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Protocol of scat collection, DNA extraction, amplification, sequencing and species identification used for non-invasively collected scats from five priority leopard reserves in northwestern Iran, June-October 2012.

SAMPLE COLLECTION AND DNA EXTRACTION

We placed each faecal sample inside a labeled paper envelope with aid of disposable gloves or fresh wooden twigs with caution paid to block any chance of contamination. Preliminary species identification of scats was made in the field. Within 12 hours 0.5-1 g of outer layer of faecal samples were independently scratched with flamed razors and preserved in 95% ethanol (1:4 ratios). Samples were kept in a dark environment at room temperature (up to four months) until initial delivery to Molecular Ecology and Evolution Lab (MEEL) of Lund University.

DNA extraction and PCR set-up were performed in a room physically isolated from DNA extracts and PCR products. We extracted faecal DNA using Qiagen QIAamp™ DNA Stool Mini Kit (Qiagen Inc.) according to manufacture's recommendations, with the minor modification that we lysed DNA samples in 3-6 mL ASL buffer (enough to cover sample) overnight and homogenized the mixture by vortexing for 15-20 minutes. Contamination was monitored by including one negative control in every extraction round (11-15 samples). Extracted DNA was stored at -20 ^{o}C.

MITOCHONDRIAL DNA AMPLIFICATION, SEQENCING, AND SPECIES IDENTIFICATION

A carnivore-specific primer pair was designed from alignments of published mitochondrial genomes of potential large carnivores from our study sites, in order to amplify and sequence a 189-bp fragment of *cytochrome b* (*cytb*) gene from all faecal samples. The primer sequences are:

5' primer: 5'-CTTTYTCATCAGTCACCCACATYTGC-3'
3' primer: 5'-TCAGAAGGACATTTGTCCTCABGGT-3'

We initially evaluated performance of this primer set on reference DNA extracts of tissue samples from brown bear (*Ursus arctos*), red fox (*Vulpes vulpes*), and wild cat (*Felis silvestris*). This test showed accurate identification of these species.

The 25-µL PCR reactions were consisted of 1-2 µL of template DNA, 2.5 µL 10x PCR Buffer II, 2.5 µL 10-mM dNTP's, 2.5 µL 25-mM $MgCl_2$, 1 µg Bovine serum albumin (BSA), 1 µL of each 10-µM forward and reverse primer, 0.5 units of AmpliTaq DNA Polymerase (Applied Biosystems), and 13.4-14.4 µL ddH_2O. PCRs were performed in GeneAmp PCR System 9700 (Applied Biosystems) with the following cycling temperatures: initial denaturation at 94 ^{o}C/2', followed by 35-40 cycles of denaturation at 94 ^{o}C/30'', annealing step of 58 ^{o}C/30'', extension at 72 ^{o}C/45'', and a final extension step at 72 ^{o}C/10'. A positive control and one negative sample were included in each reaction to control PCR conditions and contamination, respectively.

PCR products were visualized by electrophoresis on 2% agarose gel using GelRed™ (Biotium Inc.). DNA samples that failed to amplify were treated with 20% Concentrated Chelex and re-amplified up to 5 times in different dilutions. DNA from potential leopard samples with no PCR product were also re-extracted with extending all the vortexing steps and digestion with Proteinase K. Samples showing an amplified product of the predicted size were precipitated using 11 µL 8-M NH_4Ac and 37.5 µL 95% ethanol, and then diluted by adding 5 µL ddH_2O. 2 µL of the diluted DNA was sequenced in total reaction volume of 10 µL using 0.5 µL of the forward primer, 1 µL BigDye Terminator Ready Reaction Mix (Applied Biosystems), 1.5 µL 5x Buffer and 5 µL ddH_2O. PCR profile was 30 cycle at 96 ^{o}C/10'', 55 ^{o}C/5'', 60 ^{o}C/4'. The sequencing reaction was precipitated with 2.5 µL 125-mM EDTA and 35 µL 95%

ethanol, and dry pellets were sequenced in an ABI Prism® 3100 capillary sequencer (Applied Biosystems). Sequences were visually edited and aligned using Geneious R6 (ver. 6.1.6; Biomatters Ltd.). The identity of each sequence was assigned to species using BLAST (BLASTn) on GenBank, accepting positive identification if there was a matching sequence with ≥99% identity.