Testing PCR amplification from elephant dung using silica-dried swabs

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Abstract

Swabbing scat samples is a simple, non-invasive method of obtaining DNA samples for population genetic analysis of wild elephants. In this study, swab samples were taken from the scat of African savanna elephants inhabiting the Galana Wildlife Conservancy (GWC), bordering Tsavo East National Park in south-east Kenya. The swab samples were dried with silica as opposed to traditional methods of preservation in liquid or freezing while in the field. Furthermore, this study examined the rate of DNA degradation in scat samples in semi-arid conditions, typical of the Galana Wildlife Conservancy, by repeated sampling of scat in the field following deposition at defined time intervals over a period of two weeks. Results showed that both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) could be successfully amplified from scat samples up to 128 hours (about five days) following deposition. Significantly better results were obtained from samples taken from the outside of the scat than from the centre. Mitochondrial D-loop sequence data suitable for phylogenetic analysis was obtained from three fresh samples and one sample taken 16 hours after deposition. However, the sequence from a sample collected after 128 hours was not of sufficient quality for sequencing. Further research is required to determine at which point between 16 and 128 hours the DNA becomes too degraded for analysis. The mtDNA analysis showed the presence of the Southwest savanna subclade in two of the four samples from GWC, which has previously been found in only one other sample in Kenya. By confirming the effectiveness of silica-dried scat samples for use in DNA analysis, the results of this study will facilitate genetic analysis of African elephant populations and thereby contribute to efforts to conserve the gene pool of this keystone species. The paper contributes to the literature on collecting and preserving dung specimens with potential for conservation management.

Additional key words: eDNA, Polymerase chain reaction

Résumé

L'échantillonnage par prélèvement d'excrément est une méthode simple et non invasive d'obtention des échantillons d'ADN pour l'analyse génétique des populations d'éléphants sauvages. Dans cette étude, des échantillons ont été prélevés sur les excréments d'éléphants d'Afrique de la savane habitant le Galana Wildlife Conservancy (GWC), à la limite du Parc national de Tsavo East dans le sud-est du Kenya. Les échantillons ont été séchés avec de la silice par opposition aux méthodes traditionnelles de conservation en milieu liquide ou en congélation sur le terrain. En outre, cette étude a examiné le taux de dégradation de l'ADN des échantillons dans des conditions semi-arides, typiques du Galana Wildlife Conservancy, par échantillonnage répété sur le terrain après dépôt à des intervalles de temps définis sur une période de deux semaines. Les résultats ont montré que l'ADN nucléaire (ADNn) et l'ADN mitochondrial (ADNmt) pouvaient être amplifiés avec succès à partir d'échantillons de fragments jusqu'à 128 heures (environ cinq jours) après le dépôt. Des résultats significativement meilleurs ont été obtenus à partir d'échantillons prélevés de l'extérieur des excréments que du centre. Les données de séquence en boucle D mitochondriale appropriées pour l'analyse phylogénétique ont été obtenues à partir de trois échantillons frais et d'un échantillon prélevé 16 heures

après le dépôt. Cependant, la séquence d'un échantillon recueilli après 128 heures n'était pas de qualité suffisante pour le séquençage. D'autres recherches sont nécessaires pour déterminer à quel moment entre 16 et 128 heures l'ADN devient trop dégradé pour l'analyse. L'analyse de l'ADN mitochondrial a montré la présence de la sous-espèce de l'éléphant d'Afrique de la savane du sud-ouest, dans deux sur les quatre échantillons du GWC, ce qui ne se trouvait auparavant que dans un seul autre échantillon au Kenya. En confirmant l'efficacité des échantillons d'excréments séchés à la silice pour l'analyse de l'ADN, les résultats de l'étude faciliteront l'analyse génétique des populations d'éléphants d'Afrique et contribueront ainsi aux efforts de conservation du patrimoine génétique de cette espèce clé. Le document a apporté une contribution à la littérature sur la collecte et la préservation des spécimens d'excréments sur le terrain avec un potentiel pour la gestion de la conservation.

Mots-clés supplémentaires: Réaction en chaîne par polyméras, ADNe

Introduction

Performing population genetic analysis on wild animals requires a DNA source that can provide sufficient amounts of DNA from a large number of individuals, ideally without causing harm or stress to the target animals. Scat-derived DNA provides a non-invasive way to collect a wealth of genetic information on a population of wild animals, as previously shown with Asiatic wild ass (*Equus hemionus*, Pallas, 1775) (Renan et al. 2012), Iberian lynx (*Lynx pardinus*, Temminck, 1827) (Ramón-Laca et al. 2015) and African savanna elephants (*Loxodonta africana*, Blumenbach, 1797) (Wasser et al. 2004; Ishida et al. 2011). For large megafauna it is logistically simpler and safer to obtain scat-derived DNA samples than tissue samples (Dangolla et al. 2004; Gannon and Sikes 2007).

Most population genetic studies on wild populations of African savanna elephants that use scat as a source of DNA, preserve a portion of the scat sample in either ethanol (Wasser et al. 2004; Okello et al. 2005; Ishida et al. 2011) or dimethyl sulphide (Okello et al. 2008; Ishida et al. 2011). In addition, previous studies most often used fresh faecal samples, where defecation had been observed or when the outer layer of the sample was still noticeably moist. Such methodologies assume that DNA will degrade rapidly due to local environmental conditions, such as high temperature (Hájková et al. 2006), humidity/moisture content (Regnaut et al. 2006), exposure to UV light (Sinha and Häder 2002), microbial activity (Regnaut et al. 2006) and the presence of certain enzymes such as DNAases (Regnaut et al. 2006). A study on maned wolf (*Chrysocyon brachyurus*, Illiger 1815) found that freshness, as indicated by odour and moisture, predicted mitochondrial DNA (mtDNA) amplification success, and that visible freshness was an important criterion for sample collection (Vynne et al. 2011). Regnaut et al. (2006) found that enzymatic and microbial factors were the main cause of DNA degradation within a 24-hour period in faecal samples belonging to capercaillie (*Tetrao urogallus*, L.), with temperature having little effect. Under ideal conditions, very dry and shielded from UV radiation (Poinar et al. 1998), DNA present in scat can be preserved for much longer, as shown by the successful extraction and sequencing of ancient DNA from 20,000-year-old palaeofaeces (coprolites) derived from Shasta ground sloth (*Nothrotheriops shastensis*, Sinclair, 1905) that had been preserved in a cave in Nevada (Poinar et al. 1998). These findings suggest that under the right conditions DNA in scat samples remains intact for longer than conventionally thought, and that perhaps drying the sample could be an effective means of preservation.

Faecal swabs can be more effective than traditional methods, such as preservation in ethanol, for collecting a DNA sample from scat (Renan et al. 2012; Ramón-Laca et al. 2015). For example Ramón-Laca et al. (2015) showed that taking a swab from the outside of the scat sample and preserving the swab in a lysis buffer resulted in a significantly higher quantity and quality of nuclear DNA (nDNA) for herbivore scat compared to preserving a portion of the scat in ethanol. Renan et al. (2012) compared three methods of collecting and preserving DNA from Asiatic wild ass scat: (1) Scraping off the outer layer of the faeces, placing the scraped layer in ethanol for 24 hours and then drying with silica, (2) swabbing the outer layer of the faeces and then freezing the swab at –20°C, and (3) swabbing the outer layer of the faeces and placing the swab in a lysis buffer. The study found that using the frozen swab

combined with a QIAamp DNA Stool Mini Kit (Qiagen) worked best, with 100% amplification success for mtDNA and 91.7% success for nDNA, while the ethanol/silica method resulted in no mtDNA or nDNA amplification using the same extraction method (Renan et al. 2012). The study also found that the amplification success dropped to 50% for mtDNA and 45% for nDNA when using the cetyl trimethylammonium bromide (CTAB) method for extraction from frozen swab samples (Renan et al. 2012).

Based on the success of studies on other herbivores using the swabbing method (Renan et al. 2012; Ramón-Laca et al. 2015) this study aimed to evaluate a method by which swab samples were dried immediately with silica beads, rather than placed in a preservative liquid or frozen whilst in the field. Specifically we tested whether this method could be used with elephant scat samples to collect and preserve DNA and, after extraction using the CTAB protocol, amplify species-specific mitochondrial and nuclear loci. Furthermore, this study aimed to test if the mtDNA sequence data derived from this modified methodology was of sufficient quality for phylogenetic analysis. No genotyping using nDNA was performed in this study; instead nDNA amplification was used as a proof of concept.

Materials and methods

Study site

All field samples were collected from the Galana Wildlife Conservancy (GWC), located in south-east Kenya (3°04.518′ S, 39°17.665′ E) bordering Tsavo East National Park. The habitat in the two protected areas consists primarily of semi-arid scrub and animals can move freely between them as there is no fencing around the Conservancy (Ottichilo 1986). The area is part of the larger Tsavo-Amboseli ecosystem, which in turn is contiguous with the Mkomazi ecosystem in neighbouring Tanzania. This huge cross-border ecosystem is one of the strongholds of the African savanna elephant in East Africa and currently home to an estimated 12,866 elephants (Ngene et al. 2017).

Sample collection and preservation

Preliminary analysis to test collection methods and develop lab protocols for swab samples was conducted using mouth and faecal swab samples obtained from six Asian elephants (*Elephas maximus* L.) in Dublin Zoo, Ireland, prior to field work in the GWC. All lab work on samples collected in the field was undertaken in University College Dublin (UCD).

Field sampling was conducted from the 18 May to the 27 May 2017. In a normal year this would be towards the end of the rainy season; however in May 2017 the area was experiencing a period of prolonged drought. Sighting surveys for adult elephants were conducted in the morning (between 06:00 and 08:00 h local time), in order to facilitate sample collection during daylight hours at exponential sampling time points (0, 1, 2, 4, 8, 16, 32, 64, and 128 h). After an animal had been located it was followed until it was seen to defecate. The time and GPS location (Garmin GPS 60™) of defecation was noted, along with the gender of the elephant. Due to limited time for field work and the large amount of time required to be spent at each faecal bolus, the number of focal individuals was limited to three, with one additional individual (Animal C) being sampled once, as the elephant did not move away from the sample. A total of 61 swab samples were successfully collected from four individuals, two males (denominated animals A and B) and two females (C and D) (Table 1).

As a faecal bolus moves through the intestines of an animal it sloughs off intestinal epithelial cells which results in most animal scat being covered in a thin layer of epithelial cells that can be used as a source of DNA (Ramón-Laca et al. 2015). Synthetic tipped sterile swabs were dipped in phosphate buffered saline (PBS) solution (pH 7.4) and then rubbed on the scat sample until the swab was visibly covered in faecal material. The PBS solution was used as older samples had dried significantly and required a liquid solution in order to swab the sample and maximise epithelial cell yield. For consistency the PBS solution was used on all samples, regardless of sample freshness. The swab tip was then cut off and placed in a 2-ml O-ring tube (Eppendorf) containing approximately six silica beads (2.5–6.0 mm)(Fisher Scientific) per sample. The O-ring was then further sealed with parafilm. Swab samples were taken at exponential time intervals, with time zero (0 h) recorded as the time the first samples were taken after the animal had moved on (Table 1). At each time point three swabs were taken from the

faecal bolus; from the upper surface (top), from the side just above where it was in contact with the ground (underside), and from the core (centre) of the sample. To reach the centre of the sample a hole was bored into the sample; the hole was resealed between sampling times by placing removed faecal matter in the entrance of the hole. Initial defecation by animal C took place at 17:30 h but as the animal did not move away, the first and only swab sample was taken 16 h later, the following morning at 9:20 h from the top of the scat.

Samples were not frozen in the field or during transport, which meant the swab samples taken and preserved on the first day were kept for 10 days at temperatures averaging 30°C. Once in the lab in UCD the samples were kept at –20°C.

DNA extraction

Faecal samples can carry various PCR inhibitors from soil, the animal's diet and the digestive system which need to be taken into account when choosing an extraction protocol (Ramón-Laca et al. 2015). As the GWC had been experiencing a drought at the time of the study there was likely a high percentage of fibrous plant material present in the diets of the study elephants, and therefore large amounts of PCR-inhibiting polysaccharides in the faecal samples. It was therefore decided to use a modified version of the CTAB protocol for DNA extraction, as CTAB has been shown to bind to polysaccharides (Möller et al. 1992). Each swab was first placed in a lysis mix of CTAB and proteinase K (20 mg/ml) and left to digest overnight at 56°C. RNaseA was then added to the solution and incubated at 37°C for 30 min. After this stage the swab was removed from the mix and placed back in an Eppendorf tube and stored at –20°C. DNA was then extracted using chloroform-isoamyl alcohol and following this, isopropanol and ethanol were used to precipitate the DNA. For each set of extractions, a human cheek swab was used as a positive extraction control and purified water was used as a negative extraction control. A volume of 1 µl of each DNA extract was used for DNA quantification using a BioDrop µLITE spectrophotometer.

Mitochondrial amplification

A 630-bp segment of the hypervariable left peripheral domain of the D-loop control region (Douzery and Randi 1997) was amplified using the primer pair MDL3 [CCC ACA ATT AAT GGG CCC GGA GCG] and MDL5 [TTA CAT GAA TTG GCA GCC AAC CAG] (Fernando and Lande 2000). Reagents (total volume 25 µl) were prepared for PCR in a UV sterilized hood, each containing 2.5 µl 10X Buffer (Kapa Biosystems), 2.5 µl 10 mM dNTP (Invitrogen), 0.5 µl of each primer (10 µM) (Integrated DNA Technologies, EU), 0.1 µl Taq polymerase (Kapa Biosystems), 2.0 µl BSA (20 mg/ml)(Thermo Fisher Scientific), 15.9 µl water and 1.0 µl undiluted DNA extract. PCRs were run on a ProFlex PCR system (Thermo Fisher Scientific) at 95oC for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The final stage was 72°C for 10 min, after which the temperature was reduced to 4°C. For each PCR reaction, a DNA sample derived from a mouth swab of a male Asian elephant was used a positive control, while water was used as a negative control. DNA was amplified in a separate room to where it was extracted. PCR products were then stained

Table 1. Data on four African elephants in Galana Wildlife Conservancy, Kenya, from which scat was sampled for DNA analysis. Time zero (0 h) was the time the first swab sample taken after the animal had moved away from the sample, except for animal C for which time zero corresponds to 16 h after deposition.

Animal	Sex (M/F)	Time zero (h)	No. of swabs	Sampling location
A	M	07:42	25	3°03.681′ S 39°13.738′ E
B	M	07:47	18	3°03.767′ S 39°14.071′ E
C	F	09:20	1	3°03.912′ S 39°13.914′ E
D	F	06:34	17	3°03.865′ S 39°13.821′ E

with a 6X gel loading buffer and run on a 1% agarose gel (SafeView gel stain) with a GelPilot 100 bp ladder (Qiagen). To test the significance of the results (x) for amplification rates based on sample location, the mean (μ) percentage of amplification success was calculated from the three locations (Top, Underside, Centre) and this was used to find the standard deviation (σ) and z-score (z) using the following calculation: $z = (x - \mu) / \sigma$. The z-score was then used to find the p-value for each result (* p<.1, ** p<.05, *** p<.01, **** p<.001).

Microsatellite amplification

To examine whether nDNA could be amplified, the African savanna elephant microsatellite locus Lat13, which ranges from 234 to 262 bp in length, was chosen as the target sequence (Archie et al. 2003). The primers F:[TGA GCT TCT GTA GGC TCT GA] and R:[GCA CTC GAT AAA CAG TGT TGA] were used (Archie et al. 2003). Reaction volumes of 20 μl were prepared for PCR in a UV sterilised hood, each containing 2.0 μl 10X buffer, 0.4 μl 10 mM dNTPs, 0.8 μl of each primer (10 μM), 0.08 μl Taq polymerase, 13.42 μl water and 2.5 μl DNA. To assess the most efficient annealing temperature, a gradient PCR (46–60°C) was run on a Biometra thermocycler. PCRs were then run at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 56°C (based on the gradient PCR results) for 30 s and 72°C for 1 min. The final stage was 72°C for 2 min, after which the temperature was reduced to 4°C. All samples were amplified in a separate room to where they were extracted. Microsatellite PCR products were then stained with a 6X gel loading buffer and run on a 2% agarose gel with a GelPilot 50 bp ladder. Unless otherwise stated, suppliers and equipment for this analysis were the same as for mitochondrial amplification. The Asian elephant mouth derived DNA could not be used as a positive control due to the species specificity of the microsatellite primers. Purified water was used as a negative control. The significance of the results for amplification rates based on sample location was calculated with the same method as for the mitochondrial amplification rates.

Mitochondrial sequence analysis

Five successfully amplified mtDNA PCR products (both strands) were sent for commercial Sanger sequencing(Macrogen). Only samples which had shown clear bands after gel electrophoresis of amplicon were chosen for sequencing. The sequence data were analysed using Geneious version 10.2.3 (Kearse et al. 2012), which was used to align the forward and reverse strands of each sequence, with the unaligned ends of the sequences being removed. Aligned sequences were then checked through BLAST nucleotide search (Wheeler et al. 2007). Sequence data generated by Ishida et al. (2013) were used to supplement the sequences generated in this study for a haplotype analysis. From the data generated by Ishida et al. (2013) only sequences which corresponded to samples from either Kenya or Tanzania were utilised (n = 237; GenBank accession nos. JQ438119–JQ438771). ClustalW (Larkin et al. 2007) was used to align the 630 bp D-Loop sequences. A minimum spanning network (Bandelt et al. 1999) was created in PopART (Leigh and Bryant 2015) using the sequences from Ishida et al. (2013) and four of the sequences generated in this study (see Fig. 3 below). Sequences from the GWC samples were also assigned to mtDNA subclades based on nucleotide polymorphism data published by Ishida et al. (2013).

Results

DNA extraction and concentration

The extraction protocol resulted in 58 of the 61 (98%) swab samples yielding DNA. DNA concentrations ranged from 0.21 to 12.19 μg/ml (Table 2) with the exception of two samples from animal A, namely those taken from the centre of the scat after 1 h, and from the top of the scat after 2 h which yielded DNA concentrations of 51.71 and 47.42 μg/ml, respectively. It is important to note that these figures are for total DNA, which includes any microbial and plant DNA present in addition to the target DNA, and as such is a useful but imperfect index of target DNA yield. DNA concentrations for the post-PCR samples ranged from 507.1 to 725.8 μg/ml for mitochondrial amplification and from 115.6 to 143.9 μg/ml for microsatellite amplification.

Mitochondrial DNA amplification

The D-loop gene fragment was successfully amplified at least once from one of the four animals across all time-points (Fig. 1). Within the first 8 h, the 4-h time point yielded the highest amount of successful amplifications, with 89% (8 out of 9 samples) of

Table 2. Results for swab samples collected from the top (T), underside (U) and middle (M) of scats deposited by four elephants (animals A–D) in Galana Wildlife Conservancy, Kenya, showing concentrations of total DNA and results of mtDNA and nDNA amplification (where 'Y[es]' indicates the presence of a correct size band after gel electrophoresis). Only one sample) was collected from animal C, from the top of the scat when the animal first moved away 16 h after deposition (not shown in the table, to save space). This sample yielded a DNA concentration of 0.954 µg/ml and successful amplification of mtDNA, but not nDNA.

		Animal A			Animal B			Animal D		
Time (h)	Swab location	DNA conc. (µg/ml)	mtDNA	nDNA	DNA conc. (µg/ml)	mtDNA	nDNA	DNA conc. (µg/ml)	mtDNA	nDNA
0	T	7.947	Y	Y	6.016	Y	Y	1.374	Y	Y
	U	0.825	Y	Y	2.663	-	Y	1.985	Y	Y
	M	0.932	Y	-	2.228	-	-	1.957	Y	-
1	T	0.207	Y	Y	1.284	-	Y	0.741	Y	Y
	U	10.89	Y	Y	2.552	-	-	1.701	Y	Y
	M	51.71	-	-	1.642	-	-	0	-	-
2	T	47.42	-	-	0	-	-	0.619	Y	Y
	U	12.19	Y	-	11.38	-	Y	1.451	Y	-
	M	0.566	-	-	1.358	-	-	1.268	Y	-
4	T	0.596	Y	Y	2.946	-	Y	1.81	Y	Y
	U	0.565	Y	Y	1.061	Y	Y	1.215	Y	-
	M	1.779	Y	-	3.19	Y	-	0.908	Y	-
8	T	0.924	-	Y	0.275	-	-	0.481	-	Y
	U	0.32	Y	-	0.092	-	-	4.894	Y	Y
	M	2.183	-	-	1.451	-	-	1.198	-	-
16	T	0.725	Y	Y						
	U	0.603	Y	-						
	M	2.176	Y	-						
24	T				0.42	Y	-			
	U				0.832	-	-	3.338	Y	Y
	M				0.481	-	-	0.305	-	-
32	T	0.42	Y	Y						
	U	1.093	Y	-						
	M	5.262	Y	Y						
64	U	0.444	-	Y						
	M	1.703	Y	-						
128	U	1.367	-	Y						
	M	0	Y	-						

DNA extracts showing bands after mtDNA PCR. The number of swab samples collected after 8 h dropped significantly due to difficulties in revisiting samples in the field and/or due to scat samples being dismantled by dung beetles. For animals B and D the next time point was changed to 24 h instead of 16 h due to difficulties reaching the samples at the desired time. With regards to the sample location, swabs taken from the underside of the sample recorded the highest amplification success at 67% (n = 21, p = 0.1789)(p-values represent the probability that a randomly generated result will be equal to the observed result), followed by top (upper surface) swabs at 63% (n = 19, p = 0.3099) and centre (core) swabs at 48% (n = 21, p = 0.0836) (Fig. 2).

Nuclear DNA amplification

Amplification success was based on the presence of a band (at ~250 bp) after gel electrophoresis using PCR product that had been amplified with the Lat13 microsatellite primer. Nuclear DNA was successfully amplified at least once from one of the four animals across all time points sampled (Fig. 1). There was a difference in the amplification success based on whether the swabs had been taken from the outside of the sample (top and underside) versus inside the sample (centre), with only one successful amplification of nDNA from a centre sample (Fig. 2) out of 21 samples (5% success rate, p = 0.08441). Nuclear DNA was successfully amplified from 74% of top swabs (n = 19, p = 0.1661) and 58% of underside swabs (n = 21, p = 0.3421) (Fig. 2).

Mitochondrial DNA sequencing

Four of the five samples sent for commercial Sanger sequencing resulted in a usable DNA sequence. Alignment of forward and reverse strands gave sequence segments of varying lengths: 530 bp for animal A (0 h), 520 bp for animal B (0 h), 599 bp for animal C (16 h); and 567 bp for animal D (0 h). Three samples (from animals A, B and D) which had been collected from the top of the scat when fresh all matched 100% to African savanna elephant isolates available on GenBank (Accession nos. JQ438407, JQ438674 and JQ438411). The sequence for animal C matched 100% to the same isolate as animal B. Sequence data from the fifth sample (from animal A, from the top of the scat after 128 h) was unusable and no significant similarity could be found to other sequences via BLAST analysis.

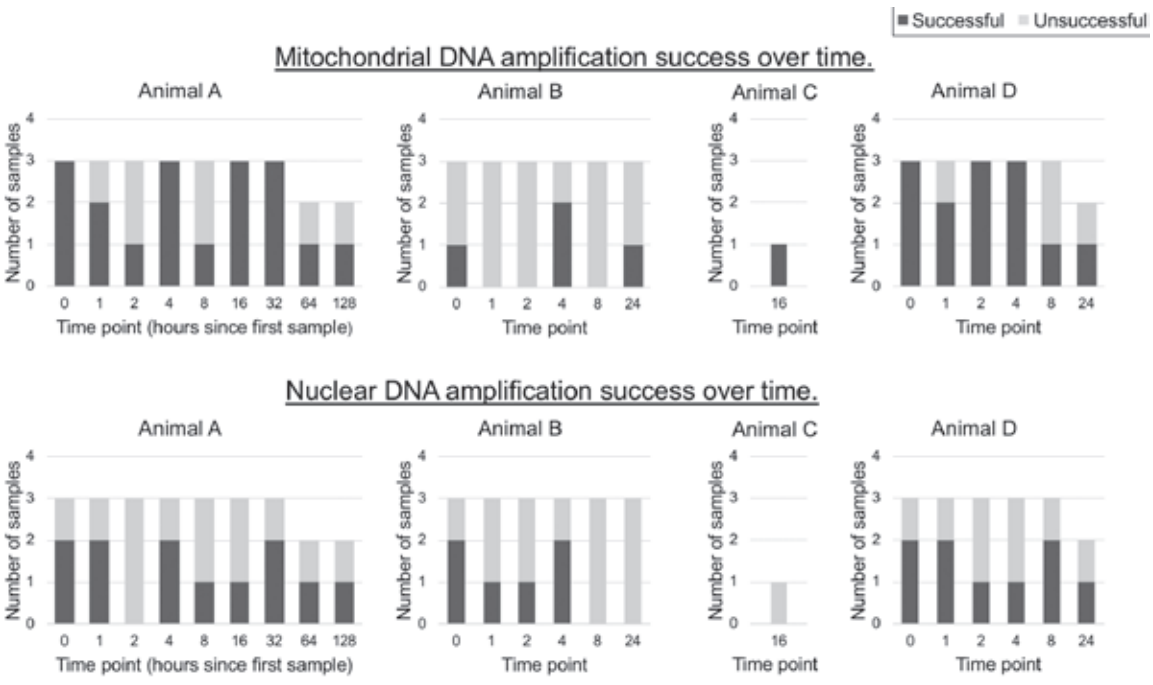

Figure 1. PCR amplification success for mtDNA and nDNA in scat samples collected from four elephants (animals A–D) in Galana Wildlife Conservancy, Kenya, based on age of scat. Time zero (0 h) is the point at which the first sample was taken, with the exception of animal C, for which the first and only sample was taken at the 16-h time point.

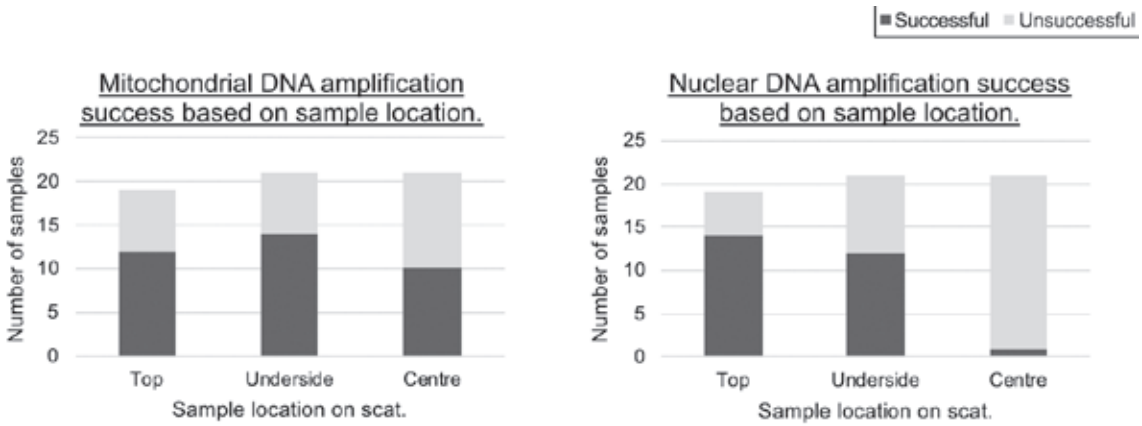

Figure 2. PCR amplification success for mtDNA and nDNA based on scat swab source (top, underside or centre), with results pooled across all four individuals and all time points.

From the four D-loop sequences obtained, three haplotypes were observed: Animals A and D each had unique haplotypes previously identified in numerous elephants in Kenya, corresponding to the savanna-wide mtDNA subclade (Figure 3; see colour plates: page ii). Animals B and C shared a haplotype which had previously only been identified in one other individual in Kenya, a male elephant in Amboseli National Park (Ishida et al. 2013), and belonging to the Southeast-savanna mtDNA subclade (Fig. 3).

Discussion

DNA analysis provides valuable information for genetic population studies of African elephants, whose results can inform continent-wide conservation strategies. Scat-derived DNA provides a non-invasive way to collect samples for DNA analysis. However sampled material deteriorates rapidly and preservation of samples taken in the field for laboratory analysis can be challenging. The combination of swabbing scat and storage of the swab samples with silica beads allows samples to be transported to the laboratory without the requirement for refrigeration or for use of preservative liquids such as ethanol. In our study, samples taken on the first day were kept at an ambient temperature that averaged 30°C for 10 days before being placed in a –20°C freezer in the UCD laboratory. Results show that storage with silica beads is a simple and effective way to preserve scat-derived DNA, as evidenced by the successful amplification and sequencing of mtDNA D-loop region, as well as amplification of nDNA. Of the swabs taken from fresh samples (0–2 h after deposition, n = 18), mtDNA and nDNA were both amplified from 61% of swab samples. Whilst the study by Renan et al. (2012) had 100% amplification success for mtDNA and 91.7% success for nDNA using the frozen swab combined with a QIAamp DNA Stool Mini Kit, the method presented in our study has the advantage of being significantly easier to prepare and transport samples, opening up the possibility for large scale studies without expensive equipment. It should be noted also that when the CTAB extraction was used by Renan et al. (2012) to extract DNA from swabs taken from fresh samples frozen in the field, mtDNA and nDNA amplification rate dropped to 50% and 45%, respectively (n = 8).

Both mtDNA and nDNA were successfully amplified from at least one sample at all time-points. While the amplified mtDNA from the fresh samples and the sample taken 16 h after deposition resulted in unambiguous sequence reads, which all matched 100% to previously identified African savanna elephant mtDNA haplotypes, mtDNA sample taken 128 h after deposition resulted in ambiguous sequence without any successful matches to sequences in GenBank, despite an indication of successful amplification based on the presence of a fragment of an appropriate size on an agarose gel. Further studies are therefore needed to determine at which point between 16 and 128 h the DNA in scats becomes too degraded for analysis. However, the successful amplification and sequencing of mtDNA from the sample taken after 16 h suggests that the time after which DNA can be obtained from a sample is likely longer than the conventionally recognised time

frame, in which scat samples are still visibly fresh. Whether the same time frame would apply to nDNA could not be determined, as this study only amplified nDNA using conventional PCR, and a full microsatellite analysis via genotyping was not conducted. It should be noted that a strongly performing microsatellite locus does not necessarily reflect potential results from other, more difficult to amplify loci. In addition, despite correct sized bands in the gels, the nDNA was not sequenced in this study so it cannot be said for definite that the correct gene locus was amplified.

With regards to which location on the scat samples was best for collecting DNA, for both mtDNA and nDNA the outside of the scat proved to be significantly more reliable than samples from the centre (with only one successful nDNA amplification out of 21 from centre swabs), while differences observed between top and underside swabs for both mtDNA and nDNA amplification success were shown to be statistically insignificant. The difference between the success of swabbing from the outside of the scat compared to the inside is likely due to the presence of intestinal cells in the mucosal layer that develops on the outside of the faecal bolus as it passes through the digestive system.

BLAST analysis identified the savanna-wide mtDNA subclade in animals A (a large male) and D (female), which was to be expected as this subclade has previously been recorded throughout Kenya (Ishida et al. 2013). However, the other two, one male and one female, were found to belong to the Southeast-savanna subclade, which is prevalent in southern Africa (Ishida et al. 2013) but previously found in Kenya only in a single male elephant in Amboseli, close to the border with Tanzania (Ishida et al. 2013). The presence of this subclade in the GWC could be the result of shared ancestral variation with northern Tanzanian populations or recent admixture acquired from elephants moving north.

In conclusion this study has shown that a combination of faecal swabbing and preservation in silica is a simple and effective means to collect scat-derived DNA samples that are of sufficient quality for phylogenetic analyses, as seen by the mtDNA sequence analysis, and that scat samples taken up to at least 16 h following deposition can be used as a source of DNA. Discovery of the presence of the Southeast-savanna subclade in GWC was a notable result of this study of just four individual elephants. It demonstrates the potential for larger studies using the same method to yield new insights into the genetic population structure of African savanna elephants and thereby contribute to efforts to conserve the gene pool of this keystone species.

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