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Using eDNA to confirm the identity of raptors that cast pellets

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ABSTRACT—Raptor pellets are regularly encountered in the field in a variety of habitats. Where multiple species of similar-sized raptors are sympatric, there can be uncertainty about which species produced a pellet based on visual assessments alone. We developed a DNA barcoding protocol that used environmental DNA (eDNA) to identify birds of prey that had produced pellets. Two different PCR primer pairs were required to successfully ascribe a pellet to the source bird species. Troubleshooting different suspension temperatures and applying multiple primer sets to the same eDNA sample provided sequences that successfully identified Red-tailed Hawk (*Buteo jamaicensis*) and Great Horned Owl (*Bubo virginianus*) pellets. Because eDNA degrades, this protocol will only work on pellets that have been in the environment for a limited time, probably <6 months. *Received 4 February 2021. Accepted 21 October 2021.*

Key words: COI gene, degradation, DNA barcoding, Great Horned Owl, Red-tailed Hawk.

Uso de eDNA para confirmar la identidad de rapaces que regurgitan egagrópilas

RESUMEN (Spanish)—Las egagrópilas de las rapaces se encuentran regularmente en el campo en una variedad de hábitats. En sitios donde múltiples especies de rapaces de tamaños similares son simpátricas, puede haber duda sobre cuál especie produjo una egagrópila con base únicamente en determinaciones visuales. Desarrollamos un protocolo de código de barras de DNA que usa DNA ambiental (eDNA) para identificar aves de presa que han producido egagrópilas. Requerimos dos pares de primers diferentes de PCR para asignar exitosamente una egagrópila a su ave de origen. La resolución de problemas de las diferentes temperaturas de suspensión y la aplicación de múltiples juegos de primers a la misma muestra de eDNA nos dio como resultado secuencias que identifican exitosamente egagrópilas del aguililla *Buteo jamaicensis* y el búho *Bubo virginianus*. Debido a que el eDNA se degrada, este protocolo solamente funcionará con egagrópilas que han estado en el ambiente por un tiempo limitado, probablemente <6 meses.

Palabras clave: *Bubo virginianus*, *Buteo jamaicensis*, código de barras de DNA, degradación, gen COI.

Raptors consume a variety of prey items that often contain indigestible materials, such as arthropod exoskeletons, feathers, fur, and bones (Ehrlich et al. 1988). To increase digestive efficiency, raptors condense these indigestible materials and regurgitate them as easily recognizable pellets. However, where multiple species of similar size are sympatric, the raptor species that produced a pellet may be difficult to ascertain.

Pollet and Shutler (2019) quantified mortality in a breeding colony of Leach's Storm-Petrels (formerly *Oceanodroma leucorhoa*, now *Hydrobates leucorhous*) on Bon Portage Island, Nova Scotia, Canada, that could be ascribed to Great Horned Owls (*Bubo virginianus*). As the only raptor at this colony hunting at night when storm-petrels are active during summer, pellets could be unambiguously assigned to this species. Subsequently, we obtained pellets from a second petrel colony, Country Island, where raptors are excluded for part of summer to protect endangered, nesting Roseate Terns (*Sterna dougallii*; Canadian Wildlife Service 2019). Raptor pellets from Country Island were of unknown age, and morphologically different from those we collected from Bon Portage Island. Based on the size of some pellets, we suspected that some had been produced by Bald Eagles (*Haliaeetus leucocephalus*), but we had limited information to make this inference.

The present study was undertaken to determine whether we could use residual environmental DNA (eDNA) to identify raptor species that had cast pellets. In a similar study, Marrero et al. (2009) collected fecal and regurgitant samples from 2 species of pigeon (Bolle's Pigeon [*Columba bollii*] and Laurel Pigeon [*C. junoniae*]) and were able to ascribe samples to these species based on DNA in cells from the birds' gastrointestinal tracts that had adhered to seed coats. Based on Marrero et al. (2009), we suspected that DNA in raptor pellets could also be derived from gastrointestinal tract cells. To test this, we used DNA barcoding, which is used to identify species by

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sequencing a 650- to 700-base pair gene region of mitochondrial cytochrome c oxidase subunit I (COI; Hebert et al. 2003).

Methods

We extracted eDNA from 10 opportunistically collected, cast pellets collected in summer 2019 from Country Island, Nova Scotia, Canada (45°06′N, 61°32′W), and 11 pellets from 2 organizations that maintain captive raptors in Nova Scotia: Hope for Wildlife, Chezzetcook, and Oaklawn Farm Zoo, Aylesford (Table 1). In all cases, we were blind to the species that produced the pellets. From each pellet, we obtained 200–700 mg of material from the interior, and avoided using parts containing bones because they were less likely to have absorbed cells or DNA from the digestive tract, and instead used parts that contained fur and/or feathers (Ellegren 1991). DNA extraction was done using a QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany).

Pellets from Country Island were collected in Ziploc brand bags (SC Johnson, Racine, Wisconsin, USA); however, because they were collected for a different study that did not anticipate the current study, they were not frozen, nor were sterile conditions used. Ages of Country Island pellets were unknown, but were a minimum of 6 months old when tested. In contrast, pellets from Hope for Wildlife and Oaklawn Farm Zoo were collected in sterile Ziploc bags while fresh and put on ice before overnight shipment to Acadia University. Samples were stored at –20 °C, and DNA extraction was conducted within 1 month of collection. One sample was extracted from each pellet, with the exception of sample numbers 5 and 6, which originated from the same pellet. The protocol for the Stool Mini Kit was slightly altered to increase DNA yield. A human DNA analysis protocol was followed (QIAGEN 2017).

In the event that DNA yield is low, either the sample contained degraded DNA or the temperature was not high enough to lyse cell walls. For cells that are difficult to lyse, the protocol advises increasing the incubation temperature at step 3 to yield more DNA, which was done at various temperatures to different samples (Table 1).

Sample DNA concentration was confirmed by nanospectroscopy using a Biodrop nanospectrometer. Polymerase chain reaction (PCR) primers AWCF1/AWCR6 (Patel et al. 2010) and BirdF1/BirdR1 (Hebert et al. 2004) were chosen as suitable for amplification of the COI region of avian species (Table 2). Each PCR reaction was set up in a 0.5 mL sterile plastic PCR tube containing 5 µL DNA, 12.5 µL Master Mix (BioRad), 1 µL forward primer, 1 µL reverse primer, and 5.5 µL sterile molecular biology grade water. A control reaction with 5 µL sterile molecular-grade water instead of DNA was also run; all other reagents were as above. Samples were all amplified with both sets of primers in 2 separate PCR reactions. PCR amplification of AWCF1/AWCR6 followed the thermocycler protocol for modern samples from Patel et al. (2010): 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 57.5 °C, 30 s at 72 °C, and a final 4 min extension at 72 °C. The thermocycler protocol for BirdF1/BirdR1 was as follows: 1 min at 94 °C followed by 5 cycles of 1 min at 94 °C, 1.5 min at 45 °C, and 1.5 min at 72 °C, followed in turn by 30 cycles of 1 min at 94 °C, 1.5 min at 51 °C, 1.5 min at 72 °C, and a final 5 min extension at 72 °C (Kerr et al. 2009).

PCR products were visualized using gel electrophoresis, which consisted of 0.5 g agarose, 50 µL of 1 x TAE, and 2 µL ethidium bromide (10 mg/mL). Two µL Thermo Scientific 6X DNA loading dye was applied to each well with 5 µL of PCR product. Gels were run at 95 V for 30 min. Once successful PCR amplifications were obtained, as confirmed by gel electrophoresis, samples 3, 5, and 8, each sample amplified with both AWCF1/AWCR6 and BirdF1/BirdR1, were sent to Genome Québec Innovation Centre (McGill University) for Sanger DNA sequencing in the forward and reverse directions. Genome Québec sent chromatograms for each of the 6 samples, and Molecular Evolutionary Genetics Analysis (MEGA-X 10.1) was used to trim sequences obtained from chromatograms. Trimmed forward and reverse sequences were aligned using the alignment tool in Geneious 2020.1.1 (https://www.geneious.com) to generate consensus sequences. Five consensus sequences were generated with a threshold above 95%. These sequences were compared with NCBI's GenBank online DNA sequence database using the Basic Local Alignment Search Tool (BLAST) for

Table 1. Raptor pellets used in this eDNA study. HFW is Hope for Wildlife and OFZ is Oaklawn Farm Zoo, Nova Scotia, Canada. Samples 5 and 6 originated from the same pellet.

Sample	Source facility	Sample wet mass (mg)	DNA concentration (μL/mL)	A260/A230 ratio	A260/A280 ratio	Incubation temperature and time (step 3 in protocol)
1	HFW	220	10	0.068	5.000	77 °C for 5 min
2	HFW	230	118	0.602	2.226	77 °C for 5 min
3	HFW	230	121	0.599	2.086	77 °C for 5 min
4	HFW	250	11	0.063	5.500	95 °C for 5 min
5	HFW	250	14	0.083	2.800	80 °C for 5 min
6	HFW	340	7	0.043	7.000	80 °C for 5 min
7	OFZ	420	13	0.068	4.333	95 °C for 5 min
8	OFZ	330	26	0.161	2.600	95 °C for 5 min
9	HFW	230	49	0.246	2.227	80 °C for 5 min
10	HFW	200	10	0.061	3.333	80 °C for 5 min
11	OFZ	240	10	0.059	2.500	80 °C for 5 min
12	OFZ	700	118	0.546	NA	80 °C for 5 min

identification, and the top 10 closest barcode sequence matches, ranked after highest bit-score, were included. Two individuals with a DNA sequence similarity of >97.3–97.6 are considered to be from a single species (Breman et al. 2011).

Results

Spectroscopy revealed that 11 fresh pellets from wildlife facilities contained DNA; however, only the 2 samples that contained raptor DNA that PCR-amplified are shown in Table 3. DNA concentrations and A260/A230 and A260/A280 ratios are given in Table 1. The top 10 closest GenBank DNA sequence matches are in Table 3. Sample 5 could be ascribed to 6 different *Buteo* species, which indicates both the current limitations of our publicly available DNA sequence databases, as well as the limitations of a single-gene approach to DNA barcode-based identifications. *Buteo jamaicensis* appeared several times and had the highest-ranking bit-scores, often accompanied by a high percentage in identical sites and query coverage. Both primer sets yielded high percentage matches with raptor species. The top 10 matches for sample 8 amplified with the primer pair BirdF1/BirdR1 included 8 *Bubo virginianus* sequences and 2 other species within the Order Strigiformes. When sample 8 was amplified with primer pair AWCF1/AWCR6, only DNA sequence matches for domestic chickens (*Gallus gallus*) were obtained, a common part of captive raptor diets. Given that the facilities from which we obtained samples do not have captive birds of the other species in Table 3, we can confidently ascribe 2 pellets to *Buteo jamaicensis* and 1 to *Bubo virginianus*. Despite our attempts to optimize lysing temperatures and thermocycler parameters, we were unable to amplify avian DNA from the Country Island pellets.

Discussion

We developed a protocol for identifying raptor species that produced pellets, and also learned about its limitations; only 2 of 21 samples yielded

Table 2. Forward and reverse COI gene primer sequences used to amplify avian eDNA from captive raptor cast pellets.

Forward primer	Reference
AWCF1: CGCYTWAACAYTCYGCCATCTTACC	Patel et al. 2010
BirdF1: TTCTCCAACCACAAAGACATTGGCAC	Hebert et al. 2004
Reverse primer	
AWCR6: ATTCCTATGTAGCCGAATGGTTCTTT	Patel et al. 2010
BirdR1: ACGTGGGAGATAATTCCAAATCCTG	Hebert et al. 2004

Table 3. GenBank BLAST search results, showing the top 10 COI sequence matches for our consensus sequences generated from 2 different samples of eDNA pellets, using 2 avian COI primer sets. "bp" is base pairs.

eDNA – ID & primers	Raptor species	Bit-score	% Identity	Query coverage (%)	Sequence length (bp)	GenBank accession
Sample 5 & AWCF1/AWCR6	*Buteo jamaicensis*	1,079.56	96.7	45.70	637	KR017962
	Buteo jamaicensis	1,064.79	99.8	41.54	579	KR017962
	Buteo jamaicensis	1,040.78	96.7	43.97	613	DQ434504
	Buteo swainsoni	992.771	97.6	41.54	579	KF525376
	Buteo buteo	976.151	97.1	41.54	579	MN122916
	Buteo buteo	976.151	97.1	41.54	579	AF380305
	Buteo buteo	970.611	96.9	41.54	579	KM364882
	Buteo swainsoni	966.918	97.2	40.89	570	KF525376
	Buteo hemilasius	959.531	96.5	41.54	579	KT935541
	Buteo lagopus	953.992	96.4	41.54	579	KP337337
Sample 5 & BirdF1/BirdR1	*Buteo jamaicensis*	390.763	92.9	62.53	267	KR017962
	Buteo jamaicensis	390.763	92.9	62.53	267	DQ434504
	Buteo albonotatus	363.064	98.1	48.48	207	JQ174206
	Buteo albonotatus	363.064	98.1	48.48	207	JN801523
	Buteo albonotatus	363.064	98.1	48.48	207	DQ432782
	Buteo hemilasius	357.524	97.6	48.48	207	GQ922627
	Buteo buteo	351.984	97.1	48.48	207	MN122916
	Buteo buteo	351.984	97.1	48.48	207	KY754488
	Buteo buteo	351.984	97.1	48.48	207	KT803616
	Buteo rufinus	351.984	97.1	48.48	207	KT803615
Sample 8 & BirdF1/BirdR1	*Bubo virginianus*	833.959	99.8	80.32	453	EU525334
	Bubo virginianus	833.959	99.8	80.32	453	DQ433382
	Bubo virginianus	833.959	99.8	80.32	453	DQ433381
	Bubo virginianus	833.959	99.8	80.32	453	DQ432775
	Bubo virginianus	833.959	99.8	80.32	453	AY666514
	Bubo virginianus	833.959	99.8	80.32	453	AY666417
	Bubo virginianus	833.959	99.8	80.32	453	AF168059
	Surnia ulula	828.419	99.6	80.32	453	KF430326
	Bubo virginianus	828.419	99.6	80.32	453	EU525335
	Bubo magellanicus	706.54	94.7	80.32	453	MG263834

PCR-amplified DNA. We suspected 2 possible reasons for the lack of DNA amplification from Country Island samples: that protocols as we applied them were inappropriate, or eDNA in samples was too degraded to amplify. If the latter explanation applied, we reasoned that protocol success rate should improve using fresh pellets. Ages of pellets from Country Island were unknown and were a minimum of 6 months old prior to barcoding; DNA had likely degraded below a threshold that would permit successful amplification. The use of fresh pellets stored at cold temperatures (Bonnet et al. 2010) facilitated successful barcoding. It would be worth repeating this study where pellets of known age were collected from captive raptors and left in wild raptor habitats for a variety of fixed intervals (e.g., a subset of them removed and analyzed every week). This would allow for testing the threshold age at which eDNA is still viable in pellets.

Samples collected for eDNA analysis often contain only small amounts of target genetic material, making subsequent PCR amplifications difficult (Duhaime et al. 2012). Other limitations include cells that are difficult to lyse during DNA extraction (but this can be overcome with the higher incubation temperatures that we used; also see Salonen et al. 2010), lack of knowledge on the taxonomic coverage of avian PCR primers used for barcoding, and finding correct thermocycler conditions (Culley et al. 2013, Rubbmark et al. 2018). Despite these challenges, we developed a protocol that identified raptor species from their egested pellets; the method should now be tested on other species.

Pellets contain DNA of both raptors and their prey, and given that a portion of prey was avian (in our case, chicken as a common food for captive raptors), both sources of DNA may be amplified, as was observed in our study (sample 8 when amplified with AWCF1/AWCR6). Both primer pairs we used targeted the COI barcoding gene region and amplified gene regions of similar length (Table 2). An eDNA sample can contain DNA from multiple organisms, and failed amplification of a particular species can be masked by other organisms present in the sample. This, age of pellets, and low amounts of raptor gut mucosa could all result in false species absences.

Rubbmark et al. (2018) highlighted a lack of knowledge on taxonomic coverage of universal primers used for metabarcoding, because most studies have been done on aquatic and invertebrate taxa. Pompanon et al. (2012) also highlighted the importance of the quality and amount of tissue in samples. For example, sample 8 contained more undigested animal tissue than other samples, which may have contributed to the success of amplifying sequences belonging to prey species. Undigested tissue has had minimal exposure to digestive enzymes in the gastrointestinal tract (Bus et al. 2014) and is therefore less likely to have suffered DNA degradation, increasing the likelihood for detection of prey DNA in pellets versus fecal samples.

Amplifying multiple gene regions from the same eDNA sample can provide greater levels of confidence in species identification by confirming taxonomic group in multiple sequence matches (Stat et al. 2017, Andujar et al. 2018). This was the case for *Buteo jamaicensis* in our study. Note that quality of sequences varied depending on the primer set used. Nevertheless, if a specific taxon is of interest, in this case the genus *Buteo* or *Bubo*, Stat et al. (2017) recommends targeting additional gene regions, such as 16S and 12S of the mitochondrial genome, to allow for more specificity.

Another factor that determines successful amplification is finding the correct thermocycler conditions. Both primer sets were applied with their own cycling conditions, but ideally each primer pair should be tested with both sets of conditions. Culley et al. (2013) suggested that certain primers perform better when a higher annealing temperature is applied, preferably around 57 °C. Future studies are needed to optimize PCR thermocycling conditions for primer sets which amplify additional gene regions, by adjusting annealing temperatures and number of cycles. Additionally, only one of our pellets was amplified in duplicate (samples 5 and 6 came from the same pellet [Table 1]); increasing the replication at the DNA extraction and PCR step for each pellet could increase confidence in the sequence results obtained.

Using universal avian primers based on the COI gene allows for detection of a variety of bird species from eDNA samples, especially when applied in combination. However, if prey species are identified in pellet DNA, as with sample 8 in combination with primers AWCF1/AWCR6, they can be ruled out because it is unlikely that the pellet was produced by the prey species itself. This is important when assessing pellets belonging to raptors that prey on or scavenge other birds of prey (i.e., superpredation or intraguild predation; Lourenco et al. 2010). Similarly, knowledge of raptor species typically found in a region or facility can be used to rule out other species identified in sequence matches.

In conclusion, using eDNA for molecular identification has many challenges, such as DNA degradation previous to and during field transport, and risk of contamination from environmental sources (Thomsen and Willerslev 2015). Several difficulties in ascribing pellets to the raptors that produced them were resolved by applying 2 PCR primer sets to increase confidence in successful eDNA amplification. Other complications, such as lysing cells derived from raptor digestive tracts, were dealt with by increasing incubation temperatures during DNA extraction. Future studies should include testing other thermocycler conditions and primers. Other primers targeting additional gene regions, such as 16S and 12S of the mitochondrial genome, should also be designed to increase specificity of the PCR assay, because the small quantities of eDNA present in pellet samples can present a challenge. Practical implications of eDNA in pellet studies include their utility in conservation of both predator and prey species, if fresh pellets can be obtained. These data can be used to determine predator effects on prey species, and in determining raptor diet.

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Literature cited

Andujar C, Arribas P, Yu DW, Vogler AP, Emerson BC. 2018. Why the COI barcode should be the community DNA metabarcode for the metazoa. Molecular Ecology. 27:3968–3975.

Bonnet J, Colotte M, Coudy D, Couallier V, Portier J, et al. 2010. Chain and conformation stability of solid-state DNA: Implications for room temperature storage. Nucleic Acids Research. 38:1531–1546.

Breman FC, Jordaens K, Sonet G, Nagy ZT, Van Houdt J, Louette M. 2011. DNA barcoding and evolutionary relationships in *Accipiter* Brisson, 1760 (Aves, Falconiformes: Accipitridae) with a focus on African and Eurasian representatives. Journal of Ornithology. 154:265–287.

Bus MM, Zmihorski M, Romanowski I, Balciauskiene L, Cichocki J, Balciauskas L. 2014. High efficiency protocol of DNA extraction from *Micromys minutus* mandibles from owl pellets: A tool for molecular research of cryptic mammal species. Acta Theriologica. 59:99–109.

Canadian Wildlife Service. 2019. Country Island field manual. Seabird monitoring: Logistics and protocols. Canadian Wildlife Service - Environment and Climate Change Canada.

Culley TM, Stamper TI, Stokes RL, Brzyski JR, Hardiman NA, et al. 2013. An efficient technique for primer development and application that integrates fluorescent labeling and multiplex PCR. Applications in Plant Sciences. 1:1300027.

Duhaime MB, Deng L, Poulos BT, Sullivan MB. 2012. Towards quantitative metagenomics of wild viruses and other ultra-low concentration DNA samples: A rigorous assessment and optimization of the linker amplification method. Environmental Microbiology. 14:2526–2537.

Ehrlich P, Dobkin DS, Wheye D. 1988. The birder's handbook. New York (NY): Simon and Schuster.

Ellegren H. 1991. DNA typing of museum birds. Nature. 354:113.

Hebert PD, Cywinska A, Ball S, Dewaard J. 2003. Biological identifications through DNA barcodes. Proceedings of the Royal Society of London B. 270:313–321.

Hebert PD, Stoeckle MY, Zemlak TS, Francis CM. 2004. Identification of birds through DNA barcodes. PLOS Biology. 2:e312.

Kerr KC, Lijtmaer DA, Barreira AS, Hebert PD, Tubaro PL. 2009. Probing evolutionary patterns in Neotropical birds through DNA barcodes. PLOS One. 4:e4379.

Lourenco R, Santos S, Rabaca J, Penteriani V. 2010. Superpredation patterns in four large European raptors. Population Ecology. 53:175–185.

Marrero P, Fregel R, Cabrera V, Nogales M. 2009. Extraction of high-quality host DNA from feces and regurgitated seeds: A useful tool for vertebrate ecological studies. Biological Research. 42:147–151.

Patel S, Waugh J, Millar CD, Lambert DM. 2010. Conserved primers for DNA barcoding historical and modern samples from New Zealand and Antarctic birds. Molecular Ecology Research. 10:431–438.

Pollet IL, Shutler D. 2019. Effects of Great Horned Owls (*Bubo virginianus*) on a Leach's Storm-Petrel (*Oceanodroma leucorhoa*) population. Wilson Journal of Ornithology. 131:152–155.

Pompanon F, Deagle BD, Symondson WO, Brown DS, Jarman SN, Taberlet P. 2012. Who is eating what: Diet assessment using next generation sequencing. Molecular Ecology. 21:1931–1950.

QIAGEN. 2017. QIAamp Fast DNA Stool Mini Kit - Quickstart protocol. Hilden (Germany): QIAGEN.

Rubbmark OR, Sint D, Horngacher N, Traugott M. 2018. A broadly applicable COI primer pair and an efficient single-tube amplicon library preparation protocol for metabarcoding. Ecology and Evolution. 8:12335–12350.

Salonen A, Nikkilä J, Jalanka-Tuovinen J, Immonen O, Rajilić-Stojanović M, et al. 2010. Comparative analysis of fecal DNA extraction methods with phylogenetic microarray: Effective recovery of bacterial and archaeal DNA using mechanical cell lysis. Ecology and Evolution. 81:127–134.

Stat M, Huggett M, Bernasconi R, DiBattista JD, Berry TE, et al. 2017. Ecosystem biomonitoring with eDNA: Metabarcoding across the tree of life in a tropical marine environment. Scientific Reports. 7:12240.

Thomsen PF, Willerslev E. 2015. Environmental DNA – An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation. 183:4–18.

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