Wildlife Biology

WLB-00583

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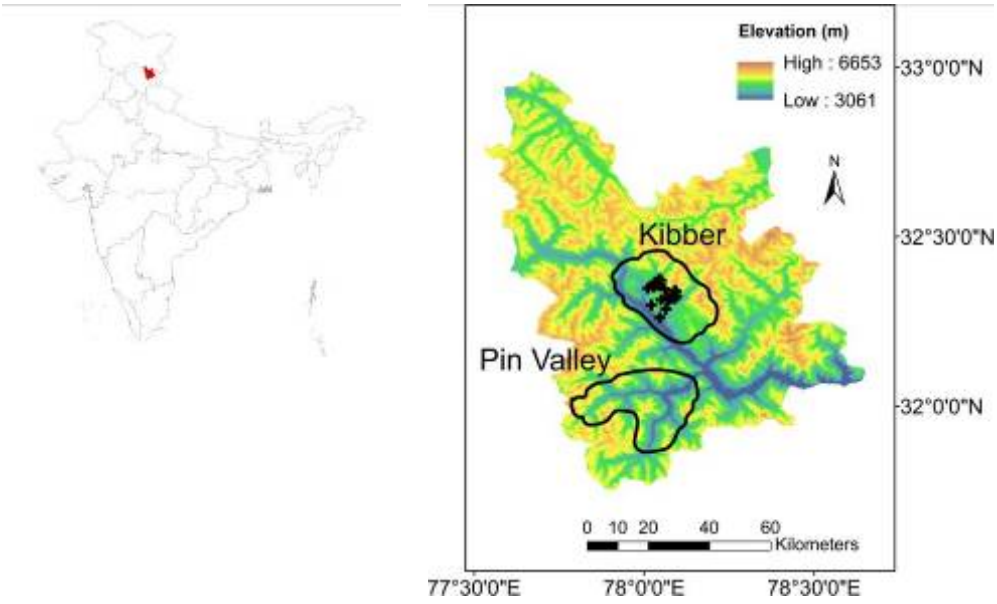
Content Appendix 1-5:

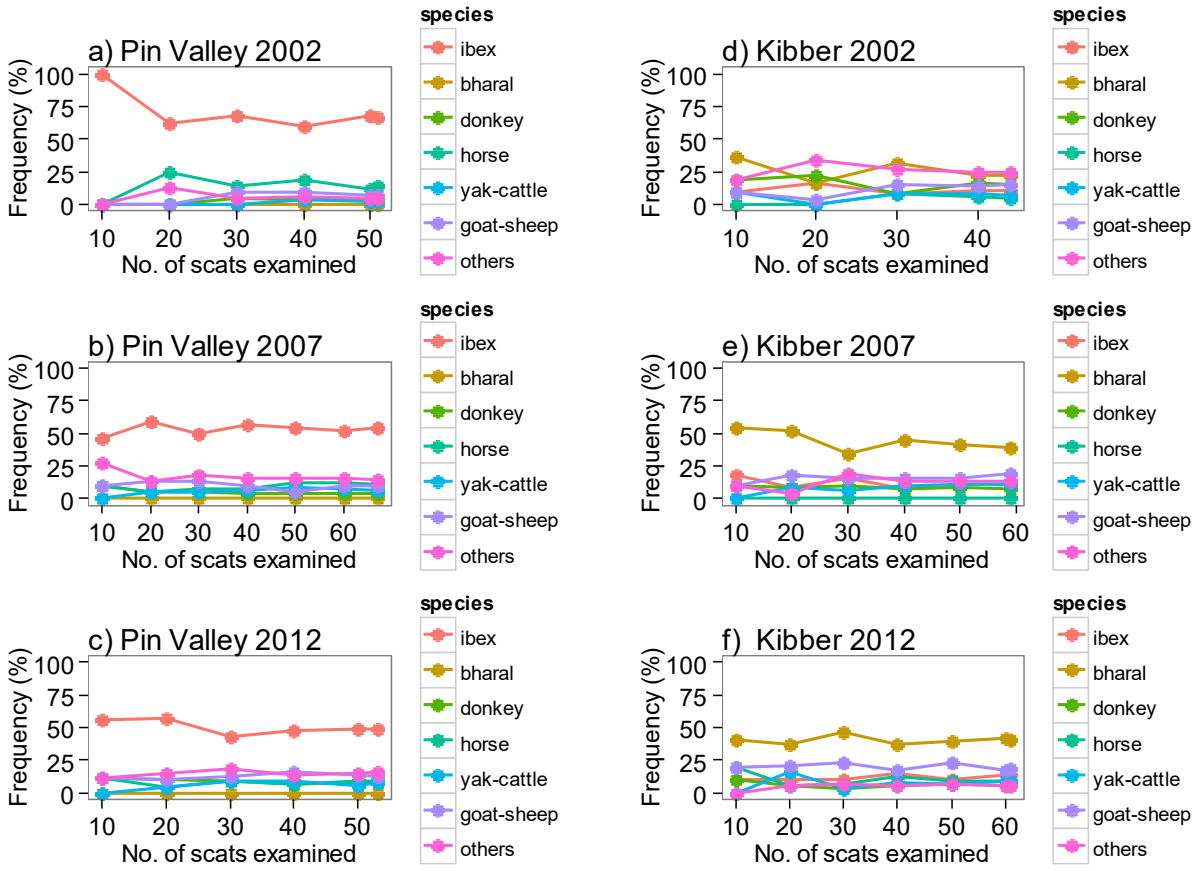
Appendix 1

Figure A1. Map of Spiti region in Trans-Himalayas of northern India. Kibber and Pin Valley are important for snow leopard conservation in this high-altitude mountainous region; these served as intervention and status-quo sites for our study. Symbols (+) represent the location of camera traps in Kibber. Digital elevation data are from USGS-SRTM http://www.cgiar-csi.org/



Appendix 2

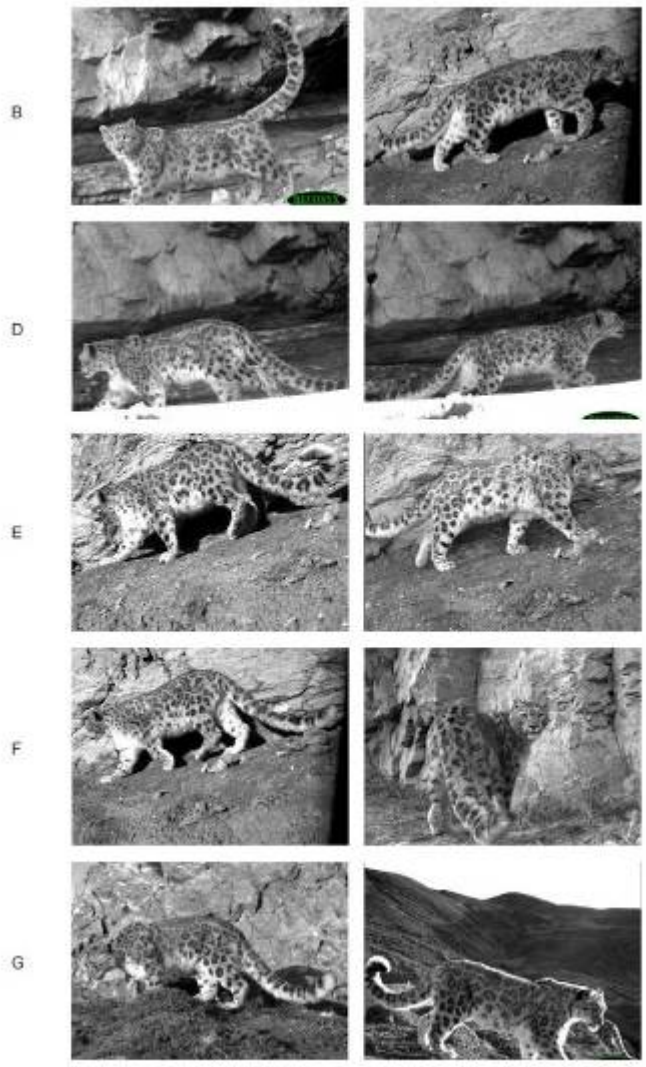
Figure A2. Variation in frequency of occurrence of different prey species in scats with number of scats examined, to determine adequacy of sampling effort. Patterns indicate that a sample of 40 scats is required to reliable estimate predator diet. Data for 2002 are from Bagchi and Mishra



Appendix 3

Figure A3. Profile pictures of individually identified snow leopards (two pictures each) from camera traps in Kibber (Spiti, northern India) during 2007. Three individuals, labeled A-C, were encountered in this period (R. K. Sharma unpubl.). Of these, individual B was photographed again during 2011-12.

Figure A4. Profile pictures of individually identified snow leopards (two pictures each) from camera traps in Kibber (Spiti, northern India) during 2011-2012. Five individuals, labeled B (also photographed in 2007 previously), and D-G, were encountered in this period (Rishi K. Sharma, unpublished data). Also see Sharma et al. (2015) for more details of camera-traps.



Appendix 4

Description of DNA-based molecular analysis for predator identification

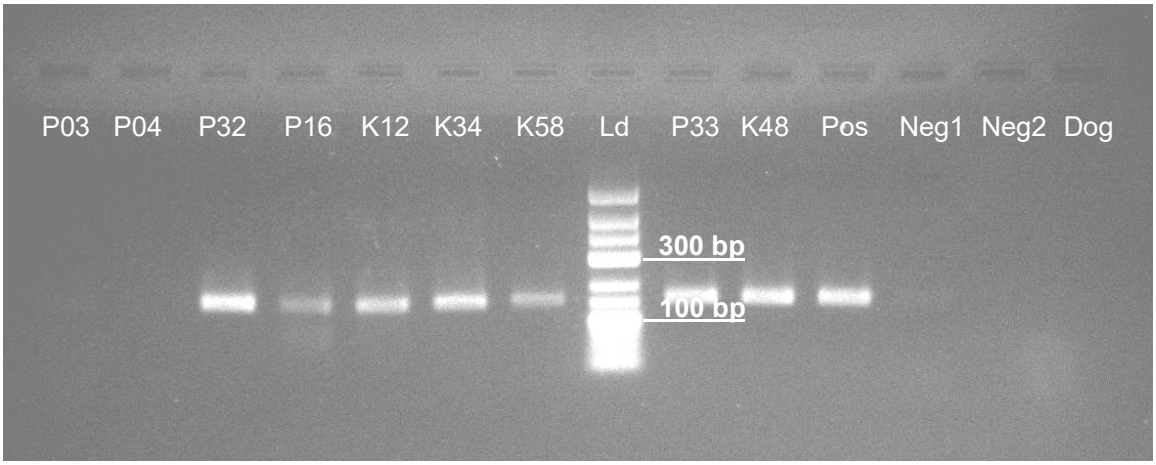
We used DNA-based molecular methods for predator identification. This is a noninvasive technique which attempts to verify whether species-specific primers can amplify regions of DNA extracted from scats with polymerase chain reaction (PCR). Previously, several studies have implemented these protocols for screening snow leopard scats (Anwar et al. 2011, Jumbay-Uulu et al. 2014). We attempted molecular analysis on all 114 scats from 2012. We extracted DNA from these scats by scraping pieces from the surface, and used QIAmp Stool Kit (Qiagen, Valancia, USA). We were able to extract 100 of these 114 scats successfully. The remaining 14 were either unsuitable for extraction, as the surface layer was damaged, or they did not yield adequate quantity of extract for use in PCR amplification. We used these extracts to amplify a 150 bp region of Cytochrome-b mitochondrial DNA which is known to be specific to snow leopards (Karmacharya et al. 2011). The forward primer was CYTB-SCT-PUN-F' (5'TGGCTGAATTATCCGATACC), and the reverse primer was CYTB-SCT-PUN-R' (5'-AGCCATGACTGCGAGCAATA). A 25 µl PCR reaction was prepared containing 2.5 µl of 10X PCR buffer, 2.5 µl of 25 mM magnesium chloride, 2 µl of 10 mM deoxynucleotide (dNTPs), 0.25 µl of 10 µg μl^{-1} bovine serum albumin (BSA), 0.01 µl of 5 U μl^{-1} Taq polymerase enzyme, 0.40 µl of each 10 µM primer, 13.85 µl of distilled water, and 3 µl of DNA extract. The PCR reaction was carried out using the following thermocycling condition: 94 °C for 30 s, and another 15 s, followed by 50 cycles of each 60 °C for 30 s for annealing, 72 °C for 1 minute for extension, and a final extension at 72°C for 7 min. If a scat extract did not test positive at first, then we re-extracted a second replicate of that particular sample and reanalyzed with PCR. When a second replicate also did not test positive, we checked the samples against

universal mammalian Cytochrome-b primer of Kocher et al. (1989). All PCR products were visualized on 4 mm thick, 2% Agarose gel stained with ethidium bromide, in 0.5 M TBE buffer, under ultraviolet light.

Of the 100 scats checked for snow leopard specific Cytochrome-b primer, 72 tested positive. Of the remaining 28 scats, 11 were tested for universal mammalian Cytochrome-b primer (Kocher et al. 1989), all 11 tested negative. From this we judged that these are too degraded for DNA analysis and inference. It is known that DNA degradation (due to high UV conditions at high elevations) can set in as little as three days after scats are deposited on soil, and exposed to the elements (Santini et al. 2007, see detailed analysis of scat-DNA degradation in Panasci et al. 2011).

We could not perform genetic screening for the older scats (2002, 2007). Retrospectively, this is not a major omission. Many primers and markers for large felids published during the 1990s and 2000s have been subsequently found to be non-specific to their purported target species (Maroju et al. 2016). It is increasingly evident that this problem of cross-species mismatch is fairly common, and is also known from other taxa (e.g., rodents, Migdal et al. 2016). So, it is unclear whether genetic screening of the older scats, with contemporary primers and markers, would have offered reliable evidence (see discussion on accuracy/precision below), or yield any DNA at all (Santini et al. 2007, Panasci et al. 2011).

Figure A5. Illustrative example of predator identification with a 150 bp region of cytochrome-b mitochondrial DNA, which is known to be specific to snow leopards. Gel electrophoresis patterns after polymerase chain reaction (PCR) of five scat samples from Pin Valley (P03, P04, P32, P16, and P33), and four from Kibber (K12, K34, K58, and K48) are shown, along with PCR-control (Neg1) and extraction-control (Neg2), positive-control (Pos), extract from dog (Dog), and ladder (L). The bands in the ladder that correspond to 100 bp and 300 bp, respectively are labeled. Seven scats show band at ca 150 bp, and two scats do not show band.



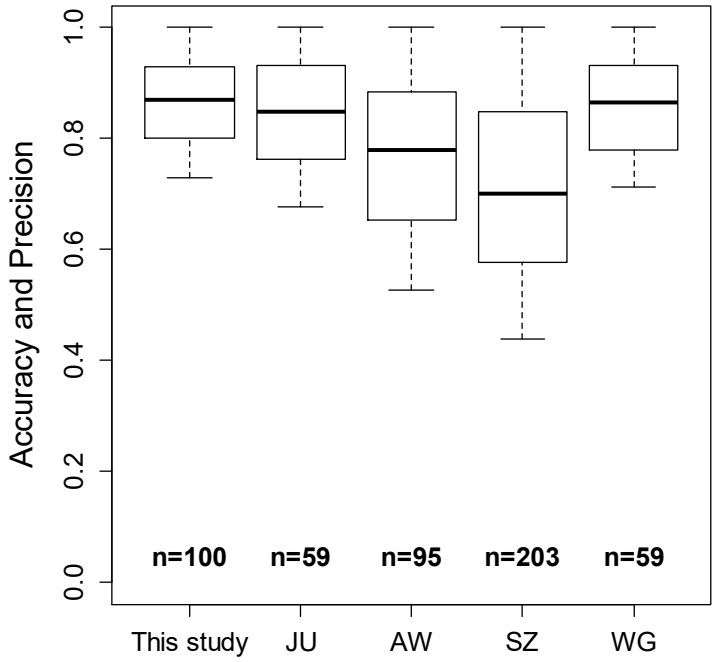
Appendix 5

Comparison of precision and accuracy of molecular identification with other studies

The DNA-based method of screening scats for predator identification is susceptible to both type-I and type-II error. Type-I error arises from a possibility of contamination of scats before they are collected by the researcher, and this contributes to false positives. A snow leopard scat can be deposited near/over a wolf scat, and subsequently both may test positive for snow leopard DNA. Even if one attempts overcome this by screening for multiple DNA matches in each scat, we still encounter problems with type-II error. Type-II errors can arise from fragmented DNA, chiefly from UV-photodegradation, and primers fail to amplify the target sequences during PCR-cycles. This contributes to false negatives, as significant UV-photodegradation of DNA can occur with 1-2 weeks (Woodruff et al. 2015). Admittedly, the likelihood of false negatives may be higher than false positives.

We explore these inherent shortcomings through randomized re-sampling iterations to evaluate accuracy and precision in our samples a posteriori, and compared them against other reports. Let p represent the number of scats testing positive for snow leopard DNA, and n represent the number of scats testing negative. Now, **accuracy** of identification is $a = \frac{p}{p+n}$. Considering false negatives (e.g. degraded scats), we examined accuracy for $72 \leq p \leq 100$ and $0 \leq n \leq 28$ in our samples. This accounts for potential false negatives, and assumes that false positives are absent. The variability among random re-sampling iterations would represent **precision**. For comparison we used the reported values of p and n in recent studies and assessed their accuracy and precision (Anwar et al. 2011, Shehzad et al. 2012, Wegge et al. 2012, Jumbay-Uulu et al. 2014). Results after 1000 random re-sampling iterations showed that accuracy and precision of our study compares favorably against other reports (Fig. A6).

Figure A6. Box-and-whisker plot (median and inter-quartile ranges) representing accuracy and precision of predator identification through DNA-screening of scats in our study, compared against other reports - (AW: Anwar et al. 2011; JU: Jumbay-Uulu et al. 2014; SZ: Shehzad et al. 2012; WG: Wegge et al. 2012). Number of scats screened in each case is also shown. Calculations are based iterative 1000 random re-samples to account for potential false negatives. The accuracy and precision of our samples compares favorably against the other reports.



Supplementary References

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