A pilot study—genetic diversity and population structure of snow leopards of Gilgit-Baltistan, Pakistan, using molecular techniques

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ABSTRACT

**Background:** The Hindu Kush and Karakoram mountain ranges in Pakistan’s northern areas are a natural habitat of the snow leopard (Panthera uncia syn. Uncia uncia) but the ecological studies on this animal are scarce since it is human shy by nature and lives in difficult mountainous tracts. The pilot study is conducted to exploit the genetic diversity and population structure of the snow leopard in this selected natural habitat of the member of the wildcat family in Pakistan.

**Method:** About 50 putative scat samples of snow leopard from five localities of Gilgit-Baltistan (Pakistan) along with a control sample of zoo maintained male snow leopard were collected for comparison. Significant quality and quantity of genomic DNA was extracted from scat samples using combined Zhang–phenol–chloroform method and successful amplification of cytochrome c oxidase I gene (190 bp) using mini-barcode primers, seven simple sequence repeats (SSR) markers and Y-linked AMELY gene (200 bp) was done.

**Results:** Cytochrome c oxidase I gene sequencing suggested that 33/50 (66%) scat samples were of snow leopard. AMELY primer suggested that out of 33 amplified samples, 21 (63.63%) scats were from male and 12 (36.36%) from female leopards. Through successful amplification of DNA of 25 out of 33 (75.75%) scat samples using SSR markers, a total of 68 alleles on seven SSR loci were identified, showing low heterozygosity, while high gene flow between population.

**Discussion:** The low gene flow rate among the population results in low genetic diversity causing decreased diversification. This affects the adaptability to climatic changes, thus ultimately resulting in decreased population size of the species.

Subjects  Biogeography, Conservation Biology, Ecology, Molecular Biology, Zoology
Keywords  Population, Genetics, Panthera uncia, Pakistan, Molecular markers

INTRODUCTION

Snow leopard or ounce (Panthera uncia) has evolved in the mountainous ranges of Asia (Bonin et al., 2004). Pakistan’s northern mountains are also among its natural habitat, specifically the Gilgit-Baltistan (GB) and Chitral (Khyber Pakhtunkhwa). It is considered an indicator of the health of the ecosystem (O’Brien et al., 1985) and an icon of
conservation (Bonin et al., 2004). Though its population in Pakistan has remained constant for the last few years it still needs conservational efforts for its future survival (Sheikh & Molur, 2005). According to recent survey reports, its population may have decreased in the country (Nawaz & Hameed, 2015). Need for studies on its population genetics is emphasized to protect this threatened species (Network, 2014; McCarthy & Chapron, 2003). According to International Union for Conservation of Nature Red List of Threatened Species, it is listed as “Vulnerable” worldwide (McCarthy et al., 2017).

Animals like snow leopards live in difficult field conditions, have small and scattered populations, nocturnal habitat, excellent camouflage in the wild and human shy nature. These factors contribute toward the difficulties faced by researchers during field sampling for conservation genetic studies (Nowell & Jackson, 1996). Researchers have found a solution by introducing noninvasive sampling which provides a gateway for molecular and genetic analysis, aiming at conservation of wildlife species (Marra et al., 2003; Wolff et al., 2007).

The DNA quantity via scat samples is usually low, but to amplify this low quantity for genetic assessment, simple sequence repeats (SSR) markers are considered excellent, being co-dominant, highly polymorphic and easily transferable between populations (Jarne & Lagoda, 1996; Luo, Hu & Li, 2003). These markers can be easily automated for high throughput screening and information on mutation rate, heterozygosity, homozygosity, number of alleles per locus and Shannon index among different populations (Nei, 1978), while the sex ratio can be determined by using sex chromosome-specific molecular markers. Inbreeding depression, loss of genetic variability and increased homozygosity are associated with smaller populations and reduced chances of species to adapt to the environmental changes (Laurenson & Caro, 1994; Eisen et al., 1998; Keller & Waller, 2002; Chisci, Rossiter & Zappa, 2001; Franklin & Frankham, 1998; Lynch, Conery & Burger, 1995; Slate et al., 2000).

According to a recent estimate, the number of snow leopard individuals is 7,463–7,980 worldwide (McCarthy et al., 2016). Retributive killing, natural prey reduction, human-snow leopard conflicts and illegal trade of bones, pelt and other body parts are the causes of population decline in snow leopards (McCarthy et al., 2017; Maheshwari & Niraj, 2018). Studies conducted on snow leopard populations in central and south–east Asian region including Kyrgyzstan and Tajikistan, India, Nepal, Bhutan, China and Mongolia, show a decreased population size, low genetic diversity and mitochondrial DNA variation making the animals vulnerable to certain environmental conditions (Janežka et al., 2008, 2011, 2017). Keeping in view the conservation status and smaller size of the population of snow leopard, this study aimed at assessing genetic diversity to evaluate the adaptive potentials of the species under environmental changes. The main objective of this study was the optimization of genomic DNA (gDNA) extraction/amplification protocols and use of SSR markers for genetic analysis using field collected scats samples of snow leopard from GB, Pakistan.

**MATERIALS AND METHODS**

**Sample collection**

Snow leopard scat samples \( n = 50 \) were collected between December, 2016 and February, 2017 from five localities of GB (Pakistan), viz., Shagarthang, Basho, Thally,
Kharkoo and Astore (Fig. 1). Each sample was packed separately in centrifuge tubes (50 mL) having silica gel to avoid desiccation, labeled and brought to the laboratory and stored at 4 °C till further processing. Samples were subsequently processed for DNA extraction without any delay to obtain good quality of gDNA. A male snow leopard scat sample was provided by Khyber Pakhtunkhwa Wildlife Department (Pakistan), which was used as control.

**DNA extraction**

Genomic DNA was extracted from scat samples, using three protocols; (1) Zhang Method: Sample (1.5 g) was washed with ethanol, purified by phenol and gDNA was isolated by binding buffer and column filter (Bernevig, Hughes & Zhang, 2006). (2) Phenol–Chloroform Method: Sample was washed by phosphate buffer saline (PBS), purified by phenol–chloroform and the gDNA was precipitated through ethanol or isopropanol (Wang et al., 2003; Pelaia et al., 2004; Glynn et al., 2004). (3) Combined Zhang and Phenyl-Chloroform Method: Sample (0.25 g) was washed with PBS, bile products were removed by starch and purified with phenol–chloroform, while gDNA was isolated by using binding buffer and filter column. Extracted gDNA was visualized on 1% agarose gel (Sambrook, Fritsch & Maniatis, 1989).
Amplification
Mini-barcode primers pair having sequence TCCACTAATCACAARGATATTGGTAC and GAAAATCATAATGAAGGCATGAGC (Vaglia et al., 2008) was used to amplify the highly conserved mitochondrial gene, cytochrome c oxidase I (COI), region of extracted gDNA using polymerase chain reaction (PCR). For sex identification, AMELY marker was used (Murphy et al., 1999) after being tested on control male scat sample.

Microsatellite analysis
Confirmed snow leopard samples were genotyped by using seven microsatellites (SSR) markers, that is, PUN82, PUN100, PUN124, PUN132, PUN225, PUN229 and PUN327 (Janečka et al., 2008). Amplified products were visualized on 2% agarose gel. Bivariate data was generated on the basis of pattern of bands, and preceded through GenAlEx to export data file in PopGen format to calculate allelic frequency, genetic distance, heterozygosity and homozygosity (Nei, 1972).

RESULTS
DNA extraction and identification
Combined Zhang–Phenol–Chloroform method was efficient, giving significant extraction success rate, average quantity and quality of gDNA (Table 1; Fig. 2). PCR amplification and sequencing of DNA extracted using 190 bp mini-barcode primer of COI gene (Fig. 3) suggested that 33 out of the total 50 (66%) samples belonged to snow leopard.

Sex determination
Polymerase chain reaction amplification of Y-linked AMELY marker (200 bp) was achieved in control male sample and in 21 field collected samples, suggesting a sex ratio of 1.75:1 male to female (21 males and 12 females, Table 2; Fig. 4). The Chi-square test shows that sex ratio in overall samples of snow leopard scats was not significantly different from 1:1 (p > 0.05).

Genetic analysis
The study could amplify 25 samples out of 33 confirmed snow leopard scat samples at annealing temperature of 55 °C for 40 cycles using seven SSR markers. It identified 68 alleles on seven loci with band sizes of 90–220 bp; falling in reasonable proximity with those suggested previously (Janečka et al., 2008). Maximum number (14) of alleles was identified for PUN225 locus and the minimum (6) for PUN82 locus (Table 3).

Highest number (44) of alleles were recorded in Shagarthang valley, followed by Thally (33), Astore (27), Basho (26) and the lowest (25) in Kharkoo valley with Shannon indices in different populations ranged between 1.17 and 1.58. Allele frequencies of different SSR loci ranged between 0.02 and 0.75 in different populations (Table 4).
**Table 1** DNA extraction from scat samples of snow leopard.

| Methods              | Extraction success rate (%) | Average quantity (ng/μL) | Quality (purity ratio) |
|----------------------|----------------------------|--------------------------|------------------------|
| Zhang                | 20                         | 1,156                    | 0.7–0.8                |
| Phenol–Chloroform    | 50                         | 986                      | <1.2                   |
| Combined             | 67                         | 1,720                    | 1.6–1.8                |

**Note:**
Success rate, average quantity and quality of extracted DNA from scat samples of snow leopard under different extraction methods.

**Figure 2** Representative gel plate showing isolated DNA from scat samples. 1–9: scat samples of snow leopard collected from wild, C: control sample from zoo. 

**Table 2** Sex ratio of Snow Leopards. Sex ratio in five populations of Snow Leopard in Gilgit-Baltistan.

| Population     | Males | Females | Sex ratio (Male:Female) |
|----------------|-------|---------|-------------------------|
| Shagarthang valley | 8     | 4       | 2:1                     |
| Basho valley    | 3     | 2       | 3:2                     |
| Thally valley   | 3     | 3       | 1:1                     |
| Kharkoo valley  | 4     | 2       | 2:1                     |
| Astore valley   | 3     | 1       | 3:1                     |
Observed heterozygosity (analyzed on PopGen) was lower compared to expected heterozygosity (Fig. 5). Gene flow (Nm), calculated using Wright’s Fs statistics, indices for seven microsatellite primers, reflected high level of gene flow (average 1.65; range 1.18–3.87 for different loci) between populations (Table 5). Nei’s diversity indices of genetic distances (Table 6) between populations were low (<1).

Dendrogram constructed using UPGMA, showed low genetic differences and high genetic similarities among three populations (Thally, Kharkoo and Astore).
Shagarthang population exhibited one degree separation from the above three populations, while Basho population appeared as a separate clade (Fig. 6). AMOVA suggested no significant variance among populations indicating no populations’ differentiation among regions. Variance contributed by variability was 46% among individuals and that within individuals was 54%, while 0% was observed among populations (Fig. 7).

Table 3  Fixation indices and gene flow between populations.

| Locus | Average deviation in populations (Fis) | Deviation in total population (Fit) | Genetic differentiation (Fst) | Gene flow (Nm) |
|-------|----------------------------------------|------------------------------------|-----------------------------|---------------|
| PUN82 | 0.26                                    | 0.32                               | 0.07                        | 3.21          |
| PUN100| 0.54                                    | 0.61                               | 0.15                        | 1.40          |
| PUN124| 0.64                                    | 0.67                               | 0.10                        | 2.21          |
| PUN225| 0.45                                    | 0.56                               | 0.19                        | 1.05          |
| PUN229| −0.00                                   | 0.05                               | 0.06                        | 3.87          |
| PUN327| 0.169                                   | 0.31                               | 0.17                        | 1.18          |
| PUN132| 0.56                                    | 0.63                               | 0.15                        | 1.33          |
| Mean  | 0.38                                    | 0.46                               | 0.13                        | 1.65          |

Note: Fixation indices along with the gene flow between five snow lioeard populations of Gilgit-Baltistan.

Table 6  Genetic distances Nei’s diversity indices (above) and genetic distances (below).

| Population    | Shagarthang | Basho | Thally | Karkoo | Astore |
|---------------|-------------|-------|--------|--------|--------|
| Shagarthang   | —           | 0.67  | 0.70   | 0.73   | 0.70   |
| Basho         | 0.39        | —     | 0.71   | 0.56   | 0.56   |
| Thally        | 0.34        | 0.33  | —      | 0.66   | 0.78   |
| Karkoo        | 0.30        | 0.57  | 0.40   | —      | 0.81   |
| Astore        | 0.35        | 0.56  | 0.24   | 0.20   | —      |

Shagarthang population exhibited one degree separation from the above three populations, while Basho population appeared as a separate clade (Fig. 6). AMOVA suggested no significant variance among populations indicating no populations’ differentiation among regions. Variance contributed by variability was 46% among individuals and that within individuals was 54%, while 0% was observed among populations (Fig. 7).
DISCUSSION

In current study DNA was extracted from 50 putative snow leopard scat samples following three protocols (Taberlet, Waits & Luikart, 1999; Frantzen et al., 1998; Murphy et al., 2002; Wasser et al., 1997). Extraction of gDNA from mucosal layer of scat samples using combined Zhang and phenyl-chloroform method (Cobellis et al., 2004; Glynn et al., 2004; Bernevig, Hughes & Zhang, 2006) yielded significant quantity and quality of DNA, which was sufficient for successful amplification of COI gene/mini-barcode, microsatellite and sex determination primers (Thornton et al., 2008; Valledor et al., 2014).

The sample from a control male was amplified by AMELY primer, suggesting the validity of the technique in determination of sex using scat samples of snow leopard. AMELY gene (200 bp) is located on Y-chromosome and hence amplified in males only.
Out of total 33 confirmed snow leopard scat samples, 21 scats were of male, whereas 12 samples were of female leopards. The possible reason for less number of female leopards could be the ease of their hunting because they usually remain attached with their cubs, so during hunting they cannot escape easily. Also, during post-parturition the vulnerability of female leopards to mortality factors increases because of an increased ecological and energetic cost of parturition and lactation leading to male-biased sex ratio. Gender-biased infanticide could be another cause of less number of females as stated in literature regarding large carnivores (Oli & Rogers, 1996; Whitman et al., 2004; Balme et al., 2013; Bailey, 2005; Balme, Slotow & Hunter, 2009; Swanepoel et al., 2015).

We successfully amplified 75.75% of the field collected scat samples using SSR markers. The lack of amplification in other samples could be due to inhibitors (including bilirubins, bile salts and complex carbohydrates) in the extracted DNA templates (Flekna et al., 2007; Monteiro et al., 2001). The effect of such inhibitors was lowered by using a smaller quantity of the extracted DNA template (Flagstad et al., 2002; Rubin et al., 2000), increasing PCR cycles from 35 to 40 (Frantz et al., 2003; Rubin et al., 2000; Levi et al., 2002; Yekta, hung Shih & Bartel, 2004; Nsubuga et al., 2004; Roon, Waits & Kendall, 2005; Bonin et al., 2004) and using 10× solution “S” as an enhancer (Frackman et al., 1998). Enhancer has the ability to dissolve polar and non-polar compounds resulting in facilitated amplification of DNA (Govinda et al., 2011).

In current study, low genetic variation/heterozygosity (0.33–0.5) was observed in snow leopard population of GB compared to the expected value (0.62–0.75). Low heterozygosity has been reported previously for many populations of snow leopards in Central Asia including Southern Mongolia, Central China, Nepal, north-western India, Pakistan, Tajikistan and Kyrgyzstan (Janjua et al., 2019; Karmacharya et al., 2011; Janečka et al., 2008, 2011, 2017).

The low genetic diversity in these areas caused by smaller isolated size of the population where higher inbreeding results in increased homozygosity and genetic fixation or loss of unexploited genetic potentials (Kotzé & Muller, 1994). It can also be attributed to bottleneck effect, genetic drift and inbreeding depression which sometimes lead to expression of deleterious recessives alleles resulting in lowered survival rate of species (Lindenmayer et al., 1993; Franklin & Frankham, 1998; O’Brien et al., 1985). Small population size resulting in inbreeding depression reduced the chances of adaptation to environmental changes (Barone et al., 1994; Crooks, 2002).

**CONCLUSION**

The findings of genetic diversity, population structure and function played a vital role in formulation of conservational strategies. The low genetic diversity in snow leopard populations of the study leads to the conclusion that the gene flow among the populations is too low and the genetic diversification of the animal is not enough to aptly adapt to the environmental changes which would not result in the efficient promotion of this species. Therefore, proper planning and management in the protected and non-protected areas is required to get the output. The illegal hunting, poaching and human-animal conflicts...
have to be stopped by arranging proper protective and safety measures to the natural habitat of the inhabitant species in order to maintain the effective population size of the threatened wild fauna.

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Competing Interests
The authors declare that they have no competing interests.

Author Contributions
- Samreen Aruge performed the experiments, analyzed the data, prepared figures and/or tables.
- Hafsa Batool performed the experiments, prepared figures and/or tables.
- Fida M. Khan analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft, collected samples.
- Fakhar-i-Abbas conceived and designed the experiments, contributed reagents/materials/analysis tools, prepared figures and/or tables, approved the final draft, collected samples.
- Safia Janjua conceived and designed the experiments, approved the final draft.

Data Availability
The following information was supplied regarding data availability:
Raw data is available in Table S1. The raw data consists of the allele frequency of SSR markers at different loci in snow leopard population samples analyzed by POP Gen.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.7672#supplemental-information.
REFERENCES

Bailey TN. 2005. *The African leopard: ecology and behavior of a solitary felid*. Caldwell: The Blackburn Press, 429.

Balme GA, Slotow R, Hunter LTB. 2009. Impact of conservation interventions on the dynamics and persistence of a persecuted leopard (Panthera pardus) population. *Biological Conservation* 142:2681–2690.

Balme GA, Batchelor A, Britz De Woronin N, Seymour G, Grover M, Hes L, Macdonald DW, Hunter LTB. 2013. Reproductive success of female leopards *Panthera pardus*: the importance of top-down processes. *Mammal Review* 43(3):221–237 DOI 10.1111/j.1365-2907.2012.00219.x.

Barone MA, Roelke ME, Howard JG, Brown JL, Anderson AE, Wildt DE. 1994. Reproductive characteristics of male Florida Panthers (*Panthera pardus*): comparative studies from Florida, Texas, Colorado, Latin America, and North American Zoos. *Journal of Mammalogy* 75(1):150–162 DOI 10.2307/1382247.

Bernevig BA, Hughes TL, Zhang S-C. 2006. Quantum spin hall effect and topological phase transition in HgTe quantum wells. *Science* 314(5806):1757–1761 DOI 10.1126/science.1133734.

Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P. 2004. How to track and assess genotyping errors in population genetics studies. *Molecular Ecology* 13(11):3261–3273 DOI 10.1111/j.1365-294X.2004.02346.x.

Chisci L, Rossiter JA, Zappa G. 2001. Systems with persistent disturbances: predictive control with restricted constraints. *Automatica* 37(7):1019–1028 DOI 10.1016/S0005-1098(01)00051-6.

Cobellis L, Razzi S, De Simone S, Sartini A, Fava A, Danero S, Gioffrè W, Mazzini M, Petraglia F. 2004. The treatment with a COX-2 specific inhibitor is effective in the management of pain related to endometriosis. *European Journal of Obstetrics & Gynecology and Reproductive Biology* 116(1):100–102 DOI 10.1016/j.ejogrb.2004.02.007.

Crooks KR. 2002. Relative sensitivities of mammalian carnivores to habitat fragmentation. *Conservation Biology* 16(2):488–502 DOI 10.1046/j.1523-1739.2002.00386.x.

Eisen MB, Spellman PT, Brown PO, Botstein D. 1998. Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences of the United States of America* 95(25):14863–14868.

Flagstad O, Roed K, Stacy JE, Jakobsen KS. 2002. Reliable noninvasive genotyping based on excremental PCR of nuclear DNA purified with a magnetic bead protocol. *Molecular Ecology* 8(5):879–883 DOI 10.1046/j.1365-294X.1999.00623.x.

Fleksa G, Schneeweiss W, Smulders FJM, Wagner M, Hein I. 2007. Real-time PCR method with statistical analysis to compare the potential of DNA isolation methods to remove PCR inhibitors from samples for diagnostic PCR. *Molecular and Cellular Probes* 21(4):282–287 DOI 10.1016/j.mcp.2007.02.001.

Frackman S, Kobs G, Simpson D, Storts D. 1998. Betaine and DMSO: enhancing agents for PCR. *Promega Notes* 65:25–29.

Franklin IR, Frankham R. 1998. How large must populations be to retain evolutionary potential? *Animal Conservation* 1(1):69–70 DOI 10.1111/j.1469-1795.1998.tb00228.x.

Frantz AC, Pope LC, Carpenter PJ, Roper TJ, Wilson GJ, Delahay RJ, Burke T. 2003. Reliable microsatellite genotyping of the Eurasian badger (*Meles meles*) using faecal DNA. *Molecular Ecology* 12(6):1649–1661 DOI 10.1046/j.1365-294X.2003.01848.x.

Frantzen MAJ, Silk JB, Ferguson JWH, Wayne RK, Kohn MH. 1998. Empirical evaluation of preservation methods for faecal DNA. *Molecular Ecology* 7(10):1423–1428 DOI 10.1046/j.1365-294X.1998.00449.x.
Glynn EF, Megee PC, Yu H-G, Mistrot C, Unal E, Koshland DE, DeRisi JL, Gerton JL. 2004. Genome-wide mapping of the cohesin complex in the yeast Saccharomyces cerevisiae. PLOS Biology 2(9):e259 DOI 10.1371/journal.pbio.0020259.

Govinda V, Attri P, Venkatesu P, Venkateswarlu P. 2011. Thermophysical properties of dimethylsulfoxide with ionic liquids at various temperatures. Fluid Phase Equilibria 304(1–2):35–43 DOI 10.1016/j.fluid.2011.02.010.

Janečka JE, Jackson R, Yuquang Z, Diqiang L, Munkhtsog B, Buckley-Beason V, Murphy WJ. 2008. Population monitoring of snow leopards using noninvasive collection of scat samples: a pilot study. Animal Conservation 11(5):401–411 DOI 10.1111/j.1469-1795.2008.00195.x.

Janečka JE, Munkhtsog B, Jackson RM, Naranbaatar G, Mallon DP, Murphy WJ. 2011. Comparison of noninvasive genetic and camera-trapping techniques for surveying snow leopards. Journal of Mammalogy 92(4):771–783 DOI 10.1644/10-MAMM-A-036.1.

Janečka JE, Zhang Y, Li D, Munkhtsog B, Bayaraa M, Galsandorj N, Wangchuk TR, Karmacharya D, Li J, Lu Z. 2017. Range-wide snow leopard phylogeography supports three subspecies. Journal of Heredity 108:597–607 DOI 10.1093/jhered/esx044.

Janjua S, Peters JL, Weckworth B, Abbas FI, Bahn V, Johansson O, Rooney TP. 2019. Improving our conservation genetic toolkit: ddRAD-seq for SNPs in snow leopards. Conservation Genetics Resources 1–5 DOI 10.1007/s12686-019-01082-2.

Jarne P, Lagoda PJL. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology & Evolution 11(10):424–429 DOI 10.1016/0169-5347(96)10049-5.

Karmacharya DB, Thapa K, Shrestha R, Dhakal M, Janecka JE. 2011. Noninvasive genetic population survey of snow leopards (Panthera uncia) in Kangchenjunga conservation area, Shey Phoksundo National Park and surrounding buffer zones of Nepal. BMC Research Notes 4:516 DOI 10.1186/1756-0500-4-516.

Keller LF, Waller DM. 2002. Inbreeding effects in wild populations. Trends in Ecology & Evolution 17:230–241 DOI 10.1016/S0169-5347(02)02489-8.

Kotzé A, Muller GH. 1994. Genetic relationship in South African cattle breeds. In: Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, Guelph. Vol. 21. Guelph: University of Guelph, 413–416.

Laurenson MK, Caro TM. 1994. Monitoring the effects of non-trivial handling in free-living cheetahs. Animal Behaviour 47:547–557 DOI 10.1006/anbe.1994.1078.

Levi F, Lucchini F, Negri E, La Vecchia CL. 2002. Trends in mortality from cardiovascular and cerebrovascular diseases in Europe and other areas of the world. Heart 88(2):119–124 DOI 10.1136/heart.88.2.119.

Lindemayer DB, Lacy RC, Thomas VC, Clark TW. 1993. Predictions of the impacts of changes in population size and environmental variability on Leadbeater’s possum, Gymnobelideus leadbeateri McCoy (Marsupialia: Petauridae) using population viability analysis: an application of the computer program VORTEX. Wildlife Research 20:67–85 DOI 10.1071/WR9930067.

Luo W-Y, Hu J, Li X-F. 2003. The evolution and application of microsatellites. Yi Chuan= Hereditas 25:615–619.

Lynch M, Conery J, Burger R. 1995. Mutation accumulation and the extinction of small populations. American Naturalist 146(4):489–518 DOI 10.1086/285812.

Maheshwari A, Niraj SK. 2018. Monitoring illegal trade in snow leopards: 2003–2014. Global Ecology and Conservation 14:e00387 DOI 10.1016/j.gecco.2018.e00387.

Marra MA, Jones SJM, Astell CR, Holt RA, Brooks-Wilson A, Butterfield YSN, Khattra J, Asano JK, Barber SA, Chan SY, Cloutier A, Coughlin SM, Freeman D, Girn N, Griffith OL, Leach SR, Mayo M, McDonald H, Montgomery SB, Pandoh PK, Petrescu AS, Robertson AG,
Schein JE, Siddiqui A, Smailus DE, Stott JM, Yang GS, Plummer F, Andonov A, Artsob H, Bastien N, Bernard K, Booth TF, Bowness D, Czub M, Drebot M, Fernando L, Flick R, Garbutt M, Gray M, Grolla A, Kabani A, Li Y, Meyers A, Normand S, Stroher U, Tyler S, Vogrig R, Ward D, Watson B, Brunham RC, Krajden M, Petric M, Skowronski DM, Upton C, Roper L. 2003. The genome sequence of the SARS-associated coronavirus. *Science* **300**(5624):1399–1404 DOI 10.1126/science.1085953.

McCarthy TM, Chapron G. 2003. Snow leopard survival strategy. *International Snow Leopard Trust and Snow Leopard Network, Seattle, USA* 1–105.

McCarthy T, Mallon D, Jackson R, Zahler P, McCarthy K. 2017. *Panthera uncia*. The IUCN red list of threatened species 2017: e.T22732A50664030.

McCarthy T, Mallon D, Sanderson EW, Zahler P, Fisher K. 2016. What is a snow leopard? Biogeography and status overview. In: McCarthy T, Mallon D, eds. *Snow Leopards: Biodiversity of the World: Conservation from Genes to Landscapes*. Amsterdam: Elsevier Academic Press, 23–42.

Monteiro L, Gras N, Vidal R, Cabrita J, Mégraud F. 2001. Detection of *Helicobacter pylori* DNA in human feces by PCR: DNA stability and removal of inhibitors. *Journal of Microbiological Methods* **45**(2):89–94 DOI 10.1016/S0167-7012(01)00225-1.

Murphy WJ, Sun S, Chen Z, Pecon-Slattery J, O'Brien SJ. 1999. Extensive conservation of sex chromosome organization between cat and human revealed by parallel radiation hybrid mapping. *Genome Research* **9**(12):1223–1230 DOI 10.1101/gr.9.12.1223.

Murphy MA, Waits LP, Kendall KC, Wasser SK, Higbee JA, Bogden R. 2002. An evaluation of long-term preservation methods for brown bear (Ursus arctos) fecal DNA samples. *Conservation Genetics* **3**(4):435–440 DOI 10.1023/A:1020503330767.

Nawaz MA, Hameed S. 2015. Research update 2008–2014 Snow leopard program: report prepared for Snow Leopard Red List Assessment Team. Pakistan.

Nei M. 1972. Genetic distance between populations. *American Naturalist* **106**(949):283–292 DOI 10.1086/282771.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**:583–590.

Network SL. 2014. *Snow leopard survival strategy*. Seattle: Snow Leopard Network, 1–145.

Nowell K, Jackson P. 1996. *Wild cats: status survey and conservation action plan*. Gland: IUCN.

Nsubuga AM, Robbins MM, Roeder AD, Morin PA, Boesch C, Vigilant L. 2004. Factors affecting the amount of genomic DNA extracted from ape faeces and the identification of an improved sample storage method. *Molecular Ecology* **13**(7):2089–2094 DOI 10.1111/j.1365-294X.2004.02207.x.

O’Brien S, Roelke M, Marker L, Newman A, Winkler C, Meltzer D, Colly L, Evermann J, Bush M, Wildt D. 1985. Genetic basis for species vulnerability in the cheetah. *Science* **227**(4693):1428–1434 DOI 10.1126/science.2983425.

Oli MK, Rogers ME. 1996. Seasonal pattern in group size and population composition of blue sheep in Manang, Nepal. *Journal of Wildlife Management* **60**(4):797–801.

Pelaia G, Cuda G, Vatrella A, Gallelli L, Fratto D, Giofrè V, D'Agostino B, Caputi M, Maselli R, Rossi F, Costanzo FS, Marsico SA. 2004. Effects of hydrogen peroxide on MAPK activation, IL-8 production and cell viability in primary cultures of human bronchial epithelial cells. *Journal of Cellular Biochemistry* **93**(1):142–152 DOI 10.1002/jcb.20124.

Roon DA, Waits LP, Kendall KC. 2005. A simulation test of the effectiveness of several methods for error-checking non-invasive genetic data. *Animal Conservation* **8**:203–215 DOI 10.1017/S1367943005001976.
Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, Fortini ME, Li PW, Apweiler R, Fleischmann W, Michael Cherry J, Henikoff S, Skupski MP, Misra S, Ashburner M, Birney E, Boguski MS, Brody T, Brokstein P, Celniker SE, Chervitz SA, Coates D, Cravchik A, Gabrielian A, Galle RF, Gelbart WM, George RA, Goldstein LSB, Gong F, Guan P, Harris NL, Hay BA, Hoskins RA, Li J, Li Z, Hynes RO, Jones SJM, Kuehl PM, Lemaître B, Littleton JT, Morrison DK, Mungall C, Farrell PH, Pickeral OK, Shue C, Vosshall LB, Zhang J, Zhao Q, Zheng XH, Lewis S. 2000. Comparative genomics of the Eukaryotes. *Science* 287(5461):2204–2215 DOI 10.1126/science.287.5461.2204.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*. New York: Cold spring harbor laboratory press.

Sheikh KM, Molur S. 2005. Status and red list of Pakistan’s mammals, based on conservation assessment and management plan for mammals. *IUCN, Pakistan 344*.

Slate J, Kruuk LEB, Marshall TC, Pemberton JM, Clutton-Brock TH. 2000. Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elaphus*). *Proceedings of the Royal Society of London. Series B: Biological Sciences* 267:1657–1662 DOI 10.1098/rspb.2000.1192.

Swanepoel LH, Somers MJ, Van WH, Schiess-Meier M, Owen C, Snyman A, Senekal C, Camacho G, Boshoff W. 2015. Survival rates and causes of mortality of leopards *Panthera pardus* in southern Africa. *Oryx* 49:595–605 DOI 10.1017/S0030605313001282.

Taberlet P, Waits LP, Luikart G. 1999. Noninvasive genetic sampling: look before you leap. *Trends in Ecology & Evolution* 14:323–327 DOI 10.1016/S0169-5347(99)01637-7.

Thornton TM, Pedraza-Alva G, Deng B, Wood CD, Aronshtam A, Clements JL, Davis RJ, Matthews DE, Doble B, Rincon M. 2008. Phosphorylation by p38 MAPK as an alternative pathway for GSK3β inactivation. *Science* 320:667 DOI 10.1126/science.1156037.

Vaglia T, Haxaire J, Kitching IJ, Meusnier I, Rougerie R. 2008. Morphology and DNA barcoding reveal three cryptic species within the *Xylophanes neoptolemus* and *loelia* species-groups (Lepidoptera: Sphingidae). *Zootaxa* 1923:18–36 DOI 10.5281/zenodo.18478.

Valledor L, Escandón M, Meijón M, Nukarinen E, María JC, Weckwerth W. 2014. A universal protocol for the combined isolation of metabolites, DNA, long RNAs, small RNAs, and proteins from plants and microorganisms. *Plant Journal* 79(1):173–180 DOI 10.1111/tpj.12546.

Wang Y, Fan W, Guo F, Peng T, Li C. 2003. Geochemistry of Mesozoic Mafic Rocks adjacent to the Chenzhou-Linwu fault, South China: implications for the Lithospheric boundary between the Yangtze and Cathaysia blocks. *International Geology Review* 45(3):263–286 DOI 10.2747/0020-6814.45.3.263.

Whitman K, Starfield AM, Quaddling HS, Packer C. 2004. Sustainable trophy hunting of African lions. *Nature* 428(6979):175–178 DOI 10.1038/nature02395.

Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, Van de Vijver M, Wheeler TM, Hayes DF, American Society of Clinical Oncology/College of American Pathologists. 2007. American society of clinical oncology/college of American pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *Archives of Pathology & Laboratory Medicine* 131:18–43.

Yekta S, hung Shih I, Bartel DP. 2004. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 304(5670):594–596 DOI 10.1126/science.1097434.