KENYA WILDLIFE SERVICE

Laboratory Progress report

Building capacity and developing tools for elephant censuring and elephant mortality monitoring in tropical montane and forest habitats in Kenya

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Summary:

This project aimed to; establish the status of the lesser known populations of elephants residing in forest landscapes in Kenya using dung counts; develop tools for elephant population estimation in montane forest dwelling elephant using dung counts, by establishing dung decay rates and dung defecation rates for these populations and habitats, and; validate elephant estimates derived from dung counts using fecal DNA capture recapture of unique elephant genotypes since this technique is known to be accurate and more precise for estimating elephant density, but routine use is expensive. Though comparatively more expensive than line transect dung density, non-invasive fecal DNA capture recapture techniques have been successfully used to provide wildlife population estimates, especially for low density populations.

Introduction:

Survey techniques using dung counts is a well-established technique for censusing elephants in forested or heavily wooded habitat where limited visibility prohibits the use of direct observation based methods such as aerial counts and road transects. However, accurate and precise population estimates are dependent on good estimates of the dung defecation rates and dung decay rates. Most estimates of dung decay and defecation rates have been estimates for lowland tropical rainforest and there is a dearth of information for elephant defecation rates in tropical montane forests or coastal forests where most elephants in Kenya reside(Walsh and White 2005, Hedges et al. 2013). Recent studies show that DNA based capture recapture methods produce elephant population estimates that are more precise and reliable (Hedges et al. 2013).

In addition to the need to regularly update elephant population trends, there is an international requirement by CITES to standardize elephant monitoring, which was to be implemented through the CITES-MIKE program. To achieve this, the MIKE program developed a monitoring protocol for elephant populations within the designated MIKE sites.

In Kenya, Mt Elgon ecosystem is one of the designated MIKE sites, alongside Tsavo, Meru and Samburu-Laikipia and hence the need for a standardized monitoring program, especially on monitoring of illegal killing of elephants. This document therefore reports on the status of implementation of the fecal DNA capture recapture, and training of monitoring rangers for the Mt. Elgon ecosystem.

Methods

Field collection

Field sample collection and training of monitoring rangers for Mt. Elgon elephant population was conducted for a period of 10 days from 5-15th February 2017, where a total of 59 elephant fecal samples (labeled MES1 to MES59), were obtained from fresh randomly selected elephant dung piles (Fig.1). We collected approximately 20g of fecal scrapping of epithelial layer from dung in every sample, with disposable plastic gloves, and stored this mass in 95% ethanol using polypropylene fecal pots at room temperature.

Fig.1 Field collection of elephant dung samples

DNA analysis

i. DNA extraction:

DNA extraction was done by using QIAamp DNA Stool mini kit. The faecal samples were weighed (180mg) of stool in 2ml microcentrifuge tube 1 ml of inhibit-EX buffer to each stool sample was added to remove PCR inhibitors. Vortexing was then done to achieve homogenization. Centrifugation was done for 1 minute to pellet stool particles. 25µl proteinase K was pipette into a new 1.5ml microcentrifuge then 600 µl of the supernatant was transferred into 1.5ml microcentrifuge tube containing proteinase K. Buffer AL (600µl) was then added and vortex for 15s. Incubation was then done for 10 minutes. The supernatant was transferred into a 1.5ml microcentrifuge tube containing proteinase K. 600 µl of ethanol (96-100%) was added to the lysate and mixed by vortexing. 600µl lysate of the lysate was put in the QIAmp spin column and centrifugation was done for 1 min. The

QIAmp spin column was placed in a new 2ml collection tube and the tube containing the filtrate was discarded. 500µl buffer AW1 was added and centrifugation done for 1 minute. The QIAamp spin column was placed in a new 2ml collection tube and the filtrate discarded. 500 µl buffer AW2 was added and centrifugation done for 3 minute. Collection tube containing the filtrate was discarded. The QIAamp spin column was placed in a new 2ml collection tube and the old collection tube discarded with the filtrate. Centrifugation was done for 3min and the QIAamp spin column transferred into a new labeled 1.5ml microcentrifuge tube and 70µl buffer ATE directly added onto the QIAmp membrane. Incubation at room temperature and centrifugation was done for 1 minute to elute DNA. DNA was stored at -20°c.

ii. PCR amplification:

A conventional Polymerase Chain Reaction (PCR) was performed on the DNA samples along with a pcr negative control with no template. MDL3 and MDL5 primers (table 1) amplifying a 630 region of the dloop region were chosen for amplification (Lee et al., 2013). The PCR reaction mix was prepared using HotStarTaq Master Mix kit (Qiagen, Cat no. 203205) as indicated in table 2 and the amplification utilized the cycling conditions indicated in table 3. The polymerase chain reaction carried out in T100TM Thermal cycler. DNA amplification of mitochondrial Dloop region was carried out using Hotstartaq master mix kit (Qiagen, Germany) according to manufacturer's protocol. The quality control (QC) was ensured through the use of a negative control sample in the polymerase chain reaction for quality assurance (QA).

Amplified products were run on a 1% agarose gel in TBE buffer for 1 hour at 70Volts. The gel was visualized under UV to observe amplicon bands and confirm they are the right size.

Samples were then sent to Inqaba South Africa for sequencing.

Table 1: The primers used for the PCR amplification

Primer ID:	Primer sequence	Tm
MDL3	5'-CCC ACA ATT AAT GGG CCC GGA GCG-3'	63°c
MDL5	5'–CCC ACA ATT AAT GGG CCC GGA GCG-3'	63°c

Table 2: The PCR reaction mix preparation

Reagents	*1 sample	* 60
HotstarTaq	12.5	750
Forward primer	1.25	75
Reverse primer	1.25	75
Water	8	480
Template	2.0	-
Total volume	25.0	

iii. Sequence editing and Analysis

Raw traces from Inqaba were assembled on Geneious V8. The sequences were edited and trimmed.

The consensus sequences were run through the established National Center for Biotechnology Information (NCBI) nucleotide databases using the nucleotide algorithm of the Basic Local Alignment Search Tool (BLASTn).

iv. Microsatellite Analysis

Microsatellites were amplified in 10 µL reaction volumes each containing 1.5 mm $MgCl_2$, 200 µm dNTP, 10 µg of bovine serum albumin (BSA), 2.5 pm of each primer (forward primer labelled with fluorescent dyes FAM, VIC or NED; Applied Biosystems), 0.4 U of Amplitaq Gold DNA polymerase (Applied Biosystems) and 1 µL of extracted DNA. PCRs were performed in an AB Veriti Thermal Cycler. The thermal profile for PCR amplification for LaT05, 13 was 95 °C for 10 min, followed by one cycle of 95 °C for 30 s, 66 °C annealing temperature for 30 s and 72 °C for 30 s. In the subsequent 10 cycles, all conditions were the same except that the annealing temperature decreased by one degree per cycle. This was followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 30 s. The thermal profile ended with a single extension of 72 °C for 5 min. For LaT06, 16, 25 and 26 the thermal profile for PCR amplification was 95 °C for 10 min, then 40 cycles of 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s, followed by a single extension of 72 °C for 5 min.

STR Locus	Design ation	Repeat Motif	Allele range	Number of alleles	Forward Sequence	Reverse Sequence
LaT05	p17	(CCAT)2 CCAC (CCAT)14 CAT (CCAT)17	265-505	22	HEX - CACCACCCATCCATCTGT	TGGCTTCTGTGAGTTCACC
LaT06	p18	(CCAT)13	291-405	24	6FAM - AGCCAGGCACATTAAGTGT	TCTCCTAGAAAAGGTTACCACA

Preliminary Results

44 out of 59 samples collected were successfully amplified for Elephant DNA and sequenced.

Blast analysis showed 99-100% match to African Savanna Elephant (*Loxodonta africanah*)

References

1. Hedges, S., A. Johnson, M. Ahlering, M. Tyson, and L. S. Eggert. 2013. Accuracy, precision, and cost-effectiveness of conventional dung density and fecal DNA based survey methods to estimate Asian elephant (Elephas maximus) population size and structure. Biological conservation 159:101-108.
2. Ishida, Y., Oleksyk, T.K., Georgiadis, N.J., David, V.A., Zhao, K., Stephens, R.M., Kolokotronis, S.O. and Roca, A.L., 2011. Reconciling apparent conflicts between mitochondrial and nuclear phylogenies in African elephants. PloS one, 6(6), p.e20642.
3. Lee, Eun-jung, Yang-han Lee, Seo-hyun Moon, Nam-ye Kim, Soon-hee Kim, Moon-sik Yang, Dong-ho Choi, and Myun-soo Han. "The identification of elephant ivory evidences of illegal trade with mitochondrial cytochrome b gene and hypervariable D-loop region." Journal of forensic and legal medicine 20, no. 3 (2013): 174-178.
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APPENDIX 1: Purchase of Consumables and Reagents for AEF project.

AMOUNT: Ksh. 1,995,000

Introduction Brief:

The African Elephant Fund Project is aimed at building capacity and developing tools for elephant censoring and elephant mortality monitoring in tropical montane and forest habitats in Kenya. In order to carry out this mandate, fresh dung samples need to be collected and fecal DNA analyzed for individual recognition, sex identification and age determination. The items being purchase are required for this laboratory analysis.

Field sample collection		
Item	**Amount**	**Cost**
Fecal pots	1000	50,000
Qik dri paper towels	10 packets	2,000
Permanent marker pens	6	3,000
Nitrile gloves powder free (medium)	10	10,000
Nitrile gloves powder free (large)	10	10,000
Scalpels	10boxes	10,000
Garbage disposal bags	10	1,000
Ethanol	10liters	40,000
Liquid nitrogen	30litres	30,000
Lab DNA analysis		
Plastic cryoboxes	20	20,000
Eppendorf tubes (1.5ml)	5pk (500tubes)	20,000
PCR tubes (0.2ml)	5pk (1000tubes)	50,000
White pipette tips (10ul)	10 pks (1000tips)	20,000
Yellow pipette tips (200ul)	5pk (1000tips)	10,000
Blue pipette tips (1000ul)	5pk (1000tips)	10,000
Gel red DNA stain	1	35,000
Aluminum foil	5 rolls	5,000
Qik dri paper towels	20 packets	4,000
Laboratory grade ethanol	2 (5litres)	40,000
Gelpilot 100bp plus ladder	3	60,000
Nitrile glove-powder free Small	20	20,000
Nitrile glove-powder free Medium	20	20,000
Nitrile glove-powder free Large	20	20,000
Scalpels	10boxes	10,000
QIAamp DNA Stool mini kit (50preps)	5	200,000
Rotor-Gene Q Accessories: PCR Strip of 4 Tubes 0.1ml and	2 packets (1x250	20,000

Caps	strips)	
5X HOT FIREPOL® Evagreen® Mix (no Rox)	10	100,000
One Taq quickload 2X mastermix with standard buffer (500rxns)	10	200,000
fecal pots	400	20,000
Qiagen Dneasy blood and tissue kit (250preps)	1	125,000
Agarose powder	2(500gms)	150,000
Primers	20	40,000
Full sequencing service unique amplicons	200	300,000
Fragment Size Analysis service	340	340,000
TOTAL		1,995,000