Report: genetic variation in snow leopards in Spiti, Himachal Pradesh

Context: In March 2024, a snow leopard was found dead in Demul, and samples were sent for analyses to NCBS to better understand genetic variation. In order to describe genetic variation, we compared this sample to two other individuals sampled earlier from Spiti. Further, we contracted the genetic variation of snow leopards to tigers, one inbred individual and one wild individual from Central India (where genetic variation is high).

Introduction:

Snow leopards reside in some of the most extreme habitats and remain the least studied big cat species. A recent study using 41 whole genomes across the current snow leopard range demonstrated that snow leopards have the lowest genetic diversity among big cats [1]. The study also showed that snow leopards have historically had a small population size, with the global snow leopard population declining to 2,500 to 4,000 individuals nearly 20,000 years ago. Given their long-term small population sizes and low genetic diversity, snow leopard populations might have experienced strong effects of genetic drift and inbreeding.

Inbreeding results in an increased homozygosity across the genome and can lead to decreased fitness (through either reduced survival or reproduction) among individuals. This is called inbreeding depression, which eventually can lead to lower population growth rates. Inbreeding depression likely results when many harmful recessive genetic variants (genetic load) come together in a homozygous state resulting in their expression and appearance of loss-of-fitness phenotypes [2]. Inbreeding depression phenotypes such as low sperm motility have previously been reported in cheetahs [3,4], lions [5], and Florida panthers [6]. The genomic heterozygosity in snow leopards is even lower than in cheetahs [1], suggesting snow leopards might have extremely low adaptive potential. With ongoing rapid changes in their environment, snow leopards could be vulnerable to severe consequences of loss of genetic variation (inbreeding) and inbreeding depression. Health defects such as eyelid coloboma [7] which might be associated with inbreeding have already been reported in captive snow leopards. Therefore, it becomes critical to assess the level of inbreeding in wild snow leopard populations and characterize their genetic load.

A more recent study on snow leopards investigated the presence of runs of homozygosity (ROH) in their genomes[8]. ROH refers to long contiguous homozygous stretches in the genome arising from a common ancestor and are indicative of inbreeding. More recent inbreeding results in long ROH segments, whereas shorter ROH segments indicate older inbreeding which has over time partially broken down due to recombination. The study on snow leopards highlights the presence of a high number of small size ROH segments with very few large size ROH segments suggesting that the extent of inbreeding observed in modern snow leopards is because of a historically small population size and not recent inbreeding[8].

Here, we report on the genomic analysis of two snow leopard samples we received from the Himachal Pradesh Forest Department. We sequenced the two individuals at ~30X depth and investigated genetic diversity in Snow leopards. We also included another snow leopard genome form India that was earlier submitted to NCBI genomic database (SRA accession number SRR27199363). We estimated the genome-wide heterozygosity, and performed ROH analysis to estimate the level of inbreeding in these snow leopards. We find evidence of low genetic diversity in these snow leopards, in agreement with the other studies on snow leopards. We elaborate on our methods and results in the following sections.

Methods:

Samples and data sources

The two snow leopard samples used in this report (SL24T1 and SL40) were received from the Himachal Pradesh Forest Department (Letter No.2274, date 12/04/2024) for genetic analyses. These samples were collected from two dead snow leopards that died due to natural causes.

We have also used genome sequence from another Indian snow leopard in the analyses. This genome is available publically on NCBI (SRA accession number SRR27199363). For comparison, we have used genome sequence data of two tigers – one captive inbred tiger, ZSB01, from Nandankanan Biological Park, Bhubaneswar, and one Central Indian tiger (ZSS12) which was housed as a wild-caught tiger in Nandankanan Biological Park, Bhubaneswar. Both of these genomes were taken from Sagar et al., 2021[9].

DNA extraction and sequencing

The two snow leopard samples (SL24T1 and SL40) were processed for DNA extraction, quality check and sequencing at National Centre for Biological Sciences, Bangalore (NCBS). DNA extraction was done using QIAGEN DNeasy Blood & Tissue Kit following the manufacturer's instructions. We assessed the quality of samples using Qubit3.0 and Agilent Tapestation. The samples were sequenced on Illumina NovaSeq 6000 platform for a targeted depth of 30X.

Bioinformatics analyses

The raw Fastq files were first quality trimmed using *Fastp*[10]. The resulting reads from snow leopard samples were aligned to the snow leopard reference genome Puncia_PCG_1.0 (RefSeq accession GCF_023721935.1) and from tiger samples to the tiger reference genome PanTigT.MC.v3 using *Samtools*[11]. ROH and genome-wide heterozygosity were estimated using *ROHan*[12] with default settings and sliding window size of 10 kb. The results were plotted in R using the package *ggplot2*. All the bioinformatics analyses were conducted at the NCBS high-throughput computation cluster.

Results:

We analysed whole genome sequence data of the two snow leopard samples (SL24T1 and SL40). In addition, we also included previously sequenced snow leopard genome which was submitted to NCBI (SRR27199363). SL40 was an archival sample already in the NCBS laboratory. For comparison, we also included whole genome sequence data from two tigers, one central Indian wild-born tiger (ZSS12), and one captive-bred (highly inbred) tiger from Bhubaneswar zoo (ZSB01).

Sequence data statistics

We obtained a mean coverage of 99.27% and 99.31% for SL24T1 and SL40, respectively. The mean depth of sequencing was 32.65X and 29.46X (Table 1). In comparison, the mean coverage for SRR27199363 was 99.14% and mean depth of sequencing was 11.13X. However, the depth of more than 10X should be good to estimate Runs of homozygosity (ROH) using *ROHan* as suggested by the authors of the program. Therefore, we do not think that this difference of depth of sequencing is reflective of the differences in genetics results presented in further sections.

Genetic diversity or genome-wide heterozygosity

The three snow leopard samples show low levels of genome-wide heterozygosity – 0.0005, 0.0005, and 0.0006 (Figure 1 and Table 1). In comparison the tiger samples show a higher value of genome-wide heterozygosity. Surprisingly, even a highly inbred captive-bred tiger showed a higher value of genome-wide heterozygosity – 0.0009. As expected, the heterozygosity for the Central-Indian tiger was even higher – 0.0012. Central Indian tigers have previously been shown to have the highest levels of genetic diversity among Indian tigers[13]. Although, the difference in heterozygosity between the snow leopards and tigers is not significant (Wilcoxon Rank Sum Test, p-value = 0.2), a bigger sample size should be tested for certainty. Overall, the snow leopards show low levels of genetic diversity. Our findings are in agreement with other studies[1,8] which also report extremely low levels of genetic diversity in snow leopards compared to other felids.

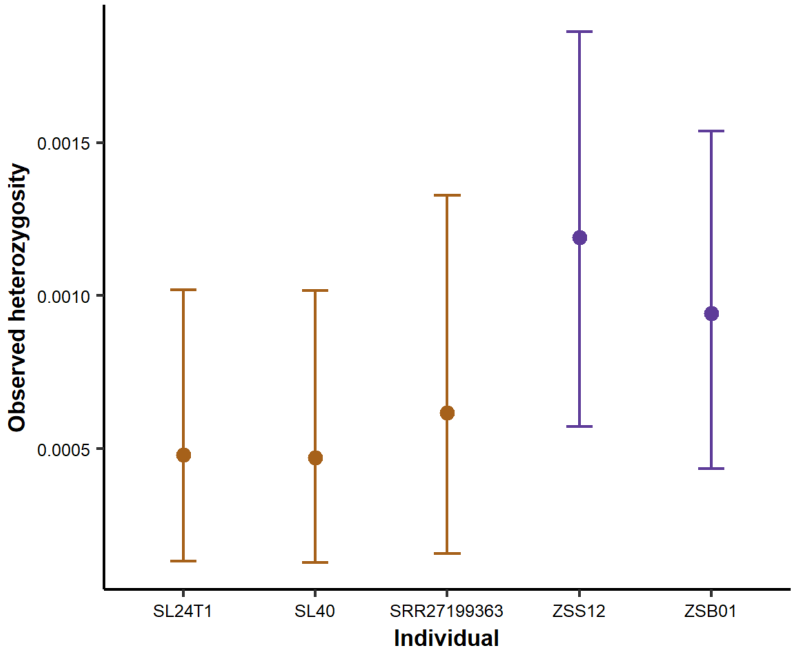

Figure 1: Genome-wide observed heterozygosity in snow-leopards (brown) and tigers (purple). Snow leopards have low genomic diversity as evidenced by lower levels of genome-wide heterozygosity as compared to an inbred tiger (ZSB01) and wild-caught Central Indian tiger.

Runs of homozygosity

We ran the ROH analysis on the snow leopard autosomes (11 autosomes characterised in the reference genome used; Puncia_PCG_1.0) for the snow leopard samples and tiger autosomes (all 18 autosomes characterised in the reference genome used, PanTigT.MC.v3) for the two tiger genomes. Therefore, the total length of genomes used for analyses was different for the two species – 1192858044 bp or 1,193 Mb for snow leopards, and 2258851657 bp or 2,258 Mb for tigers. Consequently, the absolute total ROH size is expected to be less for the snow leopards compared to tigers. We observe this difference in the total ROH between the two species (Figure 2 and Table 1), where the two tiger samples have ~374 Mb and ~687 Mb genome in ROH, while the three snow leopards show 297 MB, 322 Mb and 154 Mb total ROH size.

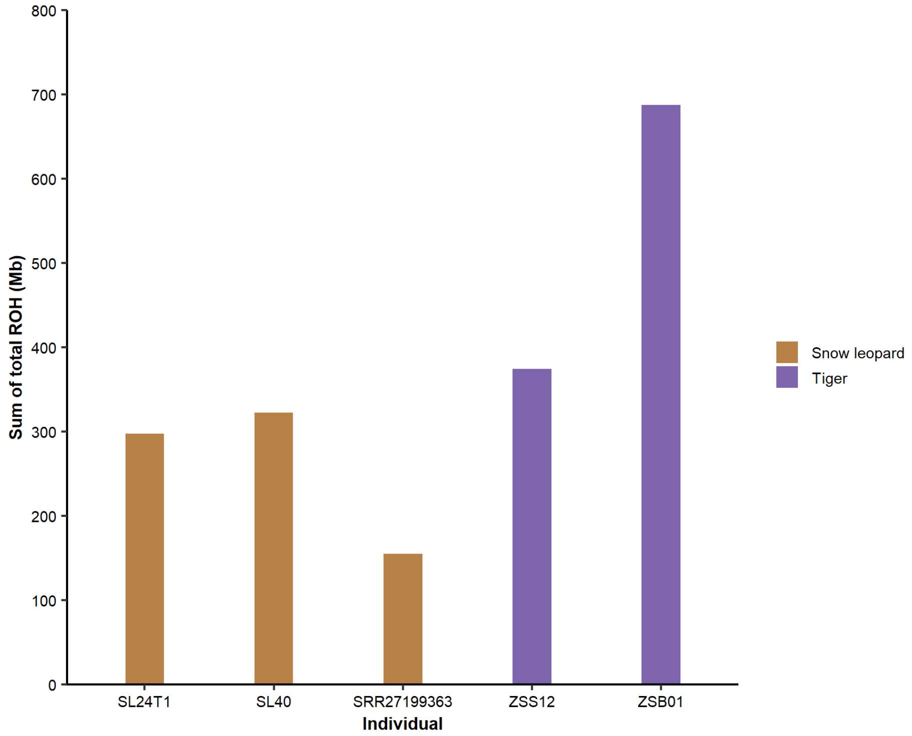

Figure 2: Bar plot showing absolute sum of ROH segments. Snow leopards (brown) show smaller size of total ROH compared to tigers (purple). However, this difference can be attributed to input autosomal size (1,193 Mb for snow leopards and 2,258 Mb for tigers).

Genomic inbreeding coefficient (F_{ROH})

For a fair comparison across species, instead of total ROH the fraction of genome in ROH segments, F_{ROH}, should be observed. We observe that while the highly inbred captive tiger (ZSB01) has a relatively high value of F_{ROH} (F_{ROH} = 0.30), the wild-caught, non-inbred tiger from Central India (ZSS12) has a low value of F_{ROH} (F_{ROH} = 0.17) (Figure 3 and Table 1). In comparison, two snow leopard genomes (SL24T1 and SL40) show F_{ROH} values closer to the inbred tiger (F_{ROH} = 0.25 and 0.27) while the third snow leopard genome (SRR27199363) shows an F_{ROH} value of 0.13, much closer to the non-inbred tiger. These results indicate that while snow leopards show signs of inbreeding, there is a remarkable range of inbreeding coefficient/levels within the individuals where some individuals are more inbred and others are less inbred. It would be interesting to see if there is correlation between inbreeding levels and geographic location or population with a higher sample size. If that is true, conservation efforts could focus on maintaining genetic diversity in highly diverse populations and maintaining their connectivity with less diverse populations.

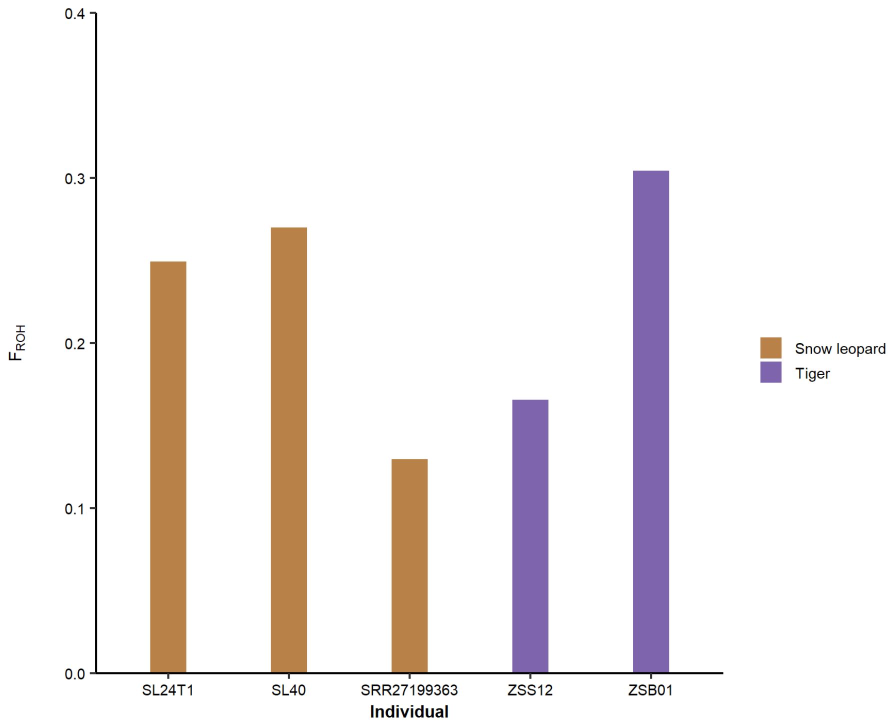

Figure 3: Inbreeding coefficient, F_{ROH}, bar plot. The total fraction of ROH in the autosome genome is comparable for snow leopards and tigers.

Distribution of ROH segments

While the sum of all ROH segments in the snow leopard genomes and F_{ROH} seem comparable to tigers (Figure 2 and Figure 3), the distribution of the length of individual ROH segments differs between the two species (Figure 4 and Table 1). The mean ROH length is 113 kb for the inbred captive tiger and 55 kb for the wild-caught tiger. In comparison, the mean ROH length for the snow leopards is 38 kb, 39 kb, and 26 kb. A similar trend is observed for the longest ROH segment for the two species (Figure 5 and Table 1), where the inbred tiger shows a longest ROH segment of size 2320 kb and the Central Indian tiger shows the longest ROH segment of size 690 kb. In comparison, the snow leopards show the longest ROH segments of size 580 kb, 560 kb, and 200 kb. These results suggest that while the snow leopards harbour long runs of homozygosity, these ROH segments are not as long as inbred tigers and hence do not suggest recent inbreeding in the snow leopard populations. As pointed out by other studies[1,8], the ROHs in the snow leopard have most likely arisen because of historically small population sizes rather than recent inbreeding.

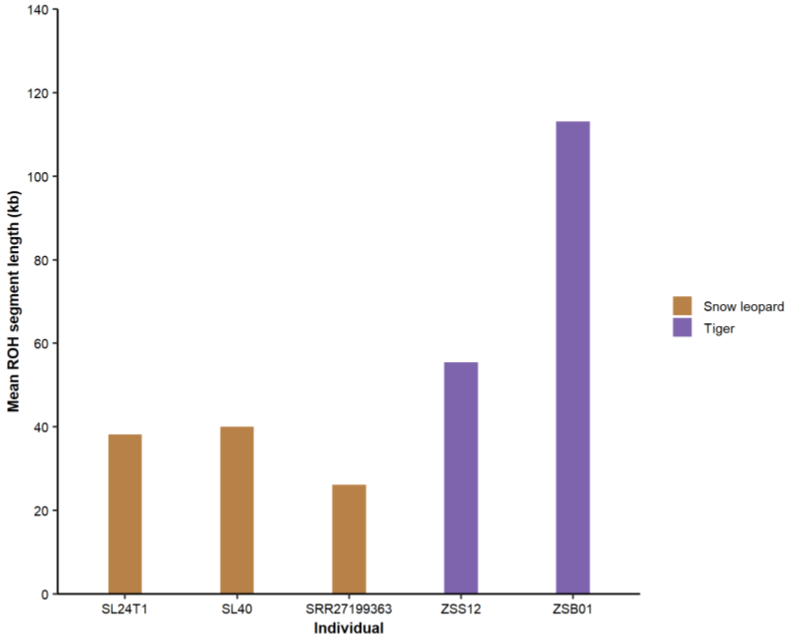

Figure 4: Mean length of ROH segments. Snow leopards show a smaller mean ROH length than tigers. Inbred captive tiger ZSB01 shows a much higher mean ROH length compared to snow leopards and the wild caught tiger, indicative of recent inbreeding in the captive tiger.

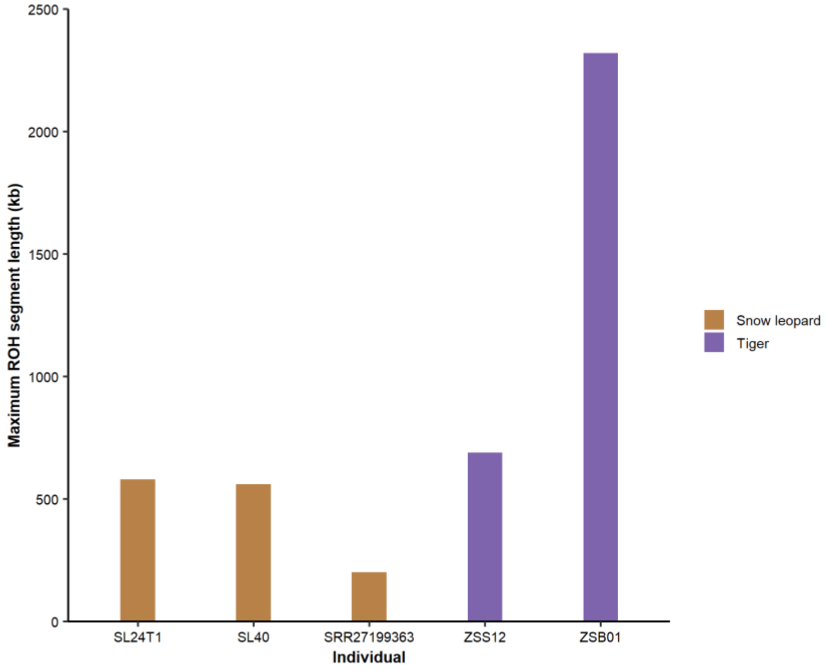

Figure 5: Longest ROH segment in snow leopards and tigers. The inbred tiger shows a much higher value of longest ROH segment compared to snow leopards and the wild caught Central Indian tiger indicative of recent inbreeding in the captive tiger.

Recent inbreeding vs historically small population size

As presented in the previous section, the length of ROH segments in snow leopards is smaller compared to tigers, and more specifically an inbred tiger. A more formal test of recent inbreeding is to look for ROH segments larger than 1 Mb which would indicate an inbreeding in the last 225 years[8]. Since we did not observe any ROH segment larger than 580 kb in our snow leopard genomes (Table 1), we decide to look at the ROH segments larger than 100 kb. While these segments will represent inbreeding events in the previous ~4000 years[8], they will help better understand the historical inbreeding (due to small effective population size) in snow leopards. We investigated the fraction of input autosomal genome in ROH > 100 kb ($F_{ROH > 100kb}$) in both tigers and snow leopards. We observed that while the overall F_{ROH} between tigers and snow leopards is comparable (Figure 3), the values of $F_{ROH > 100kb}$ show considerable differences (Figure 6 and Table 1). The $F_{ROH > 100kb}$ for the captive inbred tiger differs from the snow leopards by at least one order of magnitude ($F_{ROH > 100kb}$ for ZSB01 = 0.223 vs the $F_{ROH > 100kb}$ for the snow leopards = 0.05, 0.06, and 0.006). The lowest $F_{ROH > 100kb}$ is observed for SRR27199363 similar to other trends for this sample where it showed the maximum heterozygosity and the lowest total ROH, mean ROH length, longest ROH, and F_{ROH} among the three snow leopards.

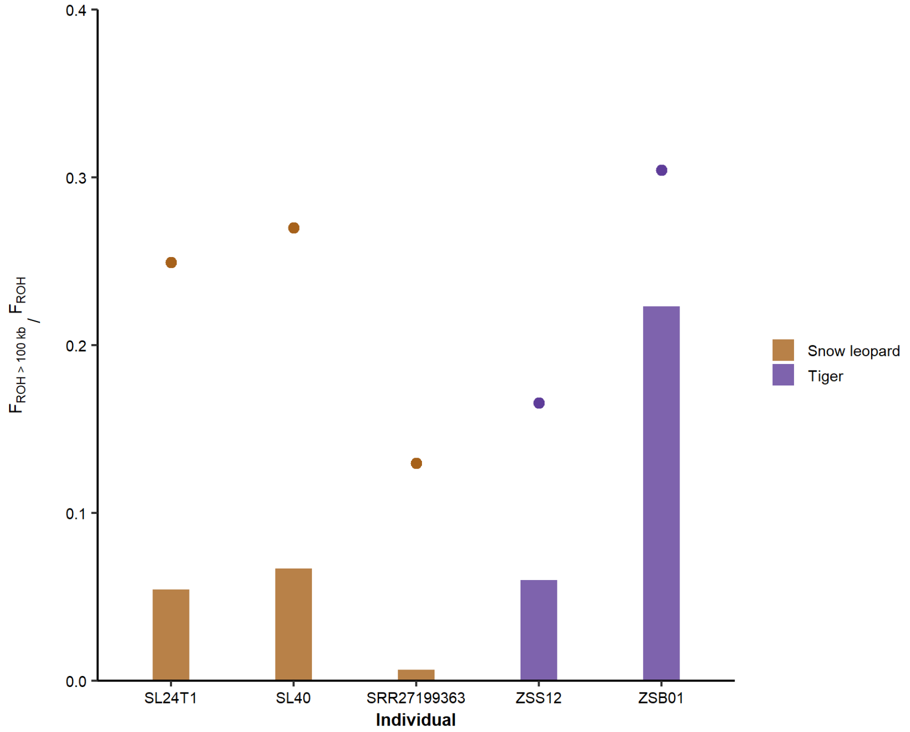

Figure 6: $F_{ROH > 100kb}$ bar plot showing the disparity between a captive inbred tiger and snow leopards. The dots show F_{ROH} values (same as depicted in Figure 3). One snow leopard, SRR27199363, shows much smaller value of $F_{ROH > 100kb}$ than the other two snow leopards, despite having comparable overall F_{ROH}, suggesting high amount of genetic diversity in this individual compared to other two snow leopards. The location of this individual might be useful for conservation management decisions.

Table 1: Sample-wise genetics results

Sample	**SL24T1 (sampled 2024)**	**SL40**	**SRR27199363**	**ZSS12**	**ZSB01**
Species	Snow leopard	Snow leopard	Snow leopard	Tiger (Wild)	Tiger (Captive)
Sequencing depth	32.65	29.46	11.13	18.40	18.81
Genome-wide heterozygosity	0.00048	0.00047	0.00062	0.00119	0.00094
Sum of total ROH (Mb)	297.33	322.21	154.60	374.26	687.89
Mean ROH length (kb)	38.14	39.97	26.13	55.46	113.14
Longest ROH segment (kb)	580	560	200	690	2320
FROH	0.25	0.27	0.13	0.17	0.30
FROH>100kb	0.05	0.07	0.01	0.06	0.22

Conclusion:

We find that snow leopards in Spiti, including the sample shared in 2024, have low genetic diversity as indicated by the low values of genome-wide heterozygosity. We also find that a significant portion of snow leopard genomes is in ROH (or homozygous) segments. However, these ROH segments are short-size stretches in the genome, indicative of historically small population size of snow leopards as opposed to long ROH segments in inbred tigers which indicate recent inbreeding. One snow-leopard sample shows much higher genetic diversity than the other two, suggesting there maybe remarkable variation in the genome-wide diversity within snow leopard populations, but this is a sample from 2011. Our results highlight the importance of genomic approaches to aid conservation, and provide important evidence to support for more rigorous genetics studies using a bigger, more spatially uniform sample size in snow leopards.

Future directions:

We would like to initiate a longer-term study where we sample 15-20 individuals identified through camera trap locations. We would like to collect varied sample types including tissue, blood, hair, saliva and scat samples to investigate genetic diversity in the Spiti population of snow leopards in collaboration with NCF and the Himachal Pradesh Forest Department.

References:

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