DNA TESTING AND GPS POSITIONING OF SNOWLEOPARD (*Panthera uncia*) GENETIC MATERIAL IN THE KHUNJERAB NATIONAL PARK NORTHERN AREAS PAKISTAN

1. Executive Summary: No more than ½ page that describes the original goals and the final results of your project. This may be used in press releases, etc. about the Snow Leopard Conservation Grants Program, so write it for the general public who may not have scientific backgrounds.

The protection of Snow Leopards in the remote and economically disadvantaged Northern Areas of Pakistan needs local people equipped with the skills to gather and present information on the number and range of individual animals in their area. It is important for the success of a conservation campaign that the people living in the area are engaged in the conservation process. Snow Leopards are elusive and range through inhospitable terrain so direct study is difficult. Consequently the major goals for this project were twofold, to gather information on snow leopard distribution in this area and to train local university students and conservation management professionals in the techniques used for locating snow leopards without the need to capture or even see the animals.

This project pioneered the use of DNA testing of field samples collected in Pakistan to determine the distribution of snow leopards and to attempt to identify individuals. These were collected in and around that country's most northerly national park, the Kunjurab National Park, which sits on the Pakistan China border.

Though the Northern Areas is not a well developed part of Pakistan, it does possess a number of institutions that can work together to strengthen snow leopard conservation.
The first of these is a newly established University with students ready to be trained in the skills needed. Secondly WWF-Pakistan has an office in the main town and a state of the art GIS laboratory in Lahore and already works closely with the Forest Department who manage the national park. All three institutions worked together in this project with WWF providing GIS expertise, the FD rangers, and the university students carrying out the laboratory work. In addition in the course of the project the University of the Punjab in Lahore also joined the effort, providing laboratory facilities for the students.

As a result of this project maps have been produced showing the location of snow leopards in two areas. Preliminary DNA evidence indicates that there is more than one animal in this relatively small area, but the greatest achievement of this project is the training and experience gained by the local students. For one student this has been life changing. Due to the opportunities provided by this study the student, Nelofar gained significant scientific training and as a consequence she is now working as a lecturer and research officer for the Center for Integrated Mountain Research, New Campus University of the Punjab, Lahore Pakistan

2. Objectives: What was the purpose of the project? How was it expected to contribute to the knowledge or conservation of snow leopards, their prey, or habitat?

This project had two major objectives. The first, was to pioneer the scientific study by noninvasive genetic sampling to identify individual snow leopards present within the Khunjurab National park. This would be the first study of this type on snow leopard samples collected from the field and would demonstrate the viability of this method in a hostile environment.
Secondly and as important as the data collected, was the opportunity this study provided for the training of students in environmental and molecular biology techniques. The KIU is a recently established university with few resources and little experience in research. The grant given provided the support for two students of the department of Biological sciences to carry out this study for their MPhil dissertation. This was the first time that an MPhil programme had been run at this university. As a result of this project the students, Sajjad and Nelofar were able to work in both newly equipped laboratory at the KIU and in the laboratories of the Molecular Microbiology department at the University of the Punjab Lahore. In addition this project also provided and opportunity for Karamat Ali a lecturer of geography at the KIU to participate in the GIS mapping of samples and funding for this project was a supporting factor in obtaining some funding for the lecturer to visit WWF labs in Lahore to receive training on GIS systems. The cost of this training was supplemented by a personal donation from myself.
The experience gained by the two MPhil students and the contacts made as a result of this work has enabled one student Ms Nelofar to gain employment at the Institute of Mountain Research where she will continue to contribute to the field of environmental science.
The sample collection and preservation were carried out by students and staff of the KIU, KNP and WWF Pakistan Gilgit under the direction of Mr Babar Khan and WWF-P Lahore. This has strengthened interaction between these organizations laying the ground for continued collaborations. In all over 50 samples were collected from two separate Nallahs (river valleys) and as a consequence detailed maps produced showing sample locations. Molecular biology work was carried out in the laboratory of Professor Shahida Hasinain in Lahore. In the time scale of the project a great deal of progress was made but due to the difficulty in obtaining reagents for detailed work only preliminary DNA studies could be made. Despite this the PCR analysis of samples indicates the presence of at least two different SL individuals in this area of the park, demonstrating that with refinement this is technique for SL analysis is a viable means of study in Pakistan.

3. Methods: Describe the methods you used in detail, so that someone else could repeat the work, or, avoid problems you later encountered.

The methods described are taken from the thesis of Miss Nelofar prepared in the spring of 2009.

Insert final MM from thesis

4. Results: Describe in detail the results of your project. Show clearly how well you did in meeting your stated goals and objectives. You may wish to include tables or graphs in this section if appropriate. This section will be very important for explaining to funders of the Snow Leopard Conservation Grant Program what value it has. Be clear, concise, and thorough.

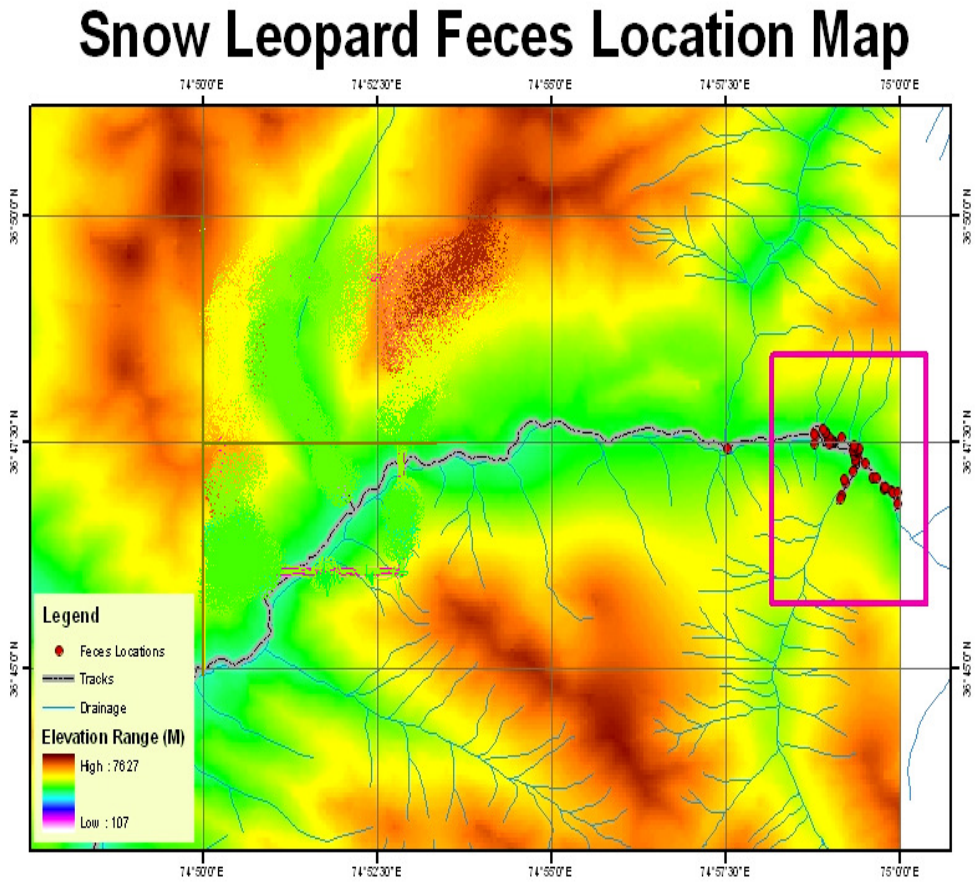
Results

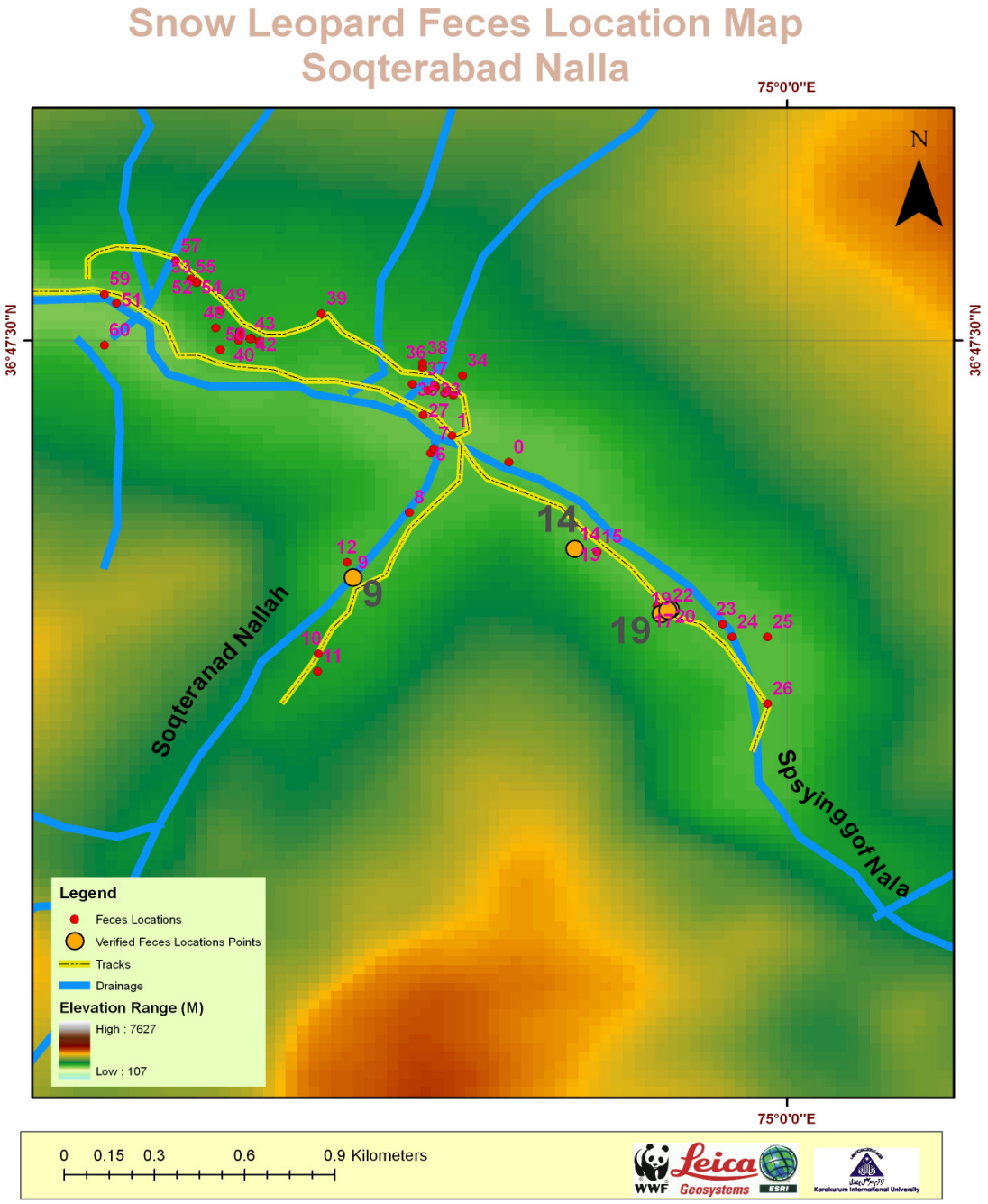
SAMPLE COLLECTION AND GPS LOCATION

Soqterabad Nallah is located in the jurisdiction of Khunjerab Village Organization (KVO) while Karachani Nallah is located in the KNP. These are considered to be most common habitats of

snow leopard. Therefore these nallahs were selected to collect the required data on snow leopard feces.
Soqterabad Nallah was surveyed on the 13th of May 2008. The Karachani Nallah survey was conducted from 26th - 28th of July. A total of 78 samples were collected. 50 from Soqterabad nallah and 28 from Karachani nallah.
The location of each sample was determined using Garmen handheld GPS.

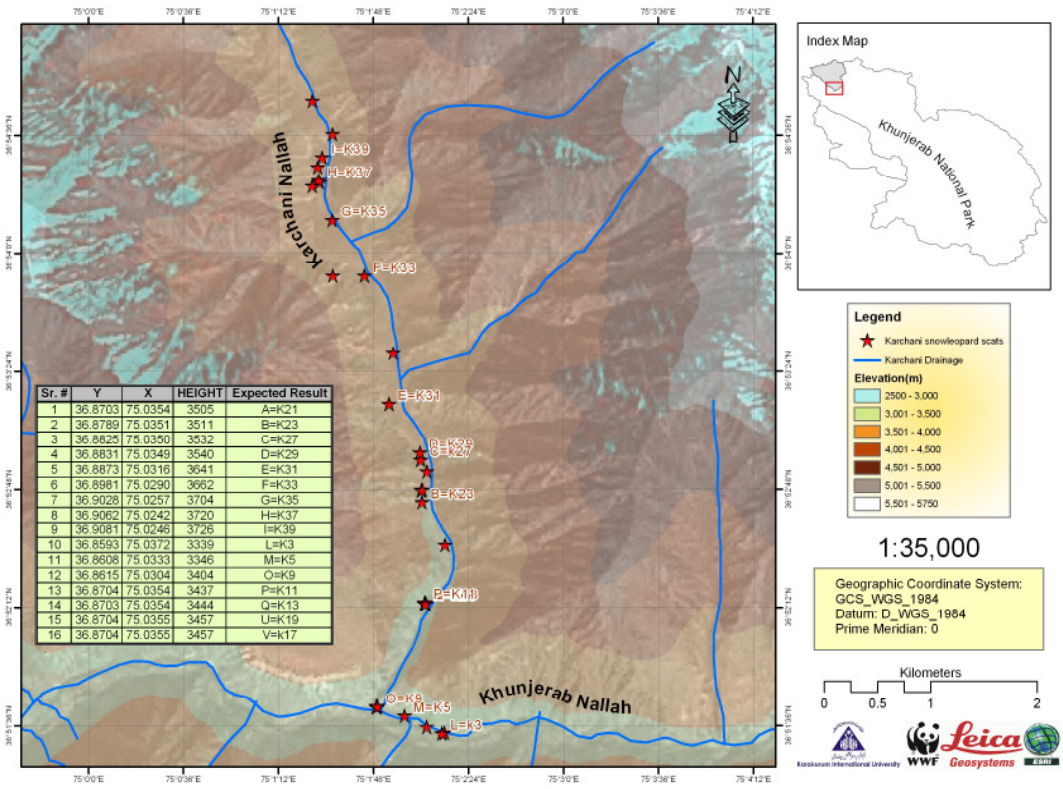
MAPS OF KNP AND SOQTERABAD SHOWING SAMPLE LOCATIONS:
FIGURE 1 (a and b) LOCATION OF SAMPLES WITHIN SOQTERABAD NALLAH





GPS-based Map of Soqterabad nallah showing locations of samples.
Samples s9,s14,s19 and s22 could produced the expected DNA fragments with three different banding patterns.

FIGURE 2 LOCATIONS OF SAMPLES WITHIN KARACHANI NALLAH



Sr. #	Y	X	HEIGHT	Expected Result
1	36.8703	75.0354	3505	A=K21
2	36.8789	75.0351	3511	B=K23
3	36.8825	75.0350	3532	C=K27
4	36.8831	75.0349	3540	D=K29
5	36.8873	75.0316	3641	E=K31
6	36.8981	75.0290	3662	F=K33
7	36.9028	75.0257	3704	G=K35
8	36.9062	75.0242	3720	H=K37
9	36.9081	75.0246	3726	I=K39
10	36.8593	75.0372	3339	L=K3
11	36.8608	75.0333	3346	M=K5
12	36.8615	75.0304	3404	O=K9
13	36.8704	75.0354	3437	P=K11
14	36.8703	75.0354	3444	Q=K13
15	36.8704	75.0355	3457	U=K19
16	36.8704	75.0355	3457	V=k17

GPS-based Map of Karachani nallah showing the location of twenty four amplified samples out of total twenty eight samples processed for PCR.

3.3. PCR PRODUCTS (GEL PHOTOS)

A total number of 48 samples were processed for PCR using Multiplexes 1, 2, and 3. Of these 32 samples were amplified using multiplex 1 and 2 whereas multiplex 3 could not work. These comprised 24 samples from Karachani and 8 samples from Soqterabad. From the study of Waits et al; 2007 the expected range of products are as shown in the table below.

TABLE-1 MULTIPLEX-1 GROUP-1 FCA 32, 100, 124.

FCA	Size range/ Expected range
32	179-199
100	112-120
124	121-133

TABLE-2 MULTIPLEX 2, GROUP 2 — FCA 96, 132, 225, 275

FCA	Size range/ Expected range
96	205-213
132	164-172
225	228-234
275	121-133

Of the 78 samples collected DNA was extracted from 48 samples in total 20 from Soqterabad Nallah and 28 from Karachani nallah.
Of these DNA extracts 48 were used as templates in the multiplex PCR reactions. 2 µl of PCR product was run on 8% polyacrylamide gel.

FIGURE 3 THE RESULTS ARE SHOWN IN FIGURES 3-5
MULTIPLEX-1

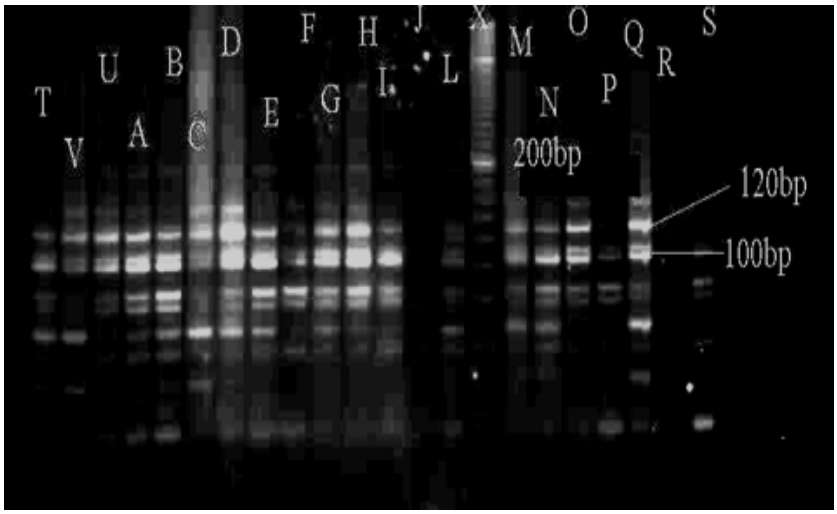


Figure 3 Shows the PCR products of 20 samples from Karachani Nallah that have been amplified using Multiplex-1.Lane label /Sample number T=k*13,V=k17,U=k19,A=k21,B=k23,C=k27,D=k29,E=k31,F=k33,G=k35,H=k37,I=k39, J=k41,L=k43,M=k45,N=k48,O=k50,P=k52,Q=k56, R=negative S=k63, X= size marker , k*= Karachani Nallah.

PRIMERS USED IN MULTIPLEX-1 AND 3.

Primers	Expected products
FCA032	179-199bp
FCA100	112-120bp
FCA124	121-133bp.

The 20 samples in Figure 3 were amplified using with Primers FCA032, FCA100 and FCA124 having expected products ranges between 112-199bp. Of these 20 amplified samples all showed products within the given expected product range of 112-200bp labeled in figure 3. Moreover, sixteen samples were identical and four (k27, k33, k43, k52) and k63 produced atypical banding.

FIGURE 4 MULTIPLEX-1

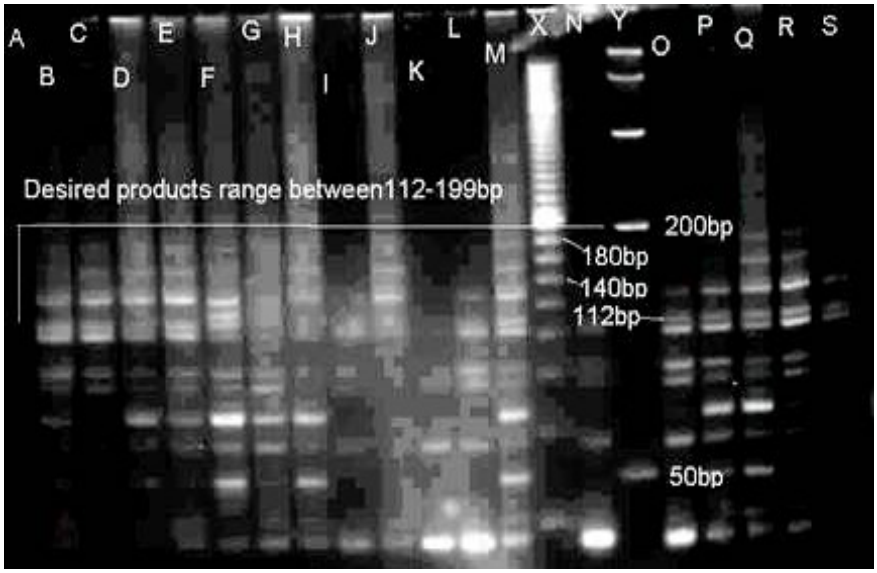


Figure 4 The PCR products of reactions using 19 samples 11 from Soqterabad Nallah and 8 from Karachani Nallah amplified using Multiplex-1
Lane label /Sample number.
s*=Soqterabad Nallah, k*=Karachani nallah.
A=s*10 B=s9, C=s13, D =s14, E=s19, F=s20, G=s22,
H=s25, I=s42, J=s50, K=s57, L=k*3, M=k5, N=k7, O=k9, P=k11, Q=k13, R=k15, S=k19,
X and Y=size markers.

Primers	Expected products
FCA032	179-199bp
FCA100	112-120bp
FCA124	121-133bp.

Figure 4 shows19 amplified samples, 11 from Soqterabad Nallah (A=s10, B=s9, C=s13, D =s14, E=s19, F=s20, G=s22, H=s25, I=s42, J=s50, K=s57) and 8 from Karachani Nallah (L=k3, M=k5, N=k7, O=k9, P=k11, Q=k13, R=k15, S=k19) using Multiplex-1. Of the 19 samples 15 produced DNA fragments in the expected range 112-199 bp. While A=s10, I=s42, K=s57, N=k7 did not produce DNA fragments in the 112-199 range. And the lane carrying sample number s20 appears to show a different banding pattern from other lanes. This could indicate that the sample is from a different animal to the others.

FIGURE 5 MULTIPLEX 2

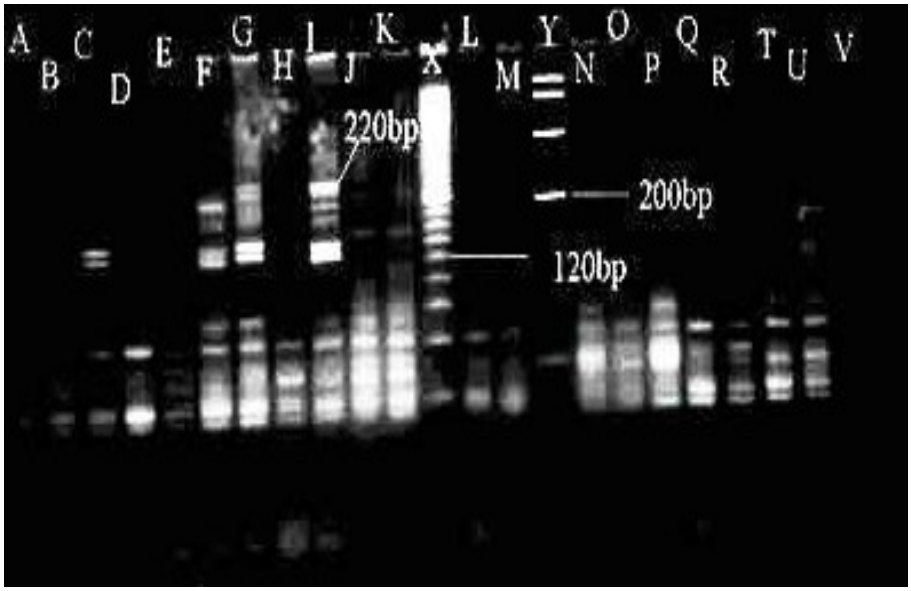


Figure 5.Multiplex-2. PCR products of 13 samples from Soqterabad nallah

A=s2, B=s7, C=s9, D=s10, E=s13, F=s14, G=s19, H=s10, I=s22, J=s25, K=s50, L=s50, M=s57 and 7 samples from Karchnai N=k3, O=k5, P=k7, Q=k9, R=k11, T=k13, U=k15.

PRIMERS USED IN MULTIPLEX 2

FCA	Size range/ expected range
96	205-213
132	164-172
225	228-234
275	121-133

The majority of samples processed for Multiplex-2 using primers FCA 96, 132, 225 and 275 did not produce PCR fragments within the expected range of 121-234bp. Only four samples s9, s14, s19, and s22 from Soqterabad nallah showed expected bands of 3 different patterns within the expected range of 121-234bp that are shown in table 5.

3.4. TABLES OF RESULTS

TABLE 3 DNA BANDING PATTERN IN FIGURE 5

Sample number	Fragments between 121 and 234bp
S9	2 bands
S14	4 bands (pattern 1)
S19	5 bands (pattern 2)
S22	5 bands (pattern 3)

Table 3: shows the samples s9, s14, s19 and s22 with three different banding patterns in (figure 5) indicating that these samples might belong to the different individuals.

TABLE 4 TOTAL AMPLIFIED SAMPLES IN KARACHANI AND SOQTERABAD USING MULTIPLEXES 1AND 2

	Nallahs	Multiplex-1 Samples producing Expected products	Multiplex-2 Samples producing Expected products	Total Amplified samples
1-	Karachani	24 samples V=k17, U=k19, A=k21, B=k23, C=k27, D=k29, E=k31, F=k33, G=k35, H=k37, I=k39, L=k43, M=k45, N=k48, O=k50, P=k52, Q=k56, S=k63 (Figure 3). L=k3, M=k5, O=k9, P=k11, Q=k13, R=k15, S=k19 (Figure 4). Sample 19 is repeated.	Nil	24
2-	Soqteraba d	8 samples B=s9, C=s13, D=s14, E=s19, F=s20, G=s22, H=s25, J=s50 (Fig.4)	4samples S22, S19, S9, S14 (Fig.5)	8

Table 4: shows samples producing expected products.
Twenty four samples from Karachani nallah showed amplification success in Multiplex-1 (Figure 3 & 4). While no sample could be amplified in Mulyiplex-2.
And eight samples could be amplified from Soqterabad nallah in Multiplex-1 (Fig.4) & four in Multiplex-2 (Fig.5). Altogether eight samples could be amplified from Soqterabad nallah.

TABLE 5: SHOWS PRELIMINARY RESULTS.

	Nallahs	Total samples collected	Total samples selected for PCR	Amplification success	Population estimation
1-	Karachani	28	28	24	2 individuals
2-	Soqterabad	50	20	8	3 individuals

Of the total 28 samples collected from Karachani nallah 24 samples showed amplification success showing two different banding patterns. And total 50 samples were collected from Soqterabad nallah only 20 best preserved samples were processed for PCR. Of these 20 samples only 8 showed expected DNA products showing three different banding patterns (Table-3). Preliminary findings indicate that there may be two individuals in Karachani and three in Soqterabad nallah.

5. Discussion: This is your chance to evaluate your own work. What did you learn that could help others wishing to do similar projects? How do you see the results being applied to conservation? What additional work is now needed based on your findings?

This study has two objectives the collection and processing of scientific data and the enhancement of skills and networks that will support conservation of snow leopards in north Pakistan.
The results of the scientific study have shown that the collection and extraction of snow leopard DNA is possible under the harsh conditions of the high altitude national park and with minimal resources. The work was successful in collection, DNA extraction and PCR DNA amplification of samples and in the generation of detailed sample location maps.
Preliminary results were obtained for the identification of individual animals but the quality of PCR product was low and there was not sufficient time to carry out refinement of the technique.

There is a major problem of supply of specialist equipment and chemicals in Pakistan especially to the KIU as it is a newly established university so does not have pre-existing lines of supply. Equipment is significantly more expensive for these types of institutes to obtain than for large organizations in countries with established research labs. Consequently funds for sampling were diverted to equipment purchase and the area sampled focused on two sites one inside and one just outside the KNP, the Nalter valley was not sampled as proposed in the application. Though the laboratory in the KIU had electrical equipment (such as gel rigs, gel viewers and PCR machines) necessary for the work the electricity supply is erratic, with frequent outages and power surges, consequently until a steady, reliable power source was fitted it was agreed that delicate laboratory equipment should not be used and alternative laboratory was found.

As a consequence another far more established university was engaged in supporting the project and Prof. Shahida Hasinan of the University of the Punjab, Lahore very kindly granted permission for students of the KIU to carry out laboratory work in the molecular microbiology laboratories. This was an unexpected additional contribution to the second objective, namely the enhancement of skills and networks in support of snow leopard conservation in Pakistan. The major success of the work undertaken has been in this area. The combined use of three organizations on the north (Karakorum International University, WWF and Forest Department) and one in central Pakistan (University of the Punjab) working towards a single goal can be taken forward to continue and strengthen conservation work. The inclusion of the Park wardens (employed by the Forest Department) at the beginning of this study was an important feature. Their assistance at the sample acquisition stage was invaluable but in addition to this their inclusion has ensured that knowledge of this new technique can be disseminated in the local communities and to park visitors.

The most significant benefit to be drawn from the project has been in the education of students of the KIU. The research undertaken by the students has enabled them to develop their investigative and analytical skills and has given them a clear understanding of how to carry out and report a study of this type. This is in addition to the specific understanding of the snow leopard conservation need in their area.

As a result of this funding the female student Miss Nelofar (who comes from the Northern Areas) has obtained a job at the Institute of Mountain Research and will be in an ideal position to develop conservation work in Pakistan in the future.

I returned to the UK a few months after the start of the project deputizing the day to day supervision to Dr Ahmed Khalil of the KIU. Throughout the year I have continued to oversee the project, although hospitalization at the end of the year prevented my working closely for a time.

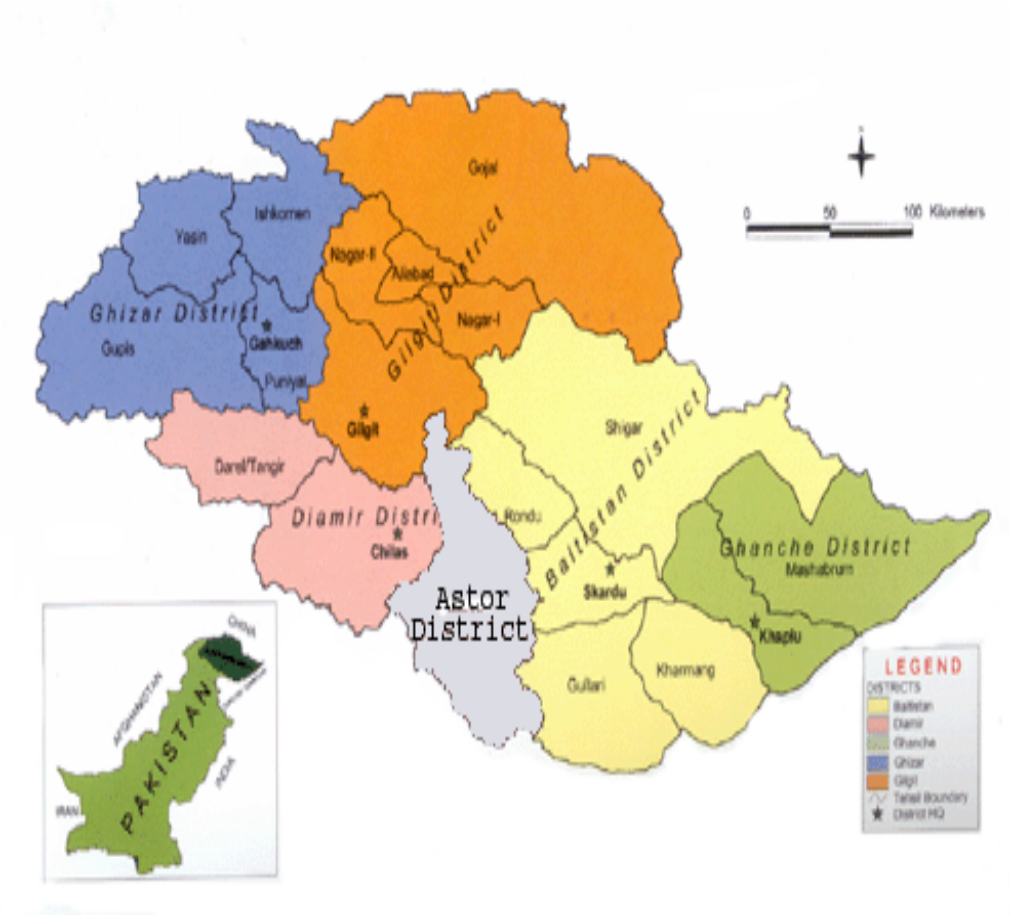
In order for non-invasive genetic sampling to be viable as a tool for conservation of the snow leopards of the Khunjurab National Park the technique would need to be refined with the use of labeled primers and more sensitive DNA separation techniques used. As I have now returned to the UK I am not in a position to carry out this work. Meanwhile through the students thesis and their reporting the knowledge of this technique could be disseminated to other students in the university. In the UK I have given a talk on the subject at the Pakistan High Commission.

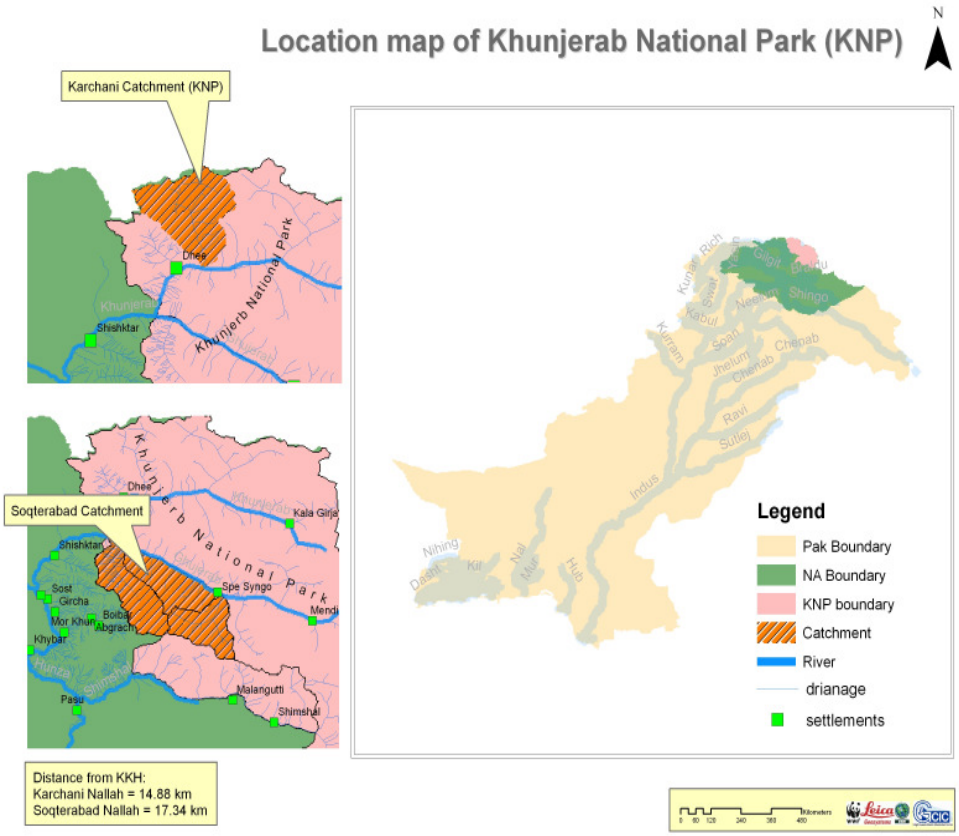
In conclusion, though there is a great deal more that can be done to put a system in place that will allow conservationists to hold a record of the genetic profiles of snow leopards in the area a significant start has been made and although I cannot carry on this work by training local students the techniques needed we have ensured that the expertise is embedded where it is most required.

6. Photographs: If you have good photographic (print, slide or digital) images of your project that we could use for advertising the Snow Leopard Conservation Grants Program, please submit them at this time. Be sure to include a brief description of the photo and provide the credits for the photographer.

Attached below are maps of the local area and a small number of photographs of individuals from the Karakurum International University who contributed to the project

Map showing the location of Gilgit district in the Northern areas of Pakistan – Courtesy of WWF Pakistan





Location Map of the KhunjuraB National Park Pakistan – WWF-Pakistan

Left to right Nelofar (MPhil student), Sumera Naz (lab assistant), Dr Naqvi (lecturer Bio), Dr Rachael Jack (Chairperson Bio), Dr Ahmed Khalil (lecturer Bio), Mr Karamat Ali (lecturer Geography)
Front row Imran (lab assistant) Fida Ali (assistant)

Imran (lab assistant) and Nelofar (MPhil student) preparing DET buffer in the labs at the KIU

Nelofar and Sajjid (MPhil Students) preparing DET buffer in the labs at the KIU

Left to right Imran, ….., (KIU Biology Department laboratory assistants) Nelofar (MPhil student)