply): squishy pliable weathered
hard moist dry shiny smooth
Color: black dark green dark brown light brown clay
Sex: M F Unk Age: Kid Yearling Adult Unk
Comments: _____
*Close envelope with sticker and place back in zip-loc

- Collect pellets from a single defecation event (see above- 6 pellets per sample is ideal for envelopes).
- Tape the envelope closed with the dots provided, DO NOT LICK the envelope flaps. Place in Ziploc bag with the desiccant envelope and close bag tightly.
- Make sure pellet samples dry completely on the day you collect them. **Mold will cause sample to be DISCARDED**. Check to see whether outside of envelope is completely dry without squishing pellets. The samples need to remain dry for optimal genotyping. If you are in the field, and it is warm/dry, you can spread them out to dry at night. Putting them on your dashboard on a warm day for a short period of time works very well.
- Otherwise, once you return from the field remove sample envelopes from Ziploc to spread out to dry. **DO NOT OPEN SAMPLE ENVELOPES**.
- Once dry, place envelopes into a dry envelope or paper bag. Store the samples at room temperature in a dry location (preferably with silica) until they are returned. Silica in bags can be dried out again by microwaving for up to 30 seconds.

How to Distinguish Goat Pellets from Other Species and Where to Look

- Where to look for fresh pellets: while entering rocky terrain with cliffs, rock outcrops or other escape terrain look for fecal pellets deposited by mountain goats.
- Examine fecal pellets for "freshness" by poking pellets with a stick. Only collect samples (toothpicks and pellets) from 'fresh' pellets that are intact (NOT that ones that you have squashed). **AVOID** moldy pellets and very dry, weathered pellets. Pellets can be difficult to identify. If you think there is a chance it is a mountain goat, please collect it.

Mountain Goat

Color: dark brown; dark green, or black

Surface Appearance: shiny, smooth

Fresh Pellets



Old Pellets



Color: light brown
Surface Appearance: weathered, cracked
Do not collect!



Goat track photos:
Luke Lamar



Bighorn Sheep



Track: Hooves are cloven. Toes are straight along the sides with rounded tips. More linear than deer or javelina. Front: 3 x 2 inches, hind: 3 x 2 inches.

Scat: When in contact with the ground, pellets dry and separate. Pellets are oval in shape with a slight point at one end. Size (pellets): 0.5 inches.

Fact: Plant species consumed vary with habitat, quality, and locality. Usually seen in mountains and hills immediately surrounding agricultural areas.

Potential Risk:

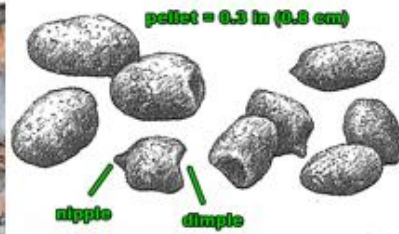


Front (F) & Hind (H) Feet



Photo Courtesy of George Andrejko – Arizona Game and Fish Department

White-tailed Deer



WHITE-TAILED DEER scat



Moose

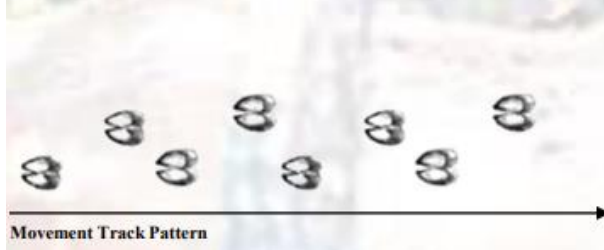
Goat

Elk

Deer

Bighorn

Elk

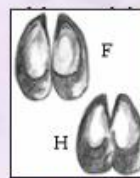


Track: Hooves are cloven. Toes are straight along the sides with rounded tips. More linear than deer or javelina. Front: 3 x 2 inches, hind: 3 x 2 inches.

Scat: When in contact with the ground, pellets dry and separate. Pellets are oval in shape with a slight point at one end. Size (pellets): 0.5 inches.

Fact: Plant species consumed vary with habitat, quality, and locality. Usually seen in mountains and hills immediately surrounding agricultural areas.

Potential Risk: RISK NOT DETERMINED



Front (F) & Hind (H) Feet



Photo Courtesy of George Andrejko – Arizona Game and Fish Department

Appendix B: Potocols for fecal pellet sample processing and analysis, provided by Jonah Ventures Laboratory (Boulder, Colorado; jonahventures.com).

Sample Processing

1.2.1

Frozen samples were thawed for 1-2 hours before processing. Sample barcodes were recorded and assigned a well within the 96 well plate or numbered extraction tube. Under a laminar flow hood, sterile cotton swabs (Fisher, cat# 22-363-173) were coated with fecal matter, and the swabs were placed in the corresponding extraction plate or tube. Sterile tweezers and pliers are used to handle cotton swabs and remove the wooden ends of the cotton swab before extraction. Plates or tubes were immediately processed or stored in -20C until the extraction process could be performed.

Extraction

2.1.2

Genomic DNA from samples was extracted using the DNeasy PowerSoil HTP 96 Kit (Cat # 12955-4) according to the manufacturer's protocol. Genomic DNA was eluted into 100µl and frozen at -20C.

PCR

3.2.1

Forward primer: CGAAATCGGTAGACGCTACG

Reverse primer: CCATTGAGTCTCTGCACCTATC

Primer reference: Taberlet P, Coissac E, Pompanon F, et al. Power and limitations of the chloroplast trnL (UAA) intron for plant DNA barcoding. Nucleic Acids Research. 2007;35(3):e14. doi:10.1093/nar/gkl938.

A portion of the chloroplast trnL intron was PCR amplified from each genomic DNA sample using the c and h trnL primers. Both forward and reverse primers also contained a 5' adaptor sequence to allow for subsequent indexing and Illumina sequencing. Each 25 µL PCR reaction was mixed according to the Promega PCR Master Mix specifications (Promega catalog # M5133, Madison, WI) which included 0.4 µM of each primer and 1 µl of gDNA. DNA was PCR amplified using the following conditions: initial denaturation at 94 °C for 3 minutes, followed by 40 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 1 minute at 72 °C, and a final elongation at 72° C for 10 minutes.

Gel

4.1.1

To determine amplicon size and PCR efficiency, each reaction was visually inspected using a 2% agarose gel with 5µl of each sample as input.

PCR Amplicon Cleanup

5.1.1

Amplicons were then cleaned by incubating amplicons with Exo1/SAP for 30 minutes at 37C following by inactivation at 95C for 5 minutes and stored at -20C.

Barcoding PCR

6.1.1

A second round of PCR was performed to complete the sequencing library construct, appending with the final Illumina sequencing adapters and integrating a sample-specific, 12-nucleotide index sequence. The indexing PCR included Promega Master mix, 0.5 μ M of each primer and 2 μ l of template DNA (cleaned amplicon from the first PCR reaction) and consisted of an initial denaturation of 95 °C for 3 minutes followed by 8 cycles of 95 °C for 30 sec, 55 °C for 30 seconds and 72 °C for 30 seconds.

PCR Normal Pool

8.1.1

Final indexed amplicons from each sample were cleaned and normalized using SequalPrep Normalization Plates (Life Technologies, Carlsbad, CA). 25 μ l of PCR amplicon is purified and normalized using the Life Technologies SequalPrep Normalization kit (cat#A10510-01) according to the manufacturer's protocol. Samples are then pooled together by adding 5 μ l of each normalized sample to the pool.

Sequencing

9.2.1

Sample library pools were sent for sequencing on an Illumina MiSeq (San Diego, CA) in the CU Boulder BioFrontiers Sequencing Center using the v2 500-cycle kit (cat# MS-102-2003). Necessary quality control measures were performed at the sequencing center prior to sequencing.

Bioinformatics

10.1.1

Sequencing success and read quality was verified using FastQC v0.11.8, and reads were demultiplexed by using Illumina-utils v2.6 (iu-demultiplex) using default settings. Sequences of each sample were then merged using the -fastq_mergepairs option in Usearch v11.0.667 [1]. The forward primer (5'-CGAAATCGGTAGACGCTACG-3') and reverse primer (5'-CCATTGAGTCTCTGCACCTATC-3') were removed using Cutadapt v1.18 [2]. Cutadapt is also used to discard sequences with length below 108 bp. Expected error filtering as implemented in Usearch is then used to discard low quality reads (max_ee=0.5)[3]. Instead of OTU clustering, reads affected by sequencing and PCR errors are then removed using the unoise3 algorithm with an alpha value of 5 [4]. This denoising is applied to each individual sample, and Exact sequence variants (ESV) compiled in a ESV table including sequences and read counts for each sample. Taxonomy is assigned to each ESV by mapping them against a GenBank reference data [5] as well as Jonah Ventures voucher sequences records, using usearch_global with -maxaccepts 0 and -maxrejects 0 to ensure mapping accuracy. Consensus taxonomy is generated from the hit tables, by first considering 100% matches, and then going down in 1% steps until hits are present for each ESV. In the respective 1% bracket, taxonomy present in at

least 90% of the hits is reported or an NA reported, if several taxa are matching the ESV. To reduce errors caused by misidentified taxa, the bracket is increased to 2% if matches of 97% or higher are present, and no family level taxonomy is returned.

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