Species Identification of Dhole Samples Using Non-Invasive Genetics

Report Submitted to:

Department of National Parks and Wildlife Conservation,

People's Trust for Endangered Species and Rufford Small Grants Foundation

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Introduction

Asiatic wild dogs or Dholes (*Cuon alpinus*) are one of the top most imperiled and the least known large carnivores worldwide. IUCN classifies these animals as endangered and estimates their population at fewer than 2500 mature individuals around the world. There is no known dhole population fully secured within a protected area and no known dhole population is above 250 anywhere. Their population is continuously declining due to the loss of habitat, prey base and possibly disease transfer from feral dogs. To address the risk that the dholes are facing, we initiated conservation actions in 2010 in the far north east region of Nepal, the Kangchenjunga Conservation Area (KCA). KCA (2035 sq.km) is one of the most remote conservation areas located in north eastern Nepal connecting Singalila National Park of India in the east and Qomolangma National Park of China in the north. KCA altitudinal ranges from 1200m at Chiruwa to the third highest peak of the world Mt. Kangchenjunga (8586m). KCA is one of a few community managed conservation areas in Nepal. The government of Nepal handed over the management responsibility of KCA to the local community establishing Kangchenjunga Conservation Area Management Council (KCAMC) in 2006.

We conducted dhole sign surveys in 2012 and collected eighty-two putative dhole scat samples and sent them to the Center for Molecular Dynamics Nepal for species identification. Sixteen samples were randomly chosen to test the effectiveness of a d-loop, dhole-specific primer. Unfortunately, all of the samples (including the positive control) failed to amplify. Nine of the original 16 samples were then tested for a general carnivore id using carnivore specific primers. All nine of the samples were shown to be carnivore positive. We then tried the dhole-specific primers again on 20 new samples and these all showed positive results. With the effectiveness of the species-specific primer shown, all the scat samples were screened using this primer. Using species-specific primers, with confirmation with sequencing, 6 out of the 82 scat samples were revealed to belong to dhole.

Methodology

SCAT DNA EXTRACTION

Scat DNA was extracted using the QiagenQIAamp DNA Stool kit (Qiagen, Germany). Around 200mg of dried scat sample was scraped on the surface with sterile scissor and tweezers and was lysed in Lysis Buffer ASL and the supernatant was subjected to InhibitEX tablet treatment to adsorb PCR inhibitors. Proteinase K was added to the remaining supernatant and buffer AL was added and incubated for 10 minutes at 70°C. DNA was eluted to a total volume of 150ul.

SPECIES IDENTIFICATION USING SPECIES-SPECIFIC PRIMERS

The dhole specific primers used for species identification and the PCR conditions are given below. The expected product size was ~319 bp.

Primers: D-LOOP-F: 5'CTACCATCAACCCCCAAAGC 3'

D-LOOP-R: 5'GCAAGGATTGATGTTTTCTCG 3'

Component	per rxn
Master Mix (2x)	7.50
Q solution (5x)	1.50
D-Loop- f	0.20
D-Loop- r	0.20
dH20 from kit	2.60
DNA	2.00
Total volume	14.00

Thermocycling		
Initial denaturation	95°C	15 min
Denaturation :	94°C	30 sec
Annealing:	**57°C**	30 sec
Elongation :	72°C	60 Sec
Number of cycles :		**45**
Final elongation	72°C	10 min
Cool Down	4°C	Hold

CARNIVORE IDENTIFICATION USING CARNIVORE-SPECIFIC PRIMERS

Carnivore identification on nine of the scat samples was done using the carnivore-specific primers and PCR conditions given below.

Primers: CYTB-SCT-F: 5' AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA 3'

CYTB-SCT-R: 5' TATTCTTTATCTGCCTATACATRCACG 3'

Component	per rxn
Master Mix (2x)	12.5
Q solution (5x)	2.5
CPrimer- f	0.63
CPrimer- r	0.63
dH20 from kit	6.74
DNA	2.00
Total volume	25.00

Thermocycling		
Initial denaturation	95°C	15 min
Denaturation :	94°C	30 sec
Annealing:	55°C	30 sec
Elongation :	72°C	60 Sec
Number of cycles :		**45**
Final elongation	72°C	10 min
Cool Down	4°C	Hold

SPECIES CONFIRMATION USING PRODUCT SEQUENCING

PCR products which amplified using Dhole-specific primers were sequenced on an ABI 3130 machine. DNA sequences were BLAST searched in the NCBI database to confirm the species.

Results

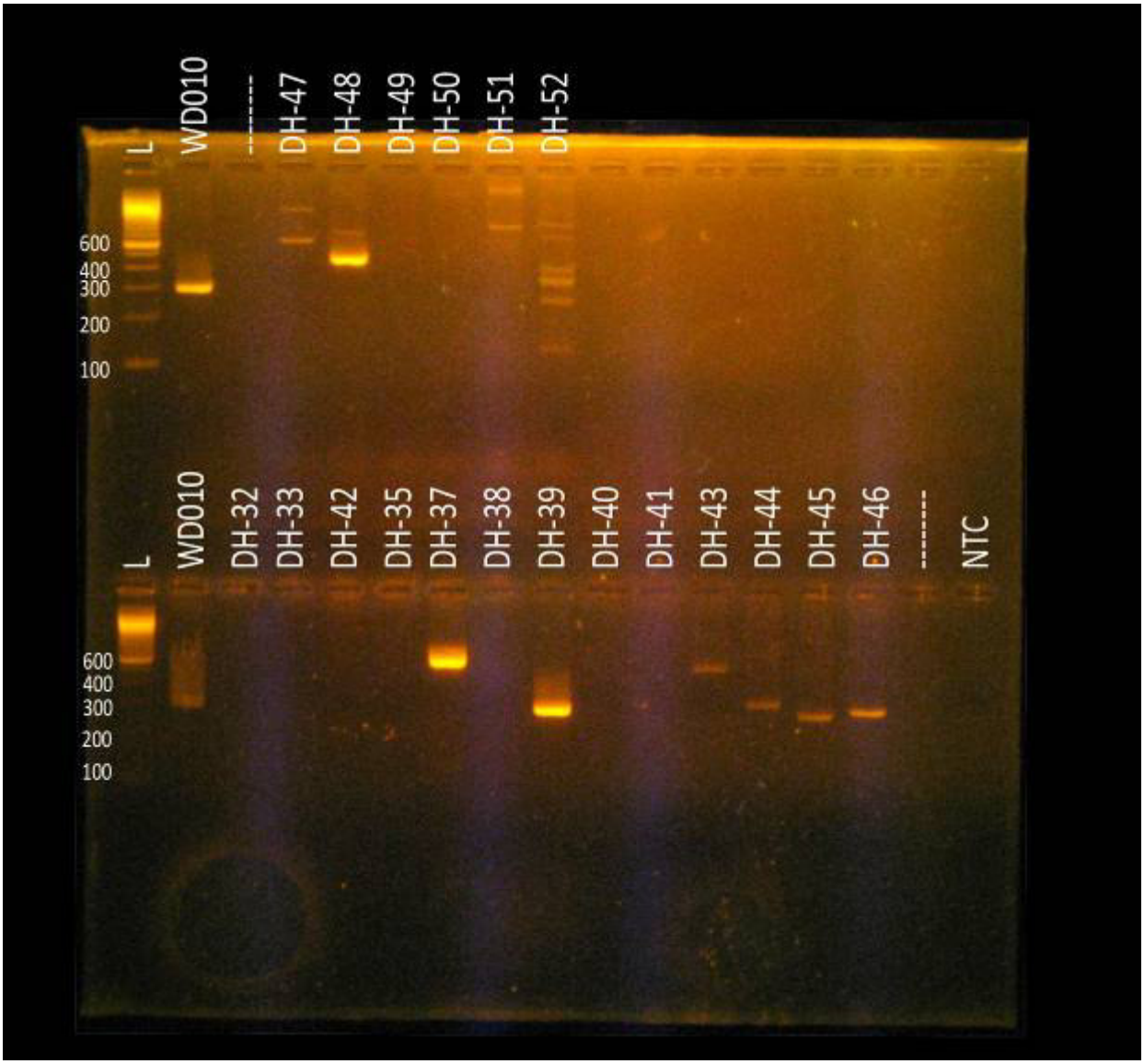

Figure 1: Results of our second attempt at species identification using Dhole-specific primers

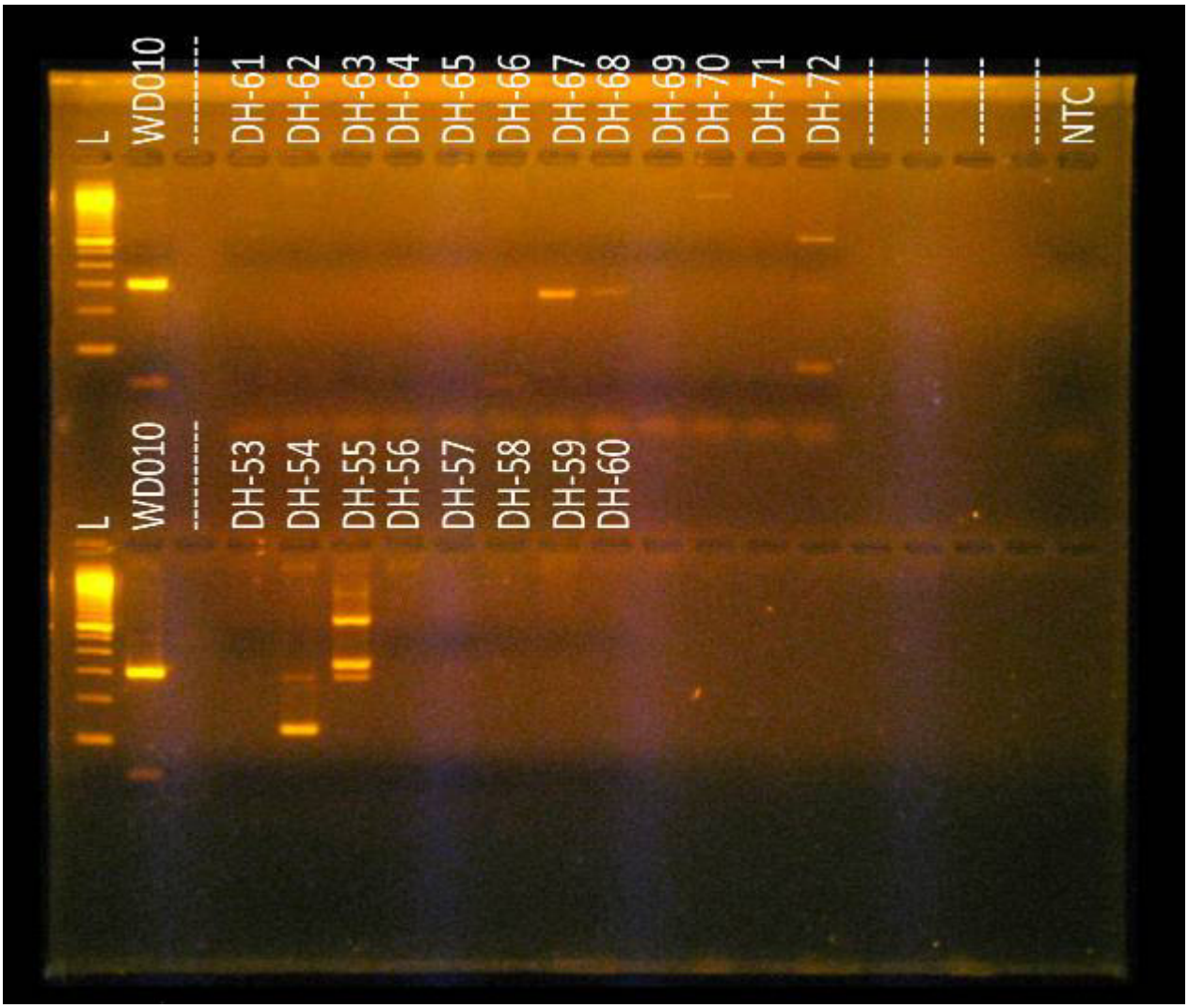

Figure 2: Results of remaining samples using Dhole-specific primers

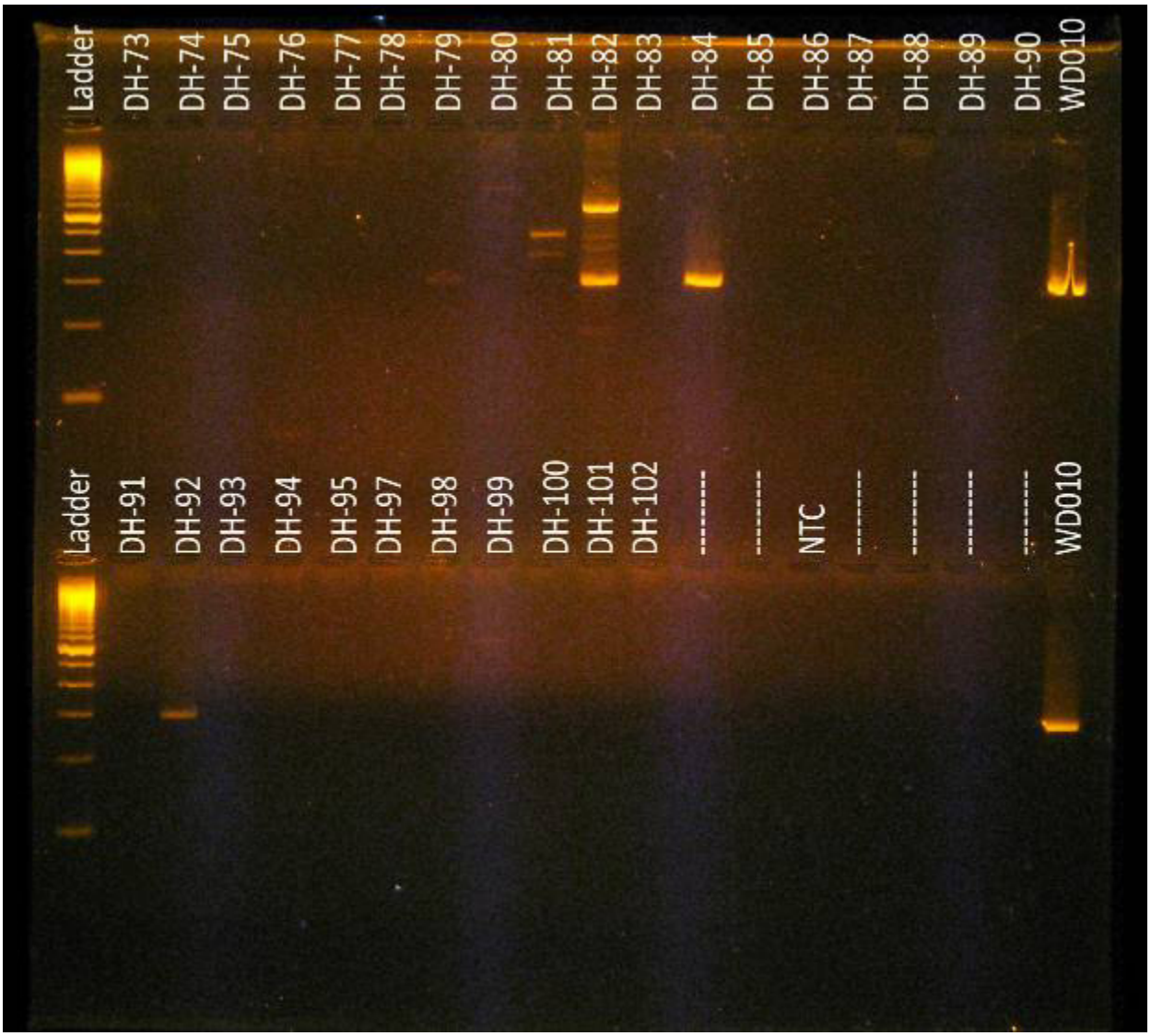

Figure 3: Results of remaining samples using Dhole-specific primers

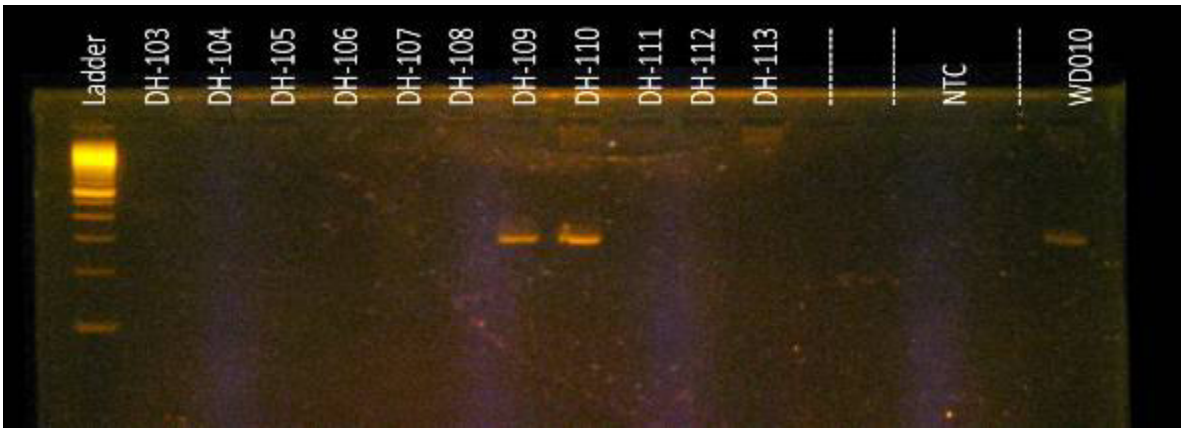

Figure 4: Results of remaining samples using Dhole-specific primers

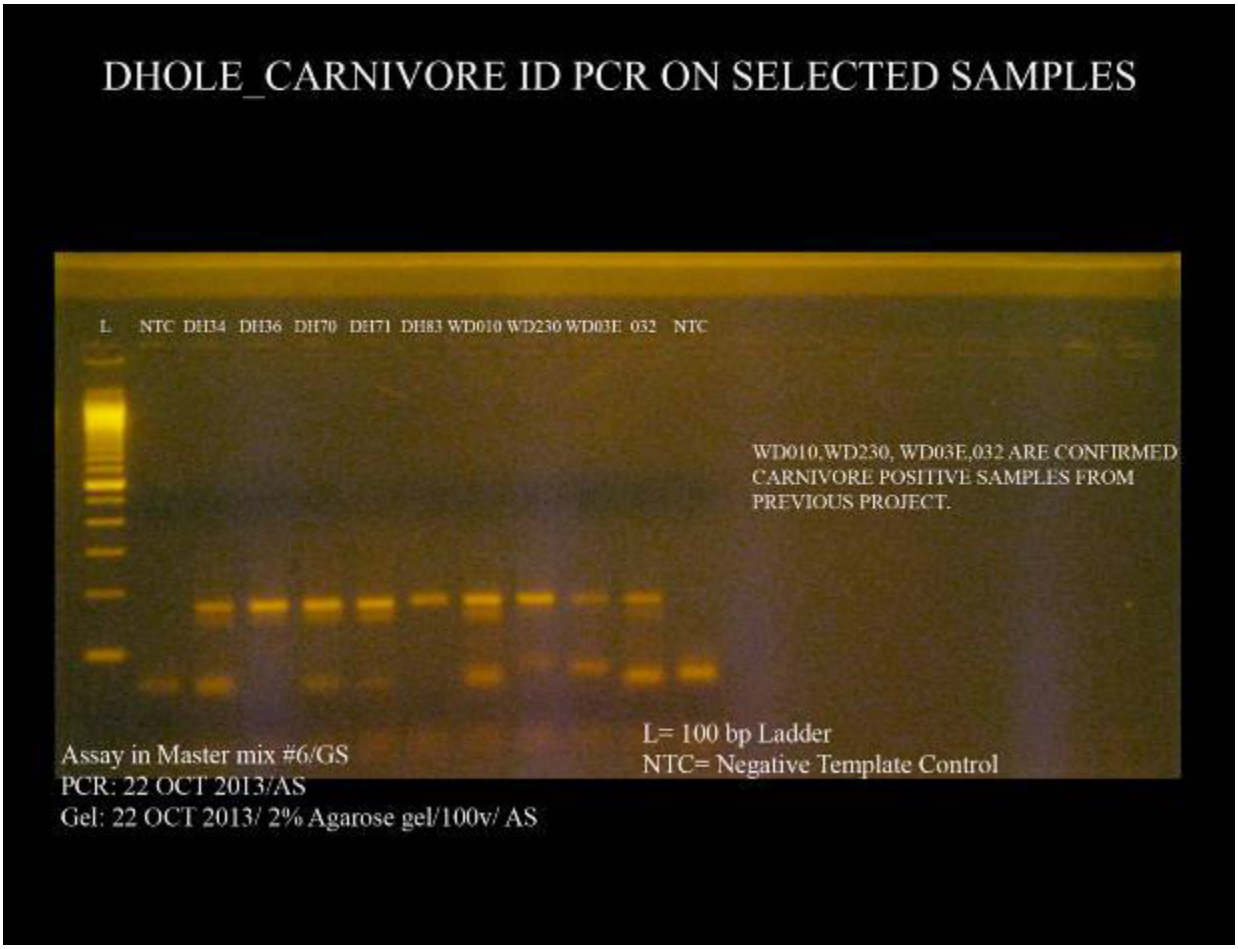

Figure 5: Results of Carnivore Id

Name	Field ID	PCR Product on Gel	Gene	Percentage Match (%)
DH-39	KO 8	Lower than positive control	*Canis lupus*	98
DH-44	KO 15	Same as positive control	-	-
DH-45	KO 16	Lower than positive control	-	-
DH-46	KO 17	Lower than positive control	*Cuonalpinus*	100
DH-52	KO 23	Higher than positive control. Multiple bands	-	-
DH-54	KO 25	Lower than positive control. Multiple bands	*Vulpesvulpes*	97
DH-55	KO 26	Same as positive control. Multiple bands	-	-
DH-67	KO 38	Lower than positive control	*Sargassum sp.*	87
DH-68	KO 39	Lower than positive control	*Martesflavigula*	96
DH-79	KO 50	Higher than positive control	*Cuonalpinus*	97
DH-82	KO 53	Same as positive control	-	-
DH-84	KO 55	Same as positive control	*Cuonalpinus*	97
DH-92	KO 63	Same as positive control	*Cuonalpinus*	98
DH-109	KO 80	Same as positive control	*Cuonalpinus*	89
DH-110	KO 81	Same as positive control	*Cuonalpinus*	98

Discussion

Out of 82 putative dhole sample tested only 6 samples were found to be dhole positive. The result was not encouraging. We searched the possible causes behind this result and concluded some points:

1. The scats were collected in the summer season and stored in a falcon tube with silica descant as a preservative.Due to the preservative the scats got dried and this made it difficult to extract the DNA properly.
2. The majority of the scats were old (more than 10 days) when collected and the outer layer of cells where the DNA lies may have been washed away due to heavy rainfall in the summer season.
3. The degradation of scats' DNA and the possibility of contamination while collecting scats may be major causes that resulted are very few samples as dhole positive.

For the next survey we are planning to conduct lab work with amplifying techniques with qualified field technicians. We will also take measures to ensure no contamination while collecting the samples. The samples for genetic tests and for diet analysis will be collected separately. We are going to collect the samples with ethanol preservative for genetic tests and the same sample will be collected with silica descant preservative in another tube for diet analysis.

Acknowledgement

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