PROGRESS REPORT

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Project Title: Developing cost-effective molecular tools to identify wild felids around Kathmandu valley through non-invasive genetic sampling
Project theme Title: Kathmandu Leopard Project

With the generous financial support (Rufford Small Grant) from the Rufford Foundation, the vision of Kathmandu Leopard Project became a reality. This project aims in unveiling mysteries of secretive cats roaming around the dense mountain forests of Kathmandu valley. With increasing urbanization and encroachment pressures around the valley, it is imperative to study the status of largest wild carnivores of this terrain, leopards (*Panthera pardus*), that plays important role as keystone species of these mountain ecosystem. The project mainly targets to study ecology of leopards and other wild carnivores including small felids. The ultimate goal of this project is to develop a simple molecular tool that can be used for accurate and quick identification of different felid species including leopard, clouded leopard (*Neofelis nebulosa*), jungle cat (*Felis chaus*), leopard cat (*Prionailurus bengalensis*) and domestic cat (*Felis catus*), which will prove beneficial in ecological studies of these carnivores.

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1 Logistics preparation

1.1 Field work logistics [July to August 2018]

Arrangement of sampling kits properly is very essential before samples could be collected. Cryovials were filled with 1.5ml DET buffer solution (prepared in lab) in order to collect and preserve fresh scat samples with sterile swab sticks. The DET buffer ensures preservation of genetic material present in scat samples for longer duration. Falcon tubes were half-filled with silica gel and capped with cotton balls, where few portions of the scat samples would be collected and stored as a backup for each sample. Other essentials such as latex gloves, stationery materials (pen, marker, ruler, cello-tape etc.) were arranged. Datasheet template for taking notes of secondary data in the field while sampling was prepared and printed out in sufficient amount.

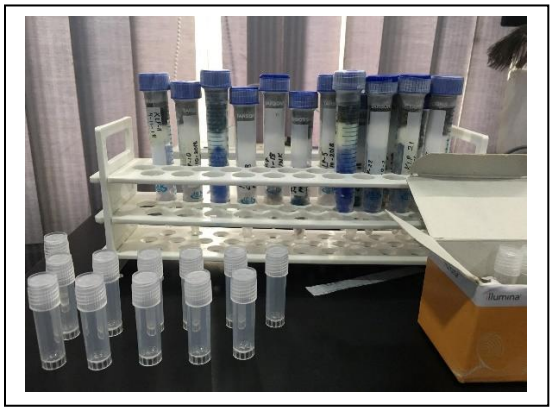

Figure 1. Sampling kits consisting of cryovials (2ml) and falcon tubes (15ml)

1.2 Legal work logistics [July to September 2018]

Government permits are required in order to conduct a wildlife research in both protected and non-protected areas of Nepal. Meetings and appointments with Department of National Park and Wildlife Conservation (DNPWC) and Department of Forest and Soil Conservation (DoFSC) in Kathmandu were set up and applications were submitted. Permit letters from the concerned organizations were obtained in due time that took about one to two months for complete processing, review and approval with multiple follow-up meetings. Cooperation with concerned offices of the national park and community forests were made following the permit and support letters were obtained from the respective departments for carrying out field research.

2 Field survey

2.1 Field site inspection during monsoon [July to September 2018]

While we were making necessary preparation of various logistics on the project's inception, we also went to various field sites for inspection. As it was in the peak of monsoon season, we found that scat samples were mostly in wet and washed form. Such conditions are usually non-feasible for obtaining good quality DNA, as host's cell debris in the scats would have been washed away by the rain water. Thus, we decided to wait for monsoon to get over before beginning our field work. We noticed some intriguing cases that seemed important to be reported to concerned bodies. There were quite a many scats of carnivores having traces of plastics in them, which was alarming. Most of the trails were also full of trashes and rubbish probably left irresponsibly by hikers and visitors.

Figure 2. Scats of carnivores observed during monsoon season, seen along with ingested plastics

2.2 Field work inception [October 2018 to ongoing]

With take-off of monsoon across Nepal's Himalayas, we started to gear up for field work. We started our field survey from community forests in south-western, southern and eastern regions (Chandragiri, Phulchoki, Nagarkot) of Kathmandu valley. The mountains were hiked through trails along ridgelines and hill slopes to search for carnivore signs specifically leopards. During the survey, we recorded numerous indirect signs of leopards such as scats, pugmarks, scratch marks on tree trunk and scrape marks on ground. Some of these signs were quite fresh when we recorded them. There are sparsely situated villages throughout these mountains, whereupon we detected promising indirect signs of carnivores including leopards. Only fresh to recently defecated scats of carnivores were sampled for genetic analysis in order to increase chances of obtaining better quality DNA materials of the host animal. Upon interacting with local people across the villages, they reported about their encounter cases with various wildlife including

Figure 3. Few images from field research.

leopards. Some people also reported about tiger (*Panthera tigris*) sighting cases. These latter reports are, however, subject to more scientific investigations.

3 Encounter cases [Dates are not disclosed here due to sensitive cases]

3.1 Village in eastern outskirt of Kathmandu valley

Three leopard cubs were found in an abandoned house in Bageswori of Bhaktapur. One of the cubs had died which was buried by officials from Division Forest Office (DFO) of Bhaktapur. With permission from the DFO officials, we took a sample from ear-tip of the dead cub before it was buried. This sample contributed to positive control sample that was later used for protocol development for the species in the lab.

Figure 4. Images of the abandoned house where three leopard cubs were found and a dead cub before it was buried.

3.2 Village in south-western outskirt of Kathmandu valley

A pet dog (*Canis lupus familiaris*) was killed from a house in Matatirtha of Kathmandu. We went there to investigate about the case and also surveyed the area to search scats. It was a cultivation area with dense crop cover and thus we couldn't easily find any scats. Later we swabbed the neck region of the dog carcass and stored it in DET buffer. This sample was used to verify the presence of leopard DNA that might have got there through saliva of the leopard while it was feeding on its kill.

Figure 5. The area where the leopard encounter occurred during which crowd gathered. A head of the dead dog (leopard's kill) found in the area during survey.

3.3 Rural village in Arghakhanchi district of western Nepal

An infant was killed by leopard in Arghakhanchi district in the western mid-hills region of Nepal. A week later, the DFO of Arghakhanchi were able to catch a leopard in cage trap, which later died due to injuries incurred from outraged local people's attack. The DFO contacted us for DNA analysis to verify if that leopard was the same which killed the child. We tested the samples and performed DNA fingerprinting and verified that it was the same leopard, which provided a sense of relief to the affected family and the local people there.

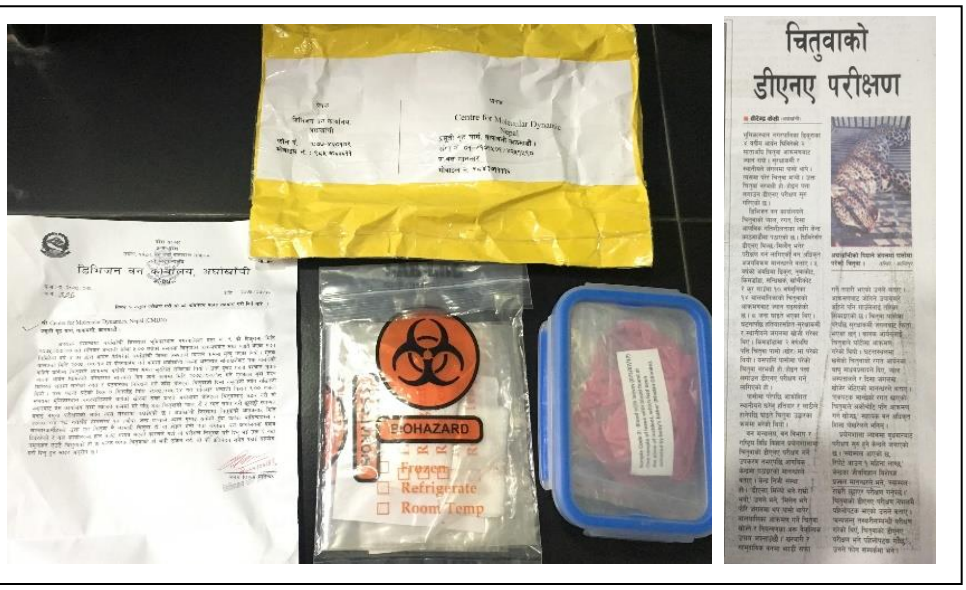

Figure 6. The official letter and forensic samples sent by Division Forest Office of Arghakhanchi, and snippet of news coverage of the case (in Nepali language) in Kantipur National Daily.

3.4 Live encounter of adult leopard in the jungle during field research

During one of our field trips, we encountered a live leopard inside a forest at about 20m distance away before it disappeared into the canopy. Two of us were hiking through trails in southern hills of Kathmandu valley. There were very fresh signs of wild boars (*Sus scrofa*) along the trail with their recent smells. As we kept following the game trail, more fresh signs such as recent pellets and rootlings of boars were observed. There were a lot of indications of boars foraging on the grounds. As I was examining a fresh pellet of boar, my peer was heading slowly ahead where there was dense vegetation cover, and there he saw an adult leopard. As soon as the leopard realized it had been spotted, it ran very swiftly and quickly into a ravine through gullies. Both of us then attempted on pursuing the big cat into the jungle but could only navigate up to a cliff which started to get steeper. Then after a few seconds, we heard loud alarm calls of macaques (*Macaca sp.*) from beneath that cliff deep inside the jungle, as the leopard might have passed through the area. We headed back and later surveyed the area carefully and found a lot of scratch marks on tree trunks.

Figure 7. The jungle habitat where an adult leopard was encountered.

3.5 Urban settlement in northern outskirt of Kathmandu valley

I got informed of a dead jungle cat in settlement area of Budhanilkantha, northern outskirt of the valley, through social media site *facebook*. I went there to investigate the case, and a local person told that the cat had died just a night before. There were injuries with unclotted blood on its head. I suspect that it might have died due to motor hit-and-run case or attacks from stray dogs. I informed the DFO officials of

Figure 8. Jungle cat lying dead in settlement area of Budhanilkantha, northern outskirt of Kathmandu valley.

Kathmandu and they arrived at the scene and buried the cat. Sample from the dead cat was taken for use as positive controls for protocol development of the species in our lab.

4 Genetic research [December 2018 to ongoing]

4.1 Phylogeography of leopards in Himalayan belt

Historically, the phylogeography of leopards throughout their global range have been accessed using mitochondrial *NADH5* and *control region* sequences together constituting about 750 bp. Previous published studies have identified biphyletic groups in Indian leopard subspecies (*P. p. fusca*) lineage, of which no further research have been attempted upon (Fig 9). One of the lineage group's geographic origin has been sourced to be from Nagarhole National Park which lies in southern peninsular India. Another lineage's geographic origin is not certain as the samples have been mentioned to be from a zoo in Gujarat which may imply it could be from anywhere in India such as the Himalayan region. Since Nepal lies completely in the Himalayan belt region of the leopard's range, we aim to investigate upon the phylogeographic lineage of leopards from this region and compare it with all other published reference sequences of leopards throughout its global range including the peninsular India and the unknown region of India. We have obtained preliminary results from *NADH5* gene which shows slight indication of the leopard from Nepal forming a lineage that is different from the peninsular Indian leopard lineage and closer to the unknown Indian leopard lineage. Before making any claims, we are performing optimization for amplifying *control region* sequences and awaiting its results. We will be investing more time into this research as this could mean a huge scientific breakthrough in terms of our current understanding about genetic lineage of leopards from the Himalayan belt.

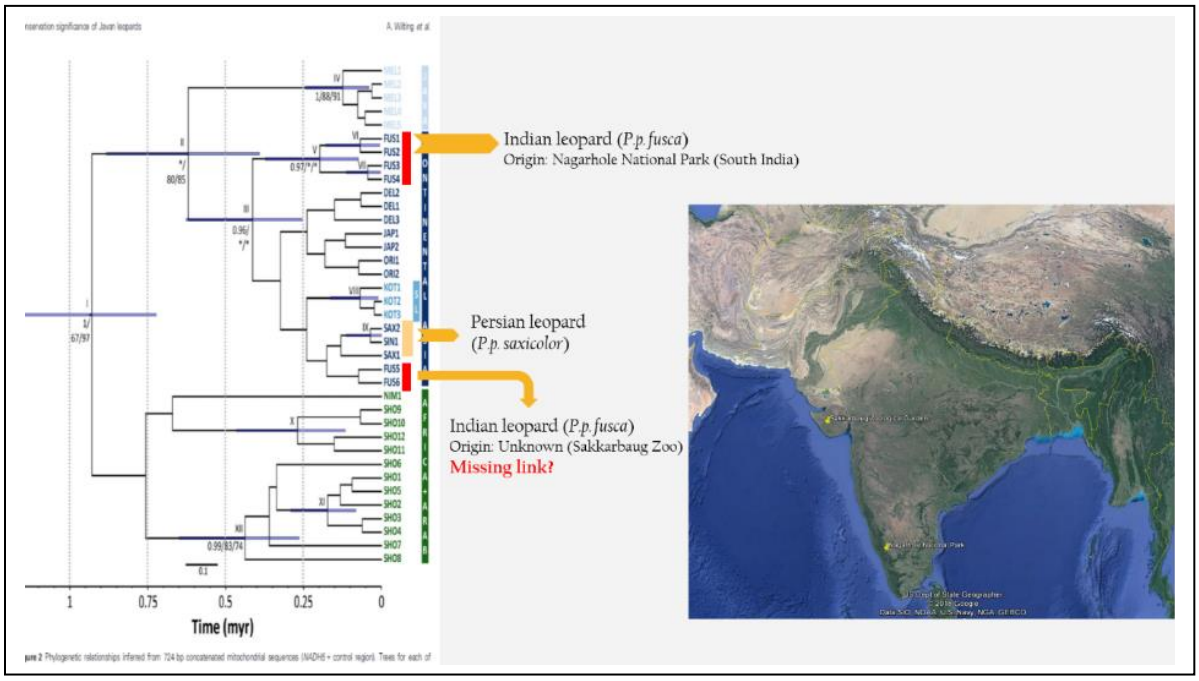

Figure 9. Infograph on phylogeny tree of leopard subspecies (adopted from Wilting et al) and map of Indian sub-continent (derived from Google Earth).

4.2 Genetic protocol development for species identification

The species-specific primers for leopard were designed using reference sequences of mtDNA *cytochrome-b* gene of leopard and comparing it with *cytochrome-b* sequences of other carnivore species. These primers would amplify ~199bp region from the leopard's mtDNA. These primers were tested against known

positive control samples of leopard and the test were verified by including positive control samples from other carnivores too (Fig 10).

We are also performing optimization assays to do species identification of three small cats: jungle cat, leopard cat and domestic cat. These cats are known to be sympatric in Kathmandu valley and are small to medium sized cats, thus the dimensions of their scats would be similar. So, this protocol will be able to accurately identify scats of these sympatric small cats.

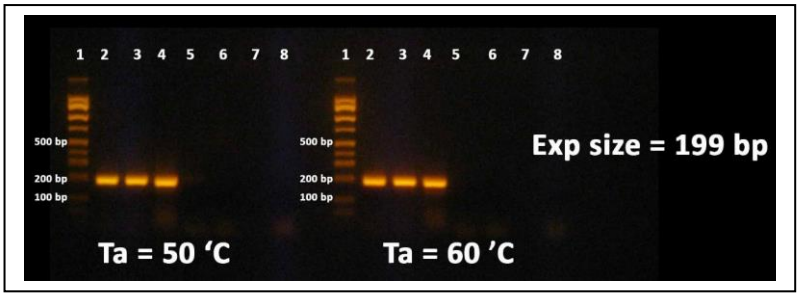

Figure 10. Electrophoresis gel image of PCR amplifications performed using leopard-specific primers, which showed bands at 199 bp for positive leopard samples.

4.3 Protocol testing on non-invasive genetic samples collected from field sites

So far, 34 noninvasive genetic samples collected from various sites in Kathmandu valley have been processed. The genomic DNA were extracted from the samples preserved in DET buffer solution using QIAamp DNA Mini Stool Kit (Qiagen). A leopard-specific PCR was performed using above mentioned self-designed primers (in Section 4.2) specific to leopard's mtDNA *cytochrome-b* region which amplifies 199 bp amplicon. Among the 34 samples, 13 samples positively amplified as leopards which was visualized through agarose gel electrophoresis. These genetically identified samples of leopards were mapped using their corresponding GPS points (Fig 12). All negative samples will be processed for small cat species identification using the protocols we are currently optimizing in our lab. Similarly, remaining samples will be processed in batches.

Figure 11. Few images of lab researchers from CMDN performing various genetic works (DNA extraction, PCR, Gel electrophoresis, Sequencing) in lab.

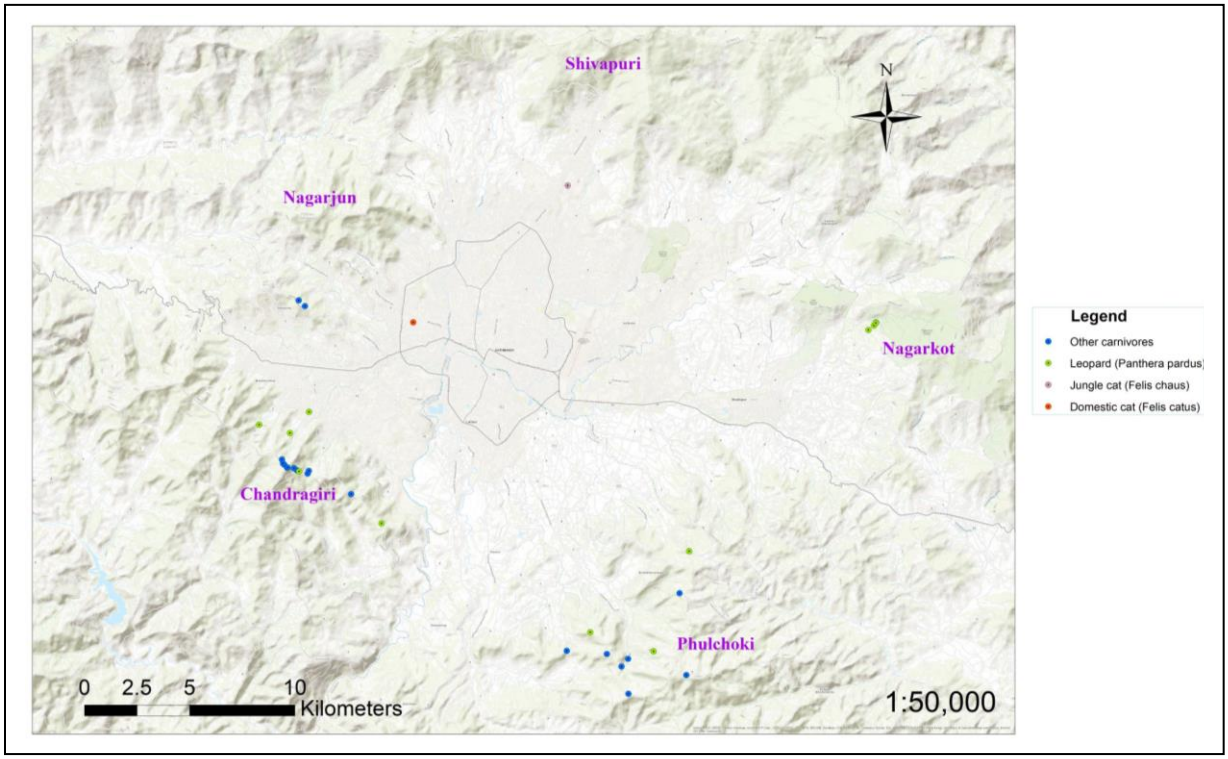

Figure 12. Distribution map of non-invasive genetic samples that have been genetically identified as Leopards. The negative samples are labelled as other carnivores, these will be further genetically tested for their species identification. Image created using ArcMap version 10.3

5 Concluding remarks

Through this mid-term update report, we want to inform that we are obtaining interesting results in terms of leopard research in Kathmandu valley. Moreover, we have been able to network through our research findings and open doors to future collaboration in leopard research field. As we are doing our remaining works of field research in Shivapuri Nagarjun National Park and development of molecular tools for small cats, we wish to continue further on this research field and expand our scopes to more ecological dynamics that seems necessary. The population dynamics and dietary ecology of the carnivores are few of those topics we are planning to investigate upon in the future. This study has helped in generating valuable baseline information about the carnivores, specifically leopards, in Kathmandu valley which is really important as the valley is undergoing massive and rapid changes in its urban planning and expansion. We hope to convey important conservation message to all concerned stakeholders through our research.

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