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Amplification of Black Vulture (*Coragyps atratus*) DNA from regurgitated food pellets

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ABSTRACT—Studies that rely on noninvasive collection of DNA for birds often use feces or feathers. Some birds, such as vultures, regurgitate undigested matter in the form of pellets that are commonly found under roost sites. Our research demonstrates that regurgitated pellets are a viable, noninvasive source of DNA for molecular ecology studies of vultures. Our objectives were to amplify 5 microsatellite loci designed for distinguishing Turkey Vultures (*Cathartes aura*) and Black Vultures (*Coragyps atratus*) in a single, multiplexed PCR, and to determine how long the target nuclear DNA persists after a vulture pellet is regurgitated and exposed to the environment. We collected pellets from captive Black Vultures and placed them in an outdoor aviary for a maximum estimated total of 12, 24, 36, or 48 h. We swabbed pellet surfaces for extraction and amplified vulture DNA using the panel of markers. All amplified alleles fell within predicted ranges of Black Vultures for all 5 loci, supporting the use of this microsatellite panel for vulture species identification. Overall amplification success for samples collected 0–12 h after regurgitation was 82.3%. Pellets collected 12–24 h, 24–36 h, and 36–48 h after regurgitation had only 18%, 10.2%, and 4.5% amplification success, respectively, which may have been due to a rain event. Our approach will be useful for noninvasive genetic sampling targeting nuclear DNA. These results should encourage noninvasive genetic sampling studies of other species that regurgitate pellets, such as raptors, water birds, or shorebirds. *Received 16 December 2021. Accepted 16 July 2022.*

Key words: amplification success, microsatellite loci, multiplex PCR, noninvasive DNA, nuclear DNA.

Amplificación de ADN de zopilote negro (*Coragyps atratus*) a partir de egagrópilas regurgitadas

RESUMEN (Spanish)—Estudios que dependen de colecta no invasiva de ADN de aves muchas veces utilizan heces o plumas. Algunas aves, como los zopilotes, regurgitan materia sin digerir en forma de egagrópilas que se encuentran comúnmente bajo los dormideros. Nuestra investigación muestra que las egagrópilas regurgitadas son una fuente de ADN viable y no invasiva para estudios de ecología molecular de zopilotes. Nuestros objetivos fueron amplificar 5 loci microsatelitales diseñados para distinguir aura gallipavo (*Cathartes aura*) y zopilote negro (*Coragyps atratus*) en un solo PCR múltiple así como determinar cuánto tiempo persiste el ADN nuclear blanco después de que una egagrópila de zopilote es regurgitada y expuesta al ambiente. Colectamos egagrópilas de zopilotes negros en cautiverio y las colocamos en un aviario al aire libre durante un tiempo máximo estimado de 12, 24, 36 y 48 h. Realizamos un frotis de la superficie de las egagrópilas para una extracción y amplificación del ADN de los zopilotes usando el panel de marcadores. Todos los alelos amplificados cayeron en los rangos predichos para los zopilotes negros para todos los 5 loci, lo que apoya el uso de este panel de microsatélites para identificación de especies de zopilotes. El éxito general de amplificación de muestras colectadas de muestras colectadas 0-12 h después de regurgitadas fue de 82.3%. Las egagrópilas colectadas 12–24 h, 24–36 h y de 36–48 h tuvieron solamente 12%, 10.2% y 4.5% de éxito de amplificación, respectivamente, lo que podría ser debido a un evento de lluvia. Nuestro enfoque será útil para muestreos genéticos no invasivos dirigidos a ADN nuclear. Estos resultados deberían fomentar los estudios de muestreo genético no invasivo en otras especies que regurgiten egagrópilas, como rapaces, aves acuáticas y aves playeras.

Palabras clave: ADN no invasivo, ADN nuclear, éxito de amplificación, loci microsatelitales, PCR mútiple.

Vultures and other birds of prey, shorebirds, and some aquatic birds such as cormorants and shags regurgitate a mass of undigested matter, referred to as a pellet. Pellets can be used as a noninvasive source of DNA in molecular ecology studies to identify diet items (Taberlet and Fumagalli 1996, Hacker et al. 2021), but another promising yet understudied tool is using pellets as a source of DNA of the regurgitating birds. One study that utilized host DNA from pellets identified mitochondrial DNA (mtDNA) of frugivorous birds from regurgitated seeds (Marrero et al. 2009), but a carnivorous diet, such as that of vulture species, could present different PCR inhibitors (e.g., proteinases and fats; Rossen et al. 1992) compared to secondary compounds found in plants/seeds (Khanuja et al. 1999).

Mitochondrial DNA metabarcoding approaches have demonstrated success in identifying raptor species from pellets (Hacker et al. 2021) but

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mtDNA is far more abundant per cell and limits the scope of inference compared to nuclear DNA. A study by van der Reis and Jeffs (2020) targeting nuclear DNA reported that nuclear DNA of New Zealand King Shags (*Leucocarbo carunculatus*) was sufficient in their extracted pellets to genetically determine sex. Feces are often used in noninvasive genetic sampling (Eggert et al. 2005, Waits and Paetkau 2005), where sloughed cells from the intestine contain an animal's DNA (Foran et al. 1997). Regurgitated pellets presumably contain sloughed cells from the esophagus or buccal cavity and are commonly found under vulture roost sites (Kelly et al. 2007). Being able to noninvasively study vultures would benefit research of these species due to challenges of capture and release, and such a method could potentially be applied to other bird species that regurgitate pellets.

Black Vultures (*Coragyps atratus*) are increasingly of interest to wildlife managers as human–wildlife conflicts with this species are increasing (Kluever et al. 2020). Microsatellite markers for Black Vultures and Turkey Vultures (*Cathartes aura*) were developed by Wostenberg et al. (2019) that could identify the 2 New World species and provide individual identification for population-level studies. However, before noninvasive genetic sampling can be employed at large spatial extents under field settings, pilot studies that determine DNA amplification success are essential (Taberlet et al. 1999, Lonsinger et al. 2015).

We chose to use Black Vultures as our targets of study due to the availability of microsatellite markers and access to an outdoor aviary with captive Black Vultures. One goal of the current study was to use these genetic markers in a single multiplexed PCR panel to identify species and assess quality of nuclear DNA on pellets. This approach could provide a noninvasive species identification of Black Vultures and Turkey Vultures and identify pellets with viable vulture DNA. Nuclear DNA from vultures could then be used for obtaining individual genotypes with additional microsatellite markers for population genetic assessments in mixed-species roosts. Additionally, it is important to know limitations when deciding if noninvasive DNA methods are feasible for a study. The second goal of our research was to determine how long the target DNA persists after a pellet is shed in the environment so that researchers may know what sampling strategies are required.

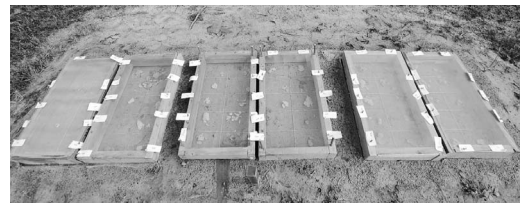

Figure 1. Regurgitated Black Vulture (*Coragyps atratus*) pellets were placed within wooden-framed boxes covered with metal window screening to limit access by scavenging invertebrates, August 2020, Gainesville, Florida, USA.

Methods

We collected 80 pellets between 28 July and 12 August 2020 from captive Black Vultures in an outdoor aviary at the USDA-APHIS-WS National Wildlife Research Center field station in Gainesville, Florida, USA. Six vultures of unknown sex and 4–16 years old contributed pellets to the study. These birds were exclusively fed feeder rats (*Rattus norvegicus*) 1 week prior to and during the duration of the study to eliminate diet composition as a variable in DNA amplification success. Vultures were kept in an open-air netted aviary with a heavy-duty vinyl membrane base. The vinyl surface was scrubbed and rinsed with a bleach solution prior to the study and after daily pellet collections. The aviary was surveyed multiple times daily to ensure all samples collected were a maximum of 12 h old. All collected pellets were placed into individual Whirl-Paks (Nasco, Fort Atkinson, Wisconsin, USA) and frozen until enough pellets were collected for the DNA persistence study. The 80 pellets were randomly selected: 20 for each experimental time treatment. Twenty pellets remained frozen to represent the first time point (pellets $<$12 h old). The other 60 pellets were placed in simulated field conditions on 25 August 2020 (Fig. 1). Pellets were placed within wooden-framed boxes covered with metal window screening to limit access by invertebrates and located within a 0.2 ha (0.5 acre) netted aviary (Fig. 1). Twenty pellets per timepoint (24, 36, or 48 h) were re-collected and placed back in a –20 °C freezer after maximum estimated exposure for their respective target timepoint. Hourly tempera-

Table 1. Multiplex of 5 microsatellite loci used to identify Black Vultures (BLVU, *Coragyps atratus*). N_A = number of alleles; *bp* = number of base pairs.

Locus name	Primer sequence (5′–3′); F, forward; R, reverse	N_A BLVU	BLVU range (*bp*)	Dye
BLVU-09	F:CCTCCATAGATGTGCCCTAACC R:ACAGCTTCTCCCTGTGTCCC	2	272–287	NED
BLVU-27	F:CCAAAACCTGCCACTGTCC R:GGTGACATTTTAATGCTGGGC	3	214–226	PET
TUVU-18	F:GGTTCTGCTGATTTCAACTTTGC R:TTCACCACAGGAAACCAAAGC	3[a]	211–216[a]	VIC
TUVU-21	F:TTGTTTGGCTCCATGTTTGG R:ACACCCATTCAAATGCAAGC	2[b]	179–180[b]	FAM
TUVU-31	F:AAGTAAATAGCTGTCTAACTGTTCATCC R:CTTTCATGCCTTGATTTCCC	1	93	VIC

[a] False allele at 229 bp excluded.
[b] False allele at 208 bp excluded.

ture and precipitation data from the field station were recorded for the duration of the study. Frozen samples were stored for up to 1 week at –20 °C, then sent overnight on ice to the USDA-APHIS-WS National Wildlife Research Center headquarters in Fort Collins, Colorado, where they were stored at –80 °C for up to 3 months until DNA extraction.

Regurgitated pellets produced from the vulture's feeder-rat diets consisted mostly of clumps of matted hair (Fig. 1) and did not have distinct shapes or discernable outer surfaces to target for extraction. We previously conducted a pilot study to compare 2 extraction methods targeting vulture DNA using 6 pellets of unknown age. One extraction method used homogenized pieces of the pellets and the Qiagen QIAamp Fast DNA Stool Mini Kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol. However, the method of swabbing throughout the pellet and extracting as described below resulted in more alleles with stronger amplification.

The surface and interior of each pellet were swabbed twice for duplicate extraction replicates using sterile Fisherbrand polyester tipped swabs (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a biological safety cabinet dedicated for use in noninvasive DNA extractions. DNA from each swab was extracted following the protocol in Piaggio et al. (2020) using a Qiagen Investigator Lyse & Spin Basket kit, Qiagen QIAamp DNA micro kit, and a Qiagen QIAcube robot for automation. Each extraction batch included 23 swabs and a negative control containing only an unused swab and reagents to monitor for contamination in the extraction process. Extracted products were stored at –20 °C for up to 3 weeks until further processing.

We performed 3 independent PCR replicates from each DNA extract using a single-panel multiplex PCR consisting of 5 microsatellite loci (Table 1; Wostenberg et al. 2019). These markers were selected because Wostenberg et al. (2019), as well as our preliminary testing, demonstrated that each of these 5 loci amplify species-specific ranges of alleles in both Black and Turkey vultures. The 10 μL PCR reaction contained 2 μL extracted DNA product, 5 μL Qiagen 2x Multiplex PCR Master Mix, 0.4 μL DEPC-treated H_2O, and 2.6 μL of the following primer mixture at a concentration of 10 μM: 0.3 μL each forward and reverse primers of BLVU-09, and BLVU-27, 0.5 μL each forward and reverse primers of TUVU-18, and 0.1 μL each forward and reverse primers of TUVU-21 and TUVU-31.

Thermocycling conditions were an initial denaturation at 95 °C for 15 min, followed by 45 cycles of 94 °C for 30 s denaturation, annealing at 58 °C for 90 s, extension at 72 °C for 60 s, and a final extension at 60 °C for 30 min. Each PCR included a negative control of PCR reagents to monitor contamination. Resulting amplification products were diluted 1:5 in DEPC-treated H_2O to prevent oversaturation of fluorescence, particularly of unincorporated primers, which can lead to size standard issues.

We performed fragment analyses on an ABI 3500 genetic analyzer (Thermo Fisher Scientific), and alleles were manually evaluated for each of the

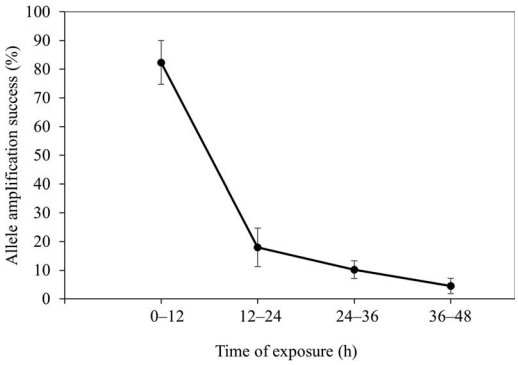

Figure 2. Nuclear DNA amplification success from regurgitated Black Vulture (*Coragyps atratus*) pellets across estimated time of field exposure. Error bars show standard deviation from the mean calculated between 5 microsatellite loci at each time period.

5 loci using GeneMapper 5.0 (Thermo Fisher Scientific). Amplification success in our study is defined as an allele with a clearly fluoresced peak (homozygote) or peaks (heterozygote) falling within our defined bin definitions based on fragment length. We calculated amplification success rates at each locus based on the number of successful amplifications out of 6 (2 DNA extractions with 3 independent PCR replicates per pellet). Amplification success was averaged across the 5 loci at each timepoint, and standard deviation was calculated to show variation between loci.

Results

All amplified alleles fell within predicted ranges of Black Vultures for all 5 microsatellite loci (Table 1; Wostenberg et al. 2019). Overall amplification success for samples collected 0–12 h after regurgitation was 82.3% (494/600 across loci; SD 7.6; Fig. 2). Amplification success at each locus BLVU-09, BLVU-27, TUVU-18, TUVU-21, and TUVU-31 was 85.8% (103/120), 73.3% (88/120), 75% (90/120), 87.5% (105/120), and 90% (108/120), respectively. Of these 20 earliest timepoint samples, 5 samples accounted for 90.6% of the failed amplifications, an indication of poor sample quality specific to those 5 samples.

Overall amplification success rapidly degraded after 12 h of field exposure, where pellets collected after 12–24 h had only 18% (108/600; SD 6.7) amplification success across loci. Sample-specific quality was again evident; 4 samples collected after 12–24 h contributed to 82.4% of the successful amplifications. Finally, amplification success after 24–36 h and after 36–48 h was 10.2% (61/600; SD 3.1) and 4.5% (27/600; SD 2.7), respectively (Fig. 2). Amplification occurred at 1 locus twice for 1 of the 8 extraction negative controls (1.7%; 2/120) and no amplification occurred in PCR negative controls.

The mean hourly temperatures for initial pellet collection (0–12 h) and additional exposure periods (24, 36, and 48 h) were 27.1 °C (80.9 °F), 26.8 °C (80.2 °F), 32.3 °C (90.2 °F), and 27.6 °C (81.7 °F), respectively. Precipitation total for the 16 d of initial pellet collection was 5.4 cm from 6 rain events. One rain event occurred during the 24 h exposure period totaling 0.25 cm.

Discussion

We determined that regurgitated pellets can be used as a source of viable vulture DNA. Our study found that this approach will be useful for noninvasive genetic sampling that targets nuclear DNA if initial field collection can occur within 12 h of pellet deposition. However, it is important to consider that this study was conducted mid-summer in Gainesville, Florida, USA. Environmental factors including heat and moisture are known to play a role in DNA degradation from noninvasive sources (Santini et al. 2007, Brinkman et al. 2010, Vili et al. 2013), therefore the rain that occurred during the 12–24 h exposure period likely contributed to the rapid and significant decline in amplification success. Additionally, rain events during initial pellet collection may have contributed to sample-specific differences in amplification success. Further, we observed digestion of our samples by insects, which may have contributed to the loss of viable vulture DNA on pellet surfaces. Thus, our 12 h collection time recommendation may be very conservative. As with all noninvasive studies it is best to trial a pilot study in a target study area to determine site-specific DNA persistence and collection challenges (Lonsinger et al. 2015).

We acknowledge that pellets exposed for each timepoint beyond 12 h received 1 additional freeze–thaw cycle and therefore may have negatively impacted results at these timepoints. How-

ever, research by Shao et al. (2012) shows that the negative effect of freeze–thaw cycles on DNA integrity is progressive, unlike the sudden loss of DNA quality we observed after 12 h. Also, the negative effect is most severe with larger fragments of DNA (Shao et al. 2012). We believe the impact of the additional freeze–thaw cycle was minimal because our amplified DNA fragments are short (Table 1).

Although collecting numerous pellets within 12 h of deposition may prove challenging for some avian species, roosting behavior of Black Vultures, which consists of birds aggregating in large conspicuous roosts, can allow for fruitful collection of pellets (Hill et al. 2021). We suggest clearing accumulated pellets from below roost sites and returning for collection of fresh pellets within 12 h. Our microsatellite amplification success rate of samples collected within 12 h (82.3%) is comparable with noninvasive genetic studies of scat (Broquet et al. 2007, Santini et al. 2007). This panel of 5 microsatellites also may serve as a prescreen of samples of unknown time since shedding. Specifically, pellets that produce ample amplification with this panel could be deemed viable and used to obtain individual identification using additional markers for the identified vulture species (Wostenberg et al. 2019).

Previous research by Wostenberg et al. (2019) identified this panel of 5 microsatellite loci to be used for species identification of Black and Turkey vultures due to different size ranges of alleles for each species within each locus. The current study confirmed this to be true, with alleles unique to Black Vultures falling within specified species-specific ranges (Wostenberg et al. 2019). In future applications, our PCR protocol can be used to differentiate North American vulture species and provide a tool for noninvasive demographic assessment of vulture roosts. Individual genotypes with additional microsatellite loci for more robust individual identification, relatedness studies, movement, and population differentiation could be gained by applying additional microsatellite vulture markers (Wostenberg et al. 2019). However, if the only goal of a study is to identify vulture species from pellets, we recommend that future research explores the use of mtDNA markers due to the far greater abundance of mtDNA per cell. Our success in obtaining bird DNA from regurgitated pellets can also open the door to noninvasive molecular ecology studies of other species of birds that also regurgitate pellets.

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