Evolutionary Genetics and Conservation of the Critically Endangered Arabian Leopard (*Panthera pardus nimr*)

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Declaration of originality

I declare that this PhD thesis entitled Evolutionary Genetics and Conservation of the Critically Endangered Arabian Leopard (*Panthera pardus nimr*) is my own research work and contains no material that has been submitted in whole or part elsewhere for the award of any academic degree. I collected all the samples from the wild and conducted the genetic laboratory work, carried out the genetic analyses, and wrote all the chapters with editorial suggestions from my PhD supervisors Jim Groombridge, Hazel Jackson and Jim Labisko. Andrew Spalton provided editorial suggestions and edits for improvement of the content and Jim Labisko provided statistical support for the phylogenetic analysis (Chapter 2). Captive and museum samples were provided by several organizations that I have acknowledged below.

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Abstract

Large carnivores are considered an important component of an ecosystem and their role as apex predators makes them crucial for maintenance of ecosystem function and biodiversity. Yet despite their important ecological value, large carnivores are among the world's most threatened species, mostly due to human persecution and loss of their habitat and prey species. The Arabian leopard (*Panthera pardus nimr*) is the region's last remaining big cat, and was once widely distributed across the Arabian Peninsula but its occupied range has contracted from ~888,300 km^2 to 17,400 km^2 since the 1970s, and it is listed as Critically Endangered on the IUCN Red List of Threatened Species. Despite its threatened status, scientific information is lacking for many aspects of the Arabian leopard, including its population and evolutionary genetics. The objectives of this study were therefore to improve the knowledge base to help develop better management strategies for the long-term persistence of the Arabian leopard. By generating a comprehensive mitochondrial DNA sequence database that included sequence data from wild Arabian leopard populations across the Arabian Peninsula my study provided evidence that the Arabian leopard is evolutionarily distinct from other leopard subspecies. Assessment of genetic diversity using a suite of microsatellite markers indicate that the Arabian leopard is genetically impoverished in comparison to other leopard subspecies. However, high levels of genetic diversity and unique alleles were discovered in wild and captive Arabian leopards of Yemeni origin, compared to the wild leopards of the Dhofar mountains of Oman, an area considered to be their last stronghold. Using genetic data from wild leopards obtained via non-invasive scat surveys, we detected fine-scale spatial genetic structure within the leopard population of Dhofar which is

likely due to recent human development in the region. DNA surveys of the Dhofar population provided robust estimates of density and population size that are comparable with those derived from camera trap estimates, indicating the reliability of genetic sampling for monitoring of the Arabian leopard. Based on these findings a number of conservation management strategies are proposed including genetic rescue via introgression of Yemen genes to restore the genetic diversity of impoverished populations and enhance the overall evolutionary potential of the Arabian leopard. Other suggested measures include strengthening legislation and enforcement in combination with community engagement to ease human-wildlife conflict as well as the protection and safeguarding of critical habitat and habitat corridors to address population fragmentation. Urgent adoption of these recommendations is required, and the novel information generated by this research provides the evidential basis for their effective implementation that will help ensure the long-term persistence of the Arabian leopard.

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Photographs of Arabian leopard taken by camera traps in the Dhofar mountains.

1. General introduction

1.1 Introduction

Large carnivores are considered an important component of an ecosystem and their role as an apex predator makes them crucial for maintenance of ecosystem function and biodiversity (Miller *et al.*, 2002; Ripple *et al.*, 2014), alongside bringing wider cultural, educational and economic benefits. However, despite their important value, large carnivores are among the world's most threatened species mostly due to human persecution and loss of their habitat and prey species (Ripple *et al.*, 2014; Wolf & Ripple, 2017). The leopard (*Panthera pardus*) was once a widely distributed carnivore, however, its global range has contracted from around 35,000,000 km^2 to ~8,500,000 km^2 (Jacobson *et al.*, 2016), meaning it is now listed as Vulnerable by the IUCN Red List for Threatened Species. Some of the regional populations/subspecies are considered Endangered or even Critically Endangered (Stein *et al.*, 2016).

Despite some highly threatened subspecies of leopard being prioritised for conservation and research efforts (Jacobson *et al.*, 2016), Jacobson *et al.* (2016) found that many of these taxa remain in need of urgent attention. To conserve the leopard and its various regional populations/subspecies, especially those that are Critically Endangered, and to ensure they continue to fulfil their important ecosystem function as an apex predator, the global conservation community needs to understand (i) their phylogenetic relationships and evolutionary history (Uphyrkina *et al.*, 2001), (ii) population genetics, connectivity and gene flow, and (iii) ecological parameters such as population size and density, to inform future conservation efforts.

Scientific information from these studies enables conservation authorities to make sound effective management strategies to protect the leopard across its range.

1.2 Leopard phylogeography

Unlike all other big cats, the leopard has the widest distribution, ranging from the tropical rainforest to woodlands in Africa, and from semi-arid deserts in the Middle East to deciduous forests of Southern Asia (Nowell & Jackson, 1996; Jacobson *et al.*, 2016). This widespread distribution and their presence in different types of habitat has resulted in morphological variations (e.g. body and skull size, coat and skin colour) between leopard populations (Nowell & Jackson, 1996; Sunquist & Sunquist, 2002). As a consequence, the taxonomic relationships for a number of leopard subspecies have been described based only on skull and coat variations (Pocock, 1932; Zukowsky, 1964; Miththapala *et al.*, 1996). However, with the advancement of molecular genetics and phylogenetic analysis some of these previously described subspecies have been re-assessed. As a result, between eight (Miththapala *et al.*, 1996) and nine distinct subspecies (Uphyrkina *et al.*, 2001) have been proposed. These subspecies are; *Panthera pardus pardus* (Africa), *P. p. nimr* (Arabia), *P. p. saxicolor* (Central Asia), *P. p. fusca* (India), *P. p. kotiya* (Sri Lanka), *P. p. delacouri* (South China), *P. p. melas* (Java), *P. p. japonensis* (North China) and *P. p. orientalis* (Russian Far East). The Cat Task Force of the Cat Specialist Group consider *P. p. japonensis* synonymous with *P. p. orientalis* on the basis of there being little molecular variation between them (Kitchener *et al.*, 2017). Further sampling of *P. p. saxicolor* (Farhadinia *et al.*, 2015) and *P. p. melas* (Wilting *et al.*, 2016) addressed the gaps of limited sample size encountered by Miththapala *et al.* (1996) and Uphyrkina

et al. (2001), and in doing so confirmed the evolutionary distinctness of these subspecies.

The leopard is thought to have originated in east Africa. The earliest fossil records of this species are from Laetoli in Tanzania and date back to 3.5 million years ago (Stein & Hayssen, 2013). However, phylogenetic studies indicate that the leopard diverged from the African lion (*Panthera leo*) 1.81-4.63 million years ago (Mya) (Uphyrkina *et al.*, 2001; Johnson *et al.*, 2006; Davis *et al.*, 2010; Wei *et al.*, 2011; Nyakatura & Bininda-Emonds, 2012; Tseng *et al.*, 2013; Zhang & Zhang, 2013).

Understanding the evolutionary history and divergence of the different leopard subspecies is very important for the conservation management of each taxa. Given the current global extinction crisis, and due to the limited resources available, conservationists need to identify priorities and ensure resources are invested to conserve those species/populations which are the most evolutionarily distinct and the most threatened. Advances in the development of molecular techniques allow us not only to identify phylogenetically distinct species, subspecies and populations but also to investigate other aspects of the rare and elusive leopard, such as identification of individuals, and estimation of population size and density.

1.3 Leopard conservation genetics

Species and populations require genetic diversity to be able to evolve and adapt to changing environments. Genetic diversity is acted upon by the process of natural selection whereby evolutionary forces determine how populations adapt and change over time in response to external influences such as changes in climate and the environment. Genetic diversity is therefore very important for the long-term

evolutionary potential of a species or population, as well as for short-term fitness and population persistence (Frankham *et al.*, 2010). Consequently, assessing and preserving genetic diversity is fundamental for conservation management, in particular for small populations which can often suffer from loss of genetic diversity due to bottleneck effects, increased levels of inbreeding, and the effects of random genetic drift (Frankham, 2003; Frankham *et al.*, 2010). Indeed, habitat fragmentation and subsequent population isolation can mean that the effects of random genetic drift are felt differently in different isolated subpopulations.

The leopard has recently disappeared from ~ 75% of its global range (Jacobson *et al.*, 2016) and five of the nine recognised subspecies are either Endangered or Critically Endangered (Stein *et al.*, 2016). Some of these subspecies (*P. p. kotiya* and *P. p. orientalis*) have been found to contain low levels of genetic diversity (Uphyrkina *et al.*, 2001). Studying the genetic aspects of endangered leopard subspecies is crucial and can provide valuable knowledge regarding population genetic diversity, population structure and gene flow, all essential for informing both *in-situ* and *ex-situ* conservation programs (Allendorf *et al.*, 2013). Genetic data can also provide information about population abundance and density and can be used for population monitoring of elusive species (Frankham *et al.*, 2010).

1.4 The Arabian leopard *Panthera pardus nimr*

The Arabian leopard, first described by Hemprich and Ehrenberg in 1833 based on skin material from Al Qunfudah in Saudi Arabia, is the only extant big cat in the Arabian Peninsula. The Asiatic lion (*Panthera leo persica)* may once have occurred in the region but no longer occurs there (Pocock, 1939) and the last Asiatic cheetah

(*Acinonyx jubatus venaticus*) on the peninsula was killed in Dhofar, Oman in 1977 (Harrison & Bates, 1991).

The Arabian leopard is found largely within mountainous areas characterised by arid to hyper-arid climate but in parts of southern Arabia, including southern Oman and south-eastern Yemen the leopard also inhabits grasslands and woodlands of mountainous areas that come under the influence of the South-West Monsoon (Spalton & Al Hikmani, 2014). It is a small generally pale coloured leopard with males weighing 30kg and females 20kg and as the top-predator its prey base commonly includes Nubian ibex (*Capra nubiana*), Arabian gazelle (*Gazella arabica)* and rock hyrax (*Procavia capensis*) in arid areas while in the monsoon habitats, where both gazelle and ibex are absent, its diet is largely composed of hyrax, other smaller mammals and birds. In all areas leopard are also known to predate on livestock including camels, cattle and goats.

In southern Oman the Arabian leopard is largely crepuscular though has been camera-trapped at most times of the day. It is largely solitary except when males and females come together for breeding and when females are accompanied by their offspring. Both sexes utilize large areas with a GPS tagged female using a range of 64 km^2 and a male using 168 km^2 (Spalton & Al Hikmani, 2014). Males share their ranges with females and male ranges overlap in space but not time – individuals presumably use scent marks to avoid each other (Spalton & Hikmani 2014).

Once widespread throughout the mountainous region of Arabia from Palestine in the north to Oman in the east (Harrison & Bates, 1991), the Arabian leopard is now only found in small isolated populations in 2% of its former range in Oman, Yemen and Saudi Arabia (Jacobson *et al.*, 2016). The current total global population is estimated

to be fewer than 250 individuals and is thought to be declining (Breitenmoser *et al.*, 2010). Human persecution, habitat fragmentation, prey loss, and genetic depletion due to small population size pose the main threats to this smallest of leopard subspecies (Breitenmoser *et al.*, 2006, 2010; Spalton & Al Hikmani, 2014).

Since the 1990s the Arabian leopard has become a conservation priority for most range states including Oman, United Arab Emirates (UAE), Saudi Arabia and Yemen. Conservation measures have focused on surveys and particularly on captive breeding; by 2011 at least nine institutions were contributing to the International Arabian Leopard Studbook (see Edmonds *et al.*, 2006; Budd, 2011; Budd & Leus, 2011 for details and location of regional leopard breeding institutions). In Oman the focus has been on *in situ* conservation.

These early conservation measures were followed by the production of a regional strategy for conservation of the Arabain leopard in 2010 and a country specific national action plan (e.g. Oman and Saudi Arabia) (Breitenmoser *et al.*, 2010; Zafar-ul Islam *et al*., 2017). However, despite over two decades of conservation efforts, little is known of the population size and density of this Critically Endangered leopard across its remaining range. An understanding of these parameters is essential for any assessment of wild populations and for the setting of conservation priorities to protect them (Sharma *et al.*, 2005; Selvan *et al.*, 2014). Furthermore, there little understanding of the evolutionary phylogenetic distinctiveness and population genetics of this leopard. Its subspecies status was reconfirmed using molecular data, but this was based on material from a single animal (Uphyrkina *et al.*, 2001), and although the Arabian leopard is considered to be genetically impoverished due to its small population size (< 250), no molecular studies have yet been conducted to

assess its conservation genetic status. The importance of genetic study for this subspecies has been highlighted in the strategy for the conservation of the Arabian leopard (Breitenmoser *et al.*, 2010). The Arabian leopard population of the Dhofar Mountains of southern Oman is considered the last stronghold for this subspecies in the wild (Breitenmoser *et al.*, 2006, 2010). This population has been the subject of many years of conservation efforts, and by 2014 there were at least 20 field staff working in the Dhofar mountains to study and safeguard the Arabian leopard. Nevertheless, its population size has recently been estimated at just 44-58 adults (Spalton & Al Hikmani, 2014) and both its habitat and principal prey species face numerous anthropogenic threats.

Just as a good understanding of population biology and of genetics is critical to the conservation of the subspecies *per se*, it is also essential for the conservation of Dhofar's remnant population. Population estimates can help conservation authorities to assess and monitor population trends, and together with density data can help to prioritise management plans and set recommendations to improve the protection status of the Dhofar Mountains, mitigate threats and also identify important habitat areas for the leopard. In addition, an understanding of the population genetics of this important subpopulation is vital to inform managers charged with maintaining genetically healthy and diverse wild and captive populations.

This PhD thesis aims to provide, for the first time, rigorous scientific information on population size and density as well as phylogenetic history, genetic diversity, population genetic structure and gene flow of the Arabian leopard. The outcomes of the PhD thesis will be shared amongst and used by regional and national institutions in the Arabian Peninsula to conserve the Arabian leopard and return it from the brink

of extinction. The results of this PhD research will provide crucial information with which to update of the Arabain leopard regional conservation strategy, country national action plans and IUCN asseement of the subspecies.

1.5 Thesis structure

Following Chapter 1 Introduction this thesis consists of five chapters which are written in manscript format for eventual publication in peer-reviewed journals in due course.

Chapter 2 investigates the evolutionary history of the Arabian leopard and its relationship to other leopard subspecies using mtDNA obtained from wild leopards in Oman, Yemen and Saudi Arabia. As some mammal species in the Arabian Peninsula were found to be distinct lineages and to have diverged a relatively long time ago from their counterparts in Africa, I hypothesised that a similar evolutionary history would hold for the Arabian leopard as opposed to an alternative scenario that the Arabian leopard is a very recent descendant. Chapter 3 assesses the levels of genetic diversity found within wild and captive populations of the Arabian leopard. Given the small population size of the Arabian taxon, I hypothesized that the Arabian leopard to have low levels of genetic diversity in comparison to other less-endangered leopard species that exist elsewhere. To test this theory, I used microsatellite DNA markers to survey—for the first time—levels of genetic diversity in both wild and captive populations of the Arabian leopard. I then use this information to (i) compare levels of genetic diversity in time and space between the different wild subpopulations and interpret observed differences in relation to the species' population size and known demographic history; and (ii) examine the genetic composition of the captive leopard population and put forward a number of

potential scenarios for future management, including the possibility of reintroduction.

Chapter 4 considers population genetic structure, and gene flow in the remnant wild leopard population of Oman's Dhofar mountains. Given, the rapid increase in development and increase of livestock numbers in this region since 1970s, I hypothesised that these factors have reduced levels of gene flow within the leopard population of Dhofar, subsequently revealed by the extent of genetic structure. We used non-invasive molecular methods to identify spatial patterns of genetic structure in order to (i) relate these patterns to physical and human barriers to leopard dispersal, and (ii) estimate gene-flow between different populations across the mountains. We interpret our findings in a way that can facilitate their incorporation into the design of appropriate conservation management strategies for the Arabian leopard.

Chapter 5 estimates density and population size of the leopards of Dhofar mountains. In this chapter we use genetic sampling and camera trapping methods to estimate density. Given the difficulties associated with individual identification from non-invasive scat sampling we use camera trapping to authenticate the use of genetic sampling for monitoring of the Arabian leopard. We use i) a Spatial Explicit Capture Recapture (SECR) approach to model capture-recapture data for the Arabian leopard from molecular scatology and ii) camera-trap surveys in the Dhofar mountains of southern Oman. We produce estimates of leopard density derived separately from camera-trap and DNA data sets and use this information to derive an estimate of the size of the Arabian leopard population. Finally, we compare our estimate of the current number of leopards to previous estimates of population size and interpret this

information in light of current conservation management actions to restore the leopard population in the Dhofar mountains.

Chapter 6 provides a general discussion of the key findings of this thesis and their contribution to our understanding of the conservation genetics and ecology of the Arabian leopard, and how these novel findings can progress future leopard conservation management and research.

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First photographs of Arabian leopard in the wild taken in Jabal Samhan in 1990s (Top: D Willis; bottom: A Spalton).

2. Evolutionary history of the Arabian leopard *Panthera pardus nimr*

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2.1 Abstract

Understanding the evolutionary history and phylogenetic relationships of threatened taxa are of critical importance for setting conservation priorities and developing appropriate management strategies. The Arabian leopard (*Panthera pardus nimr)* is the last remaining big cat in Arabia and is Critically Endangered but its phylogenetic relationships are poorly known due to limited sampling. We sequenced material from 25 leopard individuals from Oman, Yemen and Saudi Arabia for two mitochondrial DNA (mtDNA) genes (ND5 + CYTB) to reveal the evolutionary history and phylogeny of the leopards of Arabia. Our results of Maximum likelihood and Bayesian analyses confirmed that the Arabian leopards are evolutionarily distinct from other leopard subspecies, and that they could have been present in the Arabian Peninsula since the end of Lower Pleistocene. Our results also confirmed the presence of independent Oman and Yemen lineages which are likely to be a result of recent habitat fragmentation due to human pressure. Given the current situation of the Arabian leopard and its distinctive and unique evolutionary there is urgent need for regional collaboration between *in-situ* and *ex-situ* conservation organizations to conserve and ensure the long-term persistence of Arabia's last big pantherine.

Keywords: evolutionary history, phylogeny, Arabian Peninsula, Arabian leopard, *Panthera pardus nimr.*

2.2 Introduction

Understanding the evolutionary history and phylogenetic relationships of threatened taxa are of critical importance for setting conservation priorities and developing appropriate management strategies. Molecular phylogenetic information not only aids practitioners in determining taxonomic units and identifying distinct lineages, it can reveal unknown diversity and populations in need of conservation action due to their unique evolutionary history (Moritz, 1994). Evolutionary history (and therefore phylogenetic diversity) accumulates through diversification and speciation, taking place over tens, to hundreds of millions of years. Thus, when a taxon becomes extinct, swathes of unique evolutionary history and the important ecological functions it represents, are lost (Davis *et al.,* 2018). Recovery of lost evolutionary history likely requires millions of years, therefore a strong emphasis should be placed on the conservation of evolutionary distinct taxa (Davis *et al.,* 2018) and historically isolated lineages (Mortiz, 1994; Moritz, 2002) to avoid such losses. Large carnivores are examples of evolutionary distinct taxa that require conservation prioritisation not just because of their evolutionary history, but also due to their top-level ecological role in the ecosystem (Miller *et al.*, 2002). Large carnivores face serious threats, and most of them are experiencing massive declines in their populations and geographic ranges globally (Ripple *et al.*, 2014). The once widespread leopard (*Panthera pardus*) is one such species which now only occupies 25-37% of its historic range (Jacobson *et al.*, 2016).

The leopard was once widely distributed, ranging from South Africa, through the Middle East, to the Amur Valley in the Russian Far East (Figure 2.1) (Nowell & Jackson, 1996; Jacobson *et al.*, 2016). Fossil records indicate that leopards originated

in Africa, having shared a common ancestor with the African lion (*Panthera leo*) 1.81-4.63 million years ago (Mya) (Uphyrkina *et al.*, 2001; Johnson *et al.*, 2006; Davis *et al.*, 2010; Wei *et al.*, 2011; Nyakatura & Bininda-Emonds, 2012; Tseng *et al.*, 2013; Zhang & Zhang, 2013). Leopards subsequently migrated via the Middle East to Asia, possibly using similar routes to modern humans (Uphyrkina *et al.*, 2001; Hedges, 2002). However, due to continental drift and evolving climatic variations, leopard populations became geographically isolated and developed colour and body size variations, perhaps as a result of diverging evolutionary histories (Janczewski *et al.*, 1995). Determined on the basis of phenotypic and geographical variation, an assessment by Miththapala *et al.* (1996) identified 27 leopard subspecies but noted the questionable nature of some of these descriptions, with many of the subspecies having been characterised based on limited sampling of skulls and skins (see Pocock, 1932; Zukowsky, 1964; Neff, 1986). Using a molecular approach to review the 27 leopard taxa, Miththapala *et al.* (1996) revealed the presence of six geographically distinct groups: African; central Asian; Indian; Sri Lankan; Javan; and east Asian, within which further analyses combining molecular and morphological data indicated eight leopard subspecies, characterising those from the Middle East as *Panthera pardus saxicolor*. However, no sampling of Arabian leopards was included in their analyses. A later study by Uphyrkina *et al.* (2001) identified seven phylogeographic groups (now including Arabian leopards) comprising nine distinct subspecies of which eight are found in Asia and one in Africa. Similar to previous work, some designations were determined based on limited sampling and were considered tentative as a result. One of these was the Arabian leopard (*P. p. nimr*), represented by a single sample from a captive animal.

The Arabian leopard is the smallest of the leopards, and is found only in the Arabian Peninsula (Spalton & Al Hikmani, 2014). Although once widespread throughout the mountainous regions of Arabia, today it is present in just 2% of its former range (Jacobson *et al.*, 2016) (Figure 2.2), and its remaining population may number fewer than 250 individuals (Breitenmoser *et al.*, 2010; see also chapter five). Unsurprisingly, the Arabian leopard has been assessed as Critically Endangered on the IUCN Red List of Threatened Species (Stein *et al.*, 2016).

Home to the Arabian leopard, the Arabian Peninsula serves as connection between Africa and the rest of Asia. As a result, its flora and fauna have affinities with both Palaearctic and Afrotropical regions (Groucutt & Petraglia, 2012). For example, a number of species that are found on the Arabian Peninsula, such as the Asiatic cheetah (*Acinonyx jubatus venaticus*) and the Arabian tahr (*Arabitragus jayakari*) are closely related to Asian species (Charruau *et al.*, 2011; Yue *et al.*, 2013), whereas the honey badger (*Mellivora capensis*), and hamadryas baboons (*Papio hamadryas*) are more closely related to African species (Baryshnikov, 2000; Wildman *et al.*, 2004). As a consequence of the contrasting biogeographic affinities of different taxa found on the Arabian Peninsula, any phylogenetic assessment of the evolutionary origins of the Arabian leopard requires sufficient sampling of leopard taxa from across both continents.

Previous assessment of Arabian leopards and their relationship with other leopard subspecies has been hampered by limited sampling, whether molecular (Uphyrkina *et al.*, 2001) or morphological (Khorozyan *et al.*, 2006). To appropriately inform conservation management and prioritise conservation actions for the Arabian leopard, a greater understanding of its evolutionary history and clarification of

taxonomic status is required. Consequently, this study incorporated increased geographic sampling of mitochondrial (mtDNA) sequences and detailed phylogenetic analyses to (i) assess the relationship of Arabian leopards with other extant pantherine taxa within a molecular phylogenetic framework; (ii) determine species/population level differentiation of Arabian leopards; (iii) estimate the timing of divergence for Arabian leopards and extant pantherines; and (iv) determine past population dynamics, specifically to assess the evidence for population expansion or contraction within the Arabian leopard.

2.3 Methods

To provide genomic DNA we utilised blood and skin samples from wild leopards across Oman, Yemen and Saudi Arabia, in addition to three samples from captive born (offspring of wild born) leopards from Oman (Table 2.1). Genetic material was extracted following manufacturer's guidelines using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, UK). The initial digestion approach to DNA extraction varied in terms of the quantity of initial material used for the DNA extraction, depending on the type of source material. For blood samples, we used 100 µl of blood mixed with 100 µl of phosphate buffered saline (PBS) and 20 µl of proteinase K. For skin samples, pieces of skin < 25 gm were cut, then finely chopped using a sterile razor blade and placed into a 2 ml microcentrifuge with 300 µl of ATL buffer and 25 µl of proteinase K. The solution was then vortexed and incubated overnight at 56°C on a rotator. Skin samples that were especially dry were washed and soaked in PBS for 24 to 48 hours before extraction.

DNA obtained from samples was eluted into 100 µl (skin) or 200 µl (blood) of

elution buffer. For downstream PCR amplification, DNA was further diluted to an appropriate concentration using purified water. As the procedure for DNA extraction can be susceptible to contamination, we used aerosol barrier pipette tips when pipetting and conducted DNA extraction steps inside a pre-sterilized UV fume hood. Resultant template DNA was amplified via polymerase-chain reaction (PCR) using a total of six primer sets (obtained and optimised from Uphyrkina *et al.*, 2001; Ropiquet *et al.*, 2015) designed to amplify a 611 bp fragment of the mtDNA NADH dehydrogenase subunit 5 (ND5) gene, and a 1126 bp fragment of the mtDNA cytochrome b (CYTB) gene (Table 2.2). We performed PCR amplification in reaction volumes of 20 µl containing 10 µl MyTag HS Red Mix (Bioline), 3.2 µl dH_2O, 0.4 µl (0.4 µM) of each forward and reverse primer, 2 µl (0.02 µg/µL) BSA (Bovine Serum Albumin, New England Biolabs Inc.) and 4 µl of DNA. PCR cycling conditions consisted of an initial hot start of 95°C for 8 min followed by 35 cycles of 94°C for 30 s, 52°C for 1 m and 72°C for 1 s, and a final incubation period of 10 min at 72°C. To reduce the risk of contamination between DNA samples, PCR preparation was carried out separately from DNA extractions in different laboratories at the Durrell Institute of Conservation and Ecology (DICE), University of Kent. All PCRs included a negative control (with no template DNA) in each PCR batch to monitor for contamination.

PCR products were initially run out on 2% agarose gels using electrophoresis to check for amplification and to monitor for signs of contamination in the negative controls. Products from PCR were then purified and sequenced using a 3730xl analyzer (Macrogen, Amsterdam, Netherlands).

We also utilised GenBank sequence data to provide additional material for

reconstruction of evolutionary relationships (Table 2.3). Novel sequence data generated by this study will be submitted to the NCBI nucleotide database.

Sequences were quality trimmed, and visually cross-checked with trace files using Jalview v2 (Waterhouse *et al.,* 2009), then edited and aligned using MEGA7 (Kumar *et al.,* 2016) with default settings of the MUSCLE algorithm (Edgar, 2004). Sequence profiles were converted between required formats using ALTER (Glez-Peña *et al.*, 2010), DATACONVERT 1.0 (Dyer *et al.*, sd), and FORMAT CONVERTOR (Los Alamos National Security LLC, 2005). SEQUENCEMATRIX v. 1.7.8 (Vaidya *et al.,* 2011) was used to concatenate the ND5 and CYTB sequence profiles.

2.3.1 Phylogenetic analyses

Concatenated mtDNA was analysed using Bayesian inference (Huelsenbeck *et al.*, 2001) and maximum likelihood (Felsenstein, 1981). Partitioning schemes and models of nucleotide evolution for ND5 and CYTB were determined independently with linked branch lengths, and evaluated using the Akaike information criterion in PARTITIONFINDER v. 1.1.1 (Lanfear *et al.*, 2012) (Table 2.4). Bayesian analysis was performed in BEAST v. 2.4.8 (Bouckaert *et al.*, 2014) with a Markov chain of 20 million generations, sampling every 1,000 generations. BEAST input files were generated using BEAUTI v. 2.4.8 (Bouckaert *et al.*, 2014). Chain convergence and all parameters were assessed to ensure adequate mixing and effective sample size (ESS) values of 200 or greater using TRACER v. 1.7.1 (Rambaut *et al.*, 2018). Initial runs using a relaxed log-normal clock indicated a lack of among branch rate heterogeneity, we therefore applied a strict molecular clock to the data. No outgroups

were used in BEAST analyses as root position is estimated using a molecular clock (Heled & Drummond, 2010). ND5 and CYTB clock models were independent but shared the same tree partition. A substitution rate of 0.0142-0.024 per site per million years has been estimated for leopards/pantherine felids (Uphyrkina *et al.*, 2001; Wilting *et al.*, 2016) and we therefore applied a mean value of 0.0191 to the clock rate. Evaluation of well-supported clades was indicated by Bayesian posterior probabilities (PP) ≥ 0.95. TREEANNOTATOR v. 2.4.8 (Bouckaert *et al.*, 2014) was used to summarise a single maximum clade credibility tree with mean PP values after a 10% burn-in.

Maximum likelihood analyses were performed with RAXMLGUI v. 1.3.1 (Silvestro & Michalak, 2012; Stamatakis, 2014) using *P. leo* as the single outgroup. We applied 1,000 bootstrap replicates to individually optimised branch lengths under default settings using the GTRGAMMA model. Evaluation of well-supported clades was indicated by bootstrap support (BS) values ≥ 70.

2.3.2 BEAST multispecies coalescent and population-level differentiation

To infer species/population-level differentiation, we applied the multispecies coalescent to mtDNA using the StarBEAST (*BEAST) package within BEAST v. 2.4.8 (Bouckaert *et al.*, 2014). Multiple samples per lineage are recommended to accurately indicate coalescence, differentiation, and tree topology (Heled & Drummond, 2010), which necessitated the removal of *P. p. melas* from our analyses as we were unable to source additional CYTB sequence data for this taxon. Partitioning schemes are shown in Table 4. Parameters replicated those of the BEAST phylogentic analyses and we applied the ‘linear with constant root’ prior

with the Yule model distribution of prior probability. Mitochondrial DNA shared the same clock model and tree partition. Evaluation of chain convergence, model parameters, and clade support were carried out as previously described.

2.3.3 Divergence dating

To investiate divergence times between leopard taxa, we used the mtDNA sequence profile from our phylogenetic analysis, with the addition of *P. leo* as an outgroup. We applied three calibration points to the model: (i) the most recent common ancestor (TMRCA) for lions and leopards at 2.52 mya (Davis *et al.,* 2010); (ii) TMRCA for Asian and African leopards at 0.932 mya (Wilting *et al.*, 2016); and (iii) TMRCA for Javan and other Asian leopards at 0.622 mya (Wilting *et al.*, 2016). Normal distribution priors and a standard deviation of 0.3 were applied to each calibration. Partitioning schemes are shown in Table 2.4. We applied the Yule model distribution of prior probability but all other parameters replicated those of the BEAST phylogentic analyses, as did assessments of convergence, model parameters, and clade support.

Tree reconstructions for phylogenetic, coalescent, and divergence dating analyses were visualised using FIGTREE v. 1.4.4 (Rambaut, 2016).

2.3.4 Genetic variation and detecting population change

MEGA7 was used to calculate summary statistics and obtain inter-and intra-specific genetic p-distances for ND5 and CYTB, with pair-wise deletion of missing sites. To detect evidence of historical population expansion or contraction in Arabian leopards we generated an Extended Bayesian Skyline Plot (EBSP; Heled & Drummond,

2008) in BEAST v. 2.4.8 with locus-specific partitions following the EBSP tutorial (http://www.beast2.org/tutorials) and a chain length of 40 million generations. Convergence, population size changes, and ESS values were assessed using TRACER, ESPB plots were visualised using R (R Core Team, 2017)

2.4 Results

Sequence data from 25 Arabian leopards was obtained (see Table 2.5 for summary statistics), consisting of 569 bp fragments of ND5, and 200-737 bp fragments of CYTB for each individual. As missing data can provide spurious results in phylogenetic assessment (Gatesy, 2000; Lemmon *et al.*, 2009; Simmons, 2014) we removed 5 CYTB sequences — each of which represented 537 bp missing data — and the corresponding ND5 sequences for those taxa (Table 2.1). Our final concatenated mtDNA dataset therefore comprised representative sampling from 20 individuals.

In comparsion to African leopards (Ropiquet *et al.*, 2015) and mainland Asian leopards (Wilting *et al.*, 2016), we found low nucleotide diversity (π) within Arabian leopards for both ND5 and CYTB (Table 2.5).

2.4.1 Phylogenetics, evolutionary history, and population-level insights

Our Bayesian and maximum likelihood phylogenetic analyses showed highly concordant topologies (Figure 2.3, Figure 2.S1) and displayed strong support for Arabian and African leopards forming a clade sister to that of Asian leopards. Both analyses returned maximal support for the monophyly of Arabian leopards, within which are two distinct subclades; one comprised of individuals from Yemen, the

other comprised of leopards from Oman. However, only the Yemen subclade is well supported, while an Omani leopard subclade is produced with moderate support.

Between subspecies genetic distances ranged between 0.7% and 4.2% for ND5 and from zero to 4.9% for CYTB (Table 2.6). The highest observed differences for ND5 were between African and Javan, and African and Asian leopards (both 4.2%), while for CYTB it was highest between Arabian and Javan, and Arabian and Asian leopards (both 4.9%). The lowest observed differences were between Javan and Asian leopards for both ND5 (0.7%) and CYTB (zero difference). Arabian and African leopards differed between 3.0% and 2.2% for ND5 and CYTB respectively.

Genetic distances between Arabian leopard populations ranged from 0.2% to 0.4% for ND5, and 0.15% to 0.5% for CYTB (Table 2.7). The highest genetic distances were observed between leopards from Yemen and Saudi Arabia for both loci.

EBSP analyses of 25 ND5 and 20 CYTB Arabian leopard samples returned a 95% highest posterior density (HPD) interval which included zero, therefore a constant population size for Arabian leopards could not be rejected (Figure 2.S2). However, CI_{95} of kappa values for both ND5 (0.08- 8.75) and CYTB (0.17-35.42) are notably wide, suggesting too few substitutions to enable an accurate estimate.

The multispecies coalescent recovered Arabian and African leopards as a strongly supported clade, sister to that of Asian leopards (Figure 2.4), confirming the well supported relationships returned by the phylogenetic analyses.

2.4.2 Divergence dating

Time calibrated phylogenetic analysis indicates that modern leopards diverged from

African lions 2.23 Mya (CI_{95}: 1.67-2.764 My) (Figure 2.5). The Asian leopards diverged from a common ancestor that includes African and Arabian leopards 1.195 Mya (CI_{95}: 0.841-1.534 My) and was followed by a split between African and Arabian leopards at the end of Lower Pleistocene, 0.832 Mya (CI_{95}: 0.547-1.148 my). Arabian leopards are estimated to have diverged during the Late Pleistocene 0.147 Mya (CI_{95}:0.065-0.243 My). Javan leopards split from a common Asian ancestor 0.109 Mya (CI_{95}: 0.026-0.208 My). Each main ancestral node received full support (i.e. Bayesian posterior probability of 1.0).

2.5 Discussion

The Arabian leopard was described more than 180 years ago, yet little is known about the evolutionary history of the region's only extant endemic pantherine felid. To our knowledge, only two studies have attempted to clarify its phylogenetic relationships but despite employing morphological and molecular data, both were hampered by limited sampling (Uphyrkina *et al.*, 2001: n =1; Khorozyan *et al.*, 2006 n =2), although each confirmed the subspecific status of the Arabian leopard.

In this study, we generated novel mtDNA sequence data for 25 wild Arabian leopards and supplemented these data with sequences from other published sources to reconstruct the evolutionary history and inter- and intraspecific relationships of this understudied taxon. Our results support previous taxonomic hypotheses — reciprocal monophyly confirming the distinctiveness and subspecific status of the Arabian leopard — and identify a sister relationship with African leopards, an

affinity also found in previous studies (e.g. Uphyrkina *et al*., 2001; Farhadinia *et al.*, 2015; Wilting *et al.*, 2016).

2.5.1 Divergence of *Panthera pardus*

The evolutionary history of modern felids has been extensively debated, largely due to their relatively recent origin, rapid speciation, and the difficulty of determining taxonomy from an incomplete fossil record (Johnson *et al.*, 2006; Davis *et al.*, 2010; Wei *et al.*, 2011; Tseng *et al.*, 2013). The *Panthera* lineage was believed to have originated 4.47-9.32 mya (Johnson *et al.*, 2006), although the recent discovery of a fossil pantherine from the Tibetan Himalaya points to an earlier Miocene origin for big cats of 5.57-19.33 mya (Tseng *et al.*, 2013). Given the calibrations we applied, the ancestor of lions+leopards at 2.23 mya and the divergence of Asian from African+Arabian leopards at 1.195 mya fall entirely within previous estimates (Uphyrkina *et al.*, 2001; Johnson *et al.*, 2006; Davis *et al.*, 2010; Nyakatura & Bininda-Emonds, 2012; Tseng *et al.*, 2013; Zhang & Zhang, 2013; Wilting *et al.*, 2016; Paijmans *et al.*, 2018). However, we found a split more than 0.570 mya more recent than previously indicated between Javan and other Asian leopards (Wilting *et al.*, 2016), although this may be a result of sampling too few individuals from this clade and/or the increased sampling density of Arabian leopards in our analyses (Linder *et al.*, 2005; Hug & Roger, 2007; Milne, 2009; Jenny Xiang *et al.*, 2011; Schulte, 2013).

An estimate of ancestral divergence between African and Arabian leopards has been previously presented, although Wilting *et al* (2015) did not explicitly address this lineage. Their time-calibrated phylogeny indicates an origin of ~0.675 mya for

African+Arabian leopards but there are no confidence intervals presented with which to compare our results. Nevertheless, a split during the Lower Pleistocene at 0.832 mya is an estimate we consider to be broadly comparable.

Interspecific genetic distances show an expected pattern across both mtDNA loci from a biogeographic perspective, i.e. shorter distances between biogeographically closer taxa: Asian+Javan leopards, and African+Arabian leopards have the lowest uncorrected distances, and Javan leopards show the greatest distances to African, and Arabian leopards. The Arabian leopard may have originated as a result of dispersal of African leopards either via the Sinai Peninsula, across Bab al Mandab strait, or a combination of the two during low sea level stands 1.0-1.7 mya (DeMenocal, 1995; Wildman *et al.*, 2004). Although our results suggest Arabian leopards diverged from African leopards approximately 0.832 mya, studies of other mammalian taxa suggest more recent Africa-Arabia divergences (e.g Asiatic Cheetah: 32-67 kya [thousands of years ago]; Charruau *et al.*, 2011, Hamadryas baboons: 12-130 kya; Kopp *et al.*, 2014, White-tailed Mongoose:32.5 kya; Fernandes, 2011). One interpretation is that multiple opportunities for movement between these two regions have presented themselves. During the Late Pleistocene Arabia was dominated by wet periods (Parker, 2010); this may have established ideal habitat for leopards to migrate and colonize north-western and eastern Arabia. Arabian leopards could have been widespread across the region by the time connectivity with their African congeners was lost or significantly reduced toward the end of the Early Pleistocene. Combined, a subsequent rise in sea level, increasing aridity, and anthropogenic pressure would have progressively reduced connectivity between African and Arabian leopards leading to the differentiation observed in the extant population, albeit also reflecting

the lower levels of genetic diversity expected following an 'out of Africa' hypothesis (Uphyrkina *et al.*, 2001; Werdelin & Lewis, 2005; Paijmans *et al.*, 2018) and the negative human impacts on declining populations, and associated reduction in genetic diversity.

2.5.2 Divergence of leopards on the Arabian Peninsula

Our results indicate that the leopards of western (Yemen) and eastern (Oman) Arabia comprise two distinct lineages which diverged 147,000 years ago (65,000-243,000 years ago). The presence of independent lineages in the neighbouring countries of Oman and Yemen is perhaps unexpected but the mountain ranges are not contiguous and the mountains of northern Yemen (from where most Yemeni leopard DNA was obtained) are seperated from those of southern Oman by extensive plains, dry valleys (especially the mighty Wadi Hadramaut) and sand deserts. These geographic barriers could limit or prevent dispersal and gene flow between what may previously have been a single continguous population when the region was wetter.

These wetter periods, with active freshwater systems and high intensity monsoon, exisited across much of southern Arabia including the Empty Quarter 180,000–130,000, years ago (Parton *et al*., 2015). The resultant extensive freshwater lakes and pastures are likely to have presented opportunites for the dispersal of leopards while the dry periods that followed may have resulted in periods of isolation and fragmentation. The occurrence of these wetter periods coincide with our phylogenetic estimate for divergence of the Yemen and Omani Arabian leopard populations.

In addition, human populations, believed to have been present in south Arabia since *Homo sapiens* emerged from Africa 120,000 – 90,000 years ago (Bae et al., 2017) may have negatively impacted leopards leading to fragmented populations.

Future conservation management of the Arabian leopard should take into account the evolutionary split of the Arabian leopard and consider whether the two populations should be managed a single Evolutionary Significant Unit or as separate Management Units (see Chapter 6 for discussion). It would also be valuable to investigate if similar divergence occurs within other terrestrial mammals of Oman and Yemen.

Although divergence estimates are known to be influenced by choice of calibration points for dating and the priors used, and mtDNA markers often perform less well than nuDNA at resolving deep phylogenetic relationships (Springer *et al.*, 2001), the recent origin of *Panthera* may be less influenced by ancestral reconstructions using mtDNA, and with the possible exception of our Java+Asia divergence estimate, our findings are in keeping with a number of previously published dating analyses. More broadly, greater sampling across pantherine felids is likely to generate enhanced estimates and narrower confidence intervals (Tseng *et al.*, 2013), providing greater resolution in a still unresolved clade.

2.5.3 Changes in ancestral population size

Although the Arabian leopard has disappeared from almost 98% of its historical range (Jacobson *et al.*, 2016) and regional populations are considered to be in decline (Breitenmoser *et al.*, 2010), our EBSP results suggest that the population has been stable. However, the observed low nucleotide diversity and evidence from field

records of localised extinction of leopards from northern Oman (Spalton *et al.*, 2006), Jordan (Qarqz & Baker, 2006) and UAE (Edmonds *et al.*, 2006), do not support this scenario. Determining changes in population size from nucleotide sequences can be complex especially when a population has experienced a bottleneck and there is a lack of informative sequence data from prior to the bottleneck event (Heled & Drummond, 2008; Ho & Shapiro, 2011). Given this context, and the wide confidence intervals from our EBSP analyses, we advise caution in interpreting an ancestral profile of constant population size, and recommend a broader set of analyses incorporating ancient DNA, additional nuDNA, or a genome-wide sampling approach (e.g. restriction site-associated DNA sequencing [RADseq]) to infer past population fluctuations.

2.6 Conclusion

This study provides a crucial insight into the phylogeny and evolutionary history of the Arabian leopard, and confirms (i) the evolutionary distinctiveness of *P. p. nimr*, and (ii) the sister relationship with African leopards. In agreement with a previous assessment of African leopards (Mcmanus *et al.*, 2015), our finding of identifiably different Oman and Yemen lineages is suggestive of relatively recent divergence and subsequent population fragmentation, likely related to anthropogenic pressure. There is currently no documented evidence of any morphological difference between Yemen and Oman leopards, although anecdotal evidence from captive animals suggests that Yemen individuals have darker coats than those from Oman. However, it is unknown whether this is as result of diet/captivity or a true evolutionary difference. Our results are therefore highly important for regional management of this Critically Endangered leopard and should serve as an important guide for future

conservation and management of the region's last remaining big cat. Given the threatened situation of the Arabian leopard, we call for urgent regional collaboration between *in-situ* and *ex-situ* conservation organizations to conserve and ensure the long-term persistence of this unique leopard subspecies.

2.7 Tables

Table 2.1. Type, origin and source of Arabian leopard samples used this study. MECA=Ministery of Environment and Climate Affairs, OWAB=Oman Wild Animal Breeding Centre, ONHM=Oman Natural History Museum, BCEAW= Breeding Centre for Endangered Arabian Wildlife, PSAWRC= Prince Saud al-Faisal Wildlife Research Center.

Sample ID	Origin	Type	Source (obtained from)
SM4	Samhan/Oman 2002	Blood	Dr Andrew Spalton
SM8	Samhan /Oman 2002	Blood	Dr Andrew Spalton
SM11	Samhan/Oman 2006	Blood	Dr Andrew Spalton
H1	Samhan/Oman 1977	Skin	Harrison Institute, Sevenoaks, UK
H2**	Yemen 1965	Skin	Harrison Institute, Sevenoaks, UK
H3	Oman 1976	Skin	Harrison Institute, Sevenoaks, UK
H4	Musandam /Oman1979	Skin	Harrison Institute, Sevenoaks, UK
H5	Samhan/Oman 1977	Skin	Harrison Institute, Sevenoaks, UK
H6	Samhan/Oman 1979	Skin	Harrison Institute, Sevenoaks, UK
OM1	Samhan /Oman 2003	Skin	MECA, Muscat, Oman
OM3	Captive born*	Skin	OWABC, Muscat, Oman
OM5	Captive born*	Skin	OWABC, Muscat, Oman
OM6	Captive born*	Skin	OWABC, Muscat, Oman
ONHM6	Samhan 2002	Skin	ONHM, Oman
ONHM7	Samhan /Oman1997	Skin	ONHM, Oman
PP003	Samhan 1984	Blood	BCEAW, Sharjah/UAE
PP017	Wild born in Yemen	Skin	BCEAW, Sharjah/UAE
PP030	Wild born in Yemen	Skin	BCEAW, Sharjah/UAE
T5	Wild born Yemen 1995	Blood	PSAWRC, Taif, KSA
T7	Wild born Yemen 1995	Blood	PSAWRC, Taif, KSA
TT1**	Wild born Yemen	Blood	PSAWRC, Taif, KSA
TT2**	Wild born Yemen	Skin	PSAWRC, Taif, KSA
TT4**	Saudi Arabia	Skin	PSAWRC, Taif, KSA
TH7**	Wild born Yemen	Skin	PSAWRC, Taif, KSA
Yemen2	Wild born Yemen 2014	Blood	OWABC, Oman

*offspring of wild born leopards from Jabal Samhan, ** samples removed from tree building analyses due to missing data.

Table 2.2. Mitochondrial primers used to amplify Arabian leopard DNA.

Primer name	Primer sequence	Source
ND5-F	GTG CAA CTC CAA ATA AAA G	Uphyrkina *et al*. (2001)
ND5-RL2	TAA ACA GTT GGA ACA GGT T	Uphyrkina *et al*. (2001)
ND5-FL2-nimr	CGT TAC ATG GTC GAT CAT GG	Uphyrkina *et al*. (2001)
ND5-RL4	TTA GGT TTT CGT GTT GGG T	Uphyrkina *et al*. (2001)
CYTB-leo-F	GAC YAA TGA TAT GAA AAA CCA TCG TTG	Ropiquet *et al*. (2015)
CYTB-leo-R	GTT CTC CTT TTT TGG TTT ACA AGA C	Ropiquet *et al*. (2015)

Table 2.3. Sequence data acquired from GenBank for use in phylogenetic analyses. ND5 and CYTB shown in the same column row represents sampling of these regions from a single accessioned mitogenome. Analysis key: 1=BEAST and RaxML phylogenetic reconstruction; 2=BEAST multispecies coalescent; 3=BEAST divergence dating.

Genus	**Species**	**Subspecies**	**locus**	**Analyses**	**NCBI accession**	**Reference**
Panthera	*leo*		ND5, CYTB	3	KP001506	Bertola *et al.* (2016)
Panthera	*pardus*	*Melas*	ND5, CYTB	1, 3	MH588627	Paijmans *et al.* (2018)
Panthera	*pardus*	*orientalis*	ND5	1, 2, 3	HQ185550	Rozhnov *et al.* (2011)
Panthera	*pardus*	*orientalis*	ND5, CYTB	1, 2, 3	KX655614	Han et al. (unpublished)
Panthera	*pardus*	*orientalis*	CYTB	1, 2, 3	AB817078	Sugimoto *et al.* (2014)
Panthera	*pardus*	*Pardus*	ND5, CYTB	1, 2, 3	MH588632	Paijmans *et al.* (2018)
Panthera	*pardus*	*Pardus*	ND5, CYTB	1, 2, 3	MH588619	Paijmans *et al.* (2018)

Table 2.4. Partitioning schemes and substitution models for phylogenetic methods selected by PartitionFinder v1.1.1 (Lanfear et al., 2012). Codon positions in parentheses. Substitution models apply to BEAST analyses only.

Analyses	**Partitioning scheme**	**Substitution model**
BEAST & RaxML (phylogeny)	ND5 (1), CYTB (2)	HKY
	ND5 (2), CYTB (3)	TrN
	ND5 (3), CYTB (1)	HKY
*BEAST (multi-species coalescent)	ND5 (1), CYTB (2)	HKY
	ND5 (2), CYTB (3)	TrN
	ND5 (3)	HKY
	CYTB (1)	K80+I
BEAST (divergence dating)	ND5 (1), CYTB (2)	TrN+I
	ND5 (2), CYTB (3)	GTR
	ND5 (3)	HKY
	CYTB (1)	K80

Table 2.5. Summary statistics for mtDNA in Arabian leopards. N=sample size; bp=base pairs; Pi=parsimony informative sites; V=variable sites; π=nucleotide diversity.

	ND5	**CYTB**
N	25	25
Bp	569	737
Pi	1	2
V	2	3
Π	0.001	0.003

Table 2.6. Interspecific mtDNA p-distance matrix for Javan (*P. p. melas*), Arabian (*P. p. nimr*), Asian (*P. p. orientalis*), and African (*P. p. pardus*) leopards. Lower diagonal =ND5, upper diagonal =CYTB.

	Taxon			
	P. p. melas	*P. p. nimr*	*P. p. orientalis*	*P. p. pardus*
P. p. melas		0.049	0.000	0.035
P. p. nimr	0.031		0.049	0.022
P. p. orientalis	0.007	0.031		0.035
P. p. pardus	0.042	0.030	0.042	

Table 2.7. Intraspecific mtDNA p-distance matrix for Arabian leopards (*P. p. nimr*). Lower diagonal =ND5, upper diagonal =CYTB.

	Sampling locality		
	Oman	Saudi	Yemen
Oman		0.005	0.006
Saudi	0.002		0.015
Yemen	0.002	0.004	

2.8 Figures

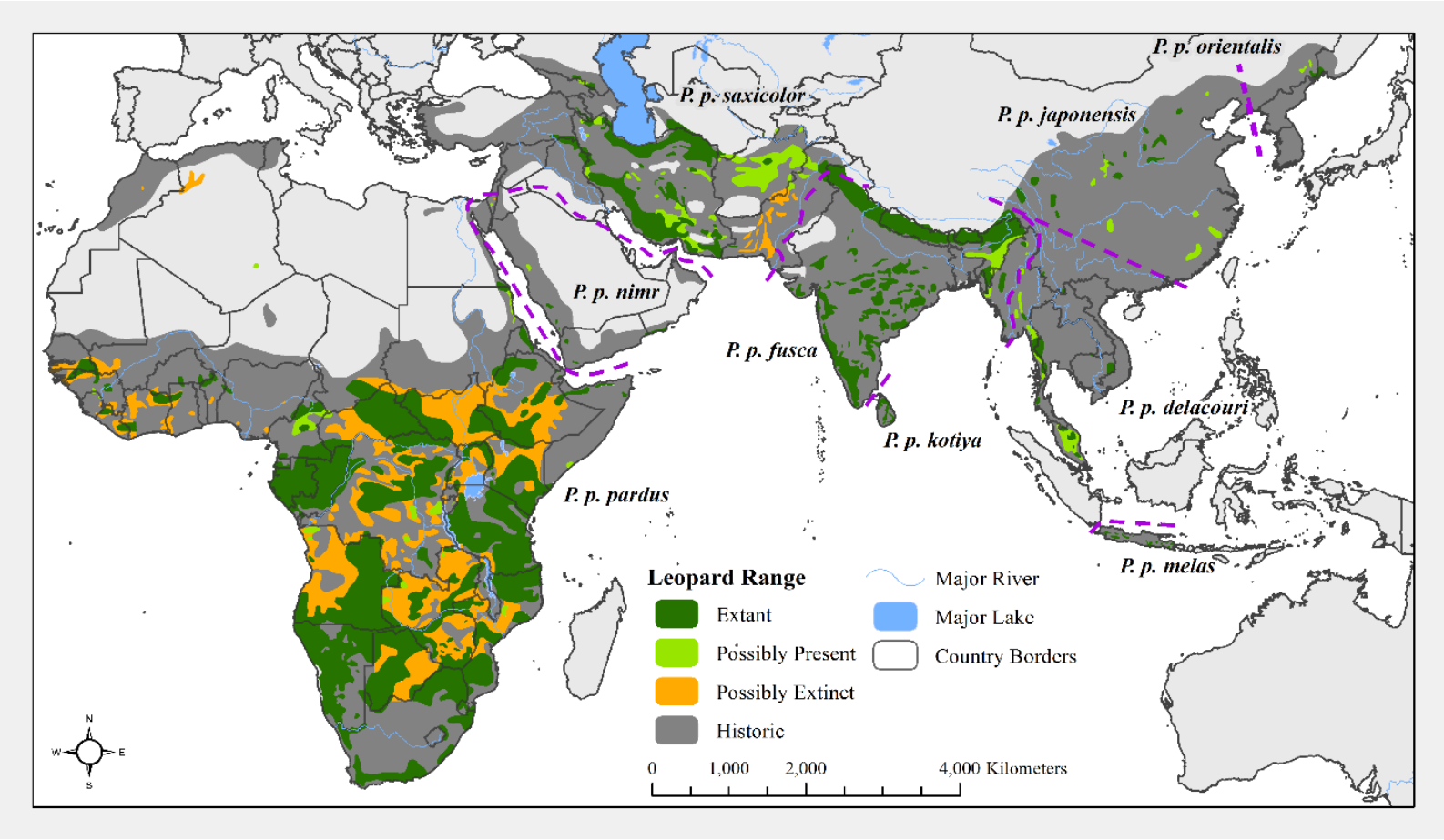

Figure 2.1~~2.1~~. *Panthera pardus* sp. global range (from Jacobson et al., 2016). Dotted purple lines indicate subspecies delineation.

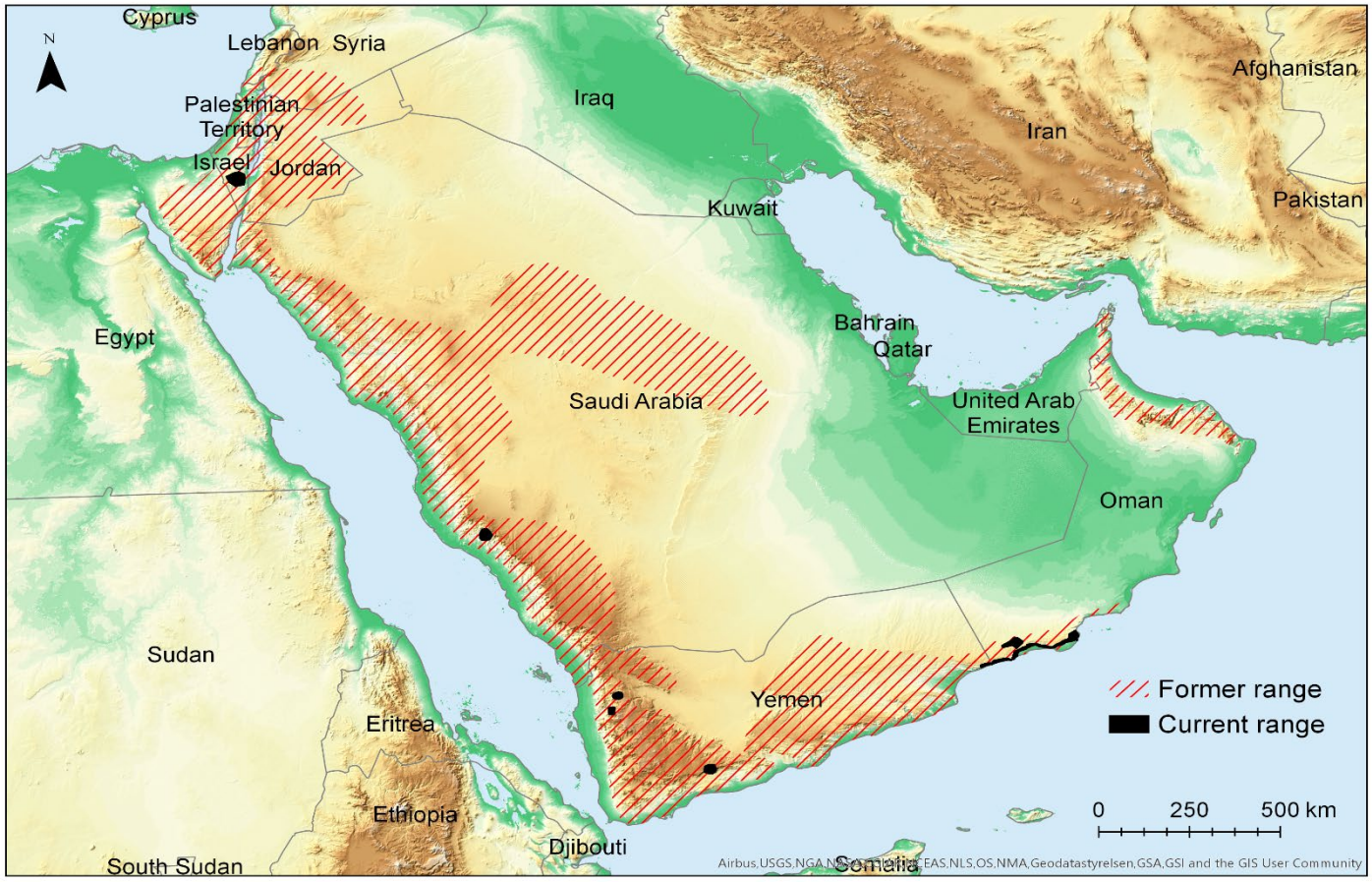

Figure 2.2. Arabian leopard *Panthera pardus nimr* historical and current range (Spalton & Al Hikmani, 2014).

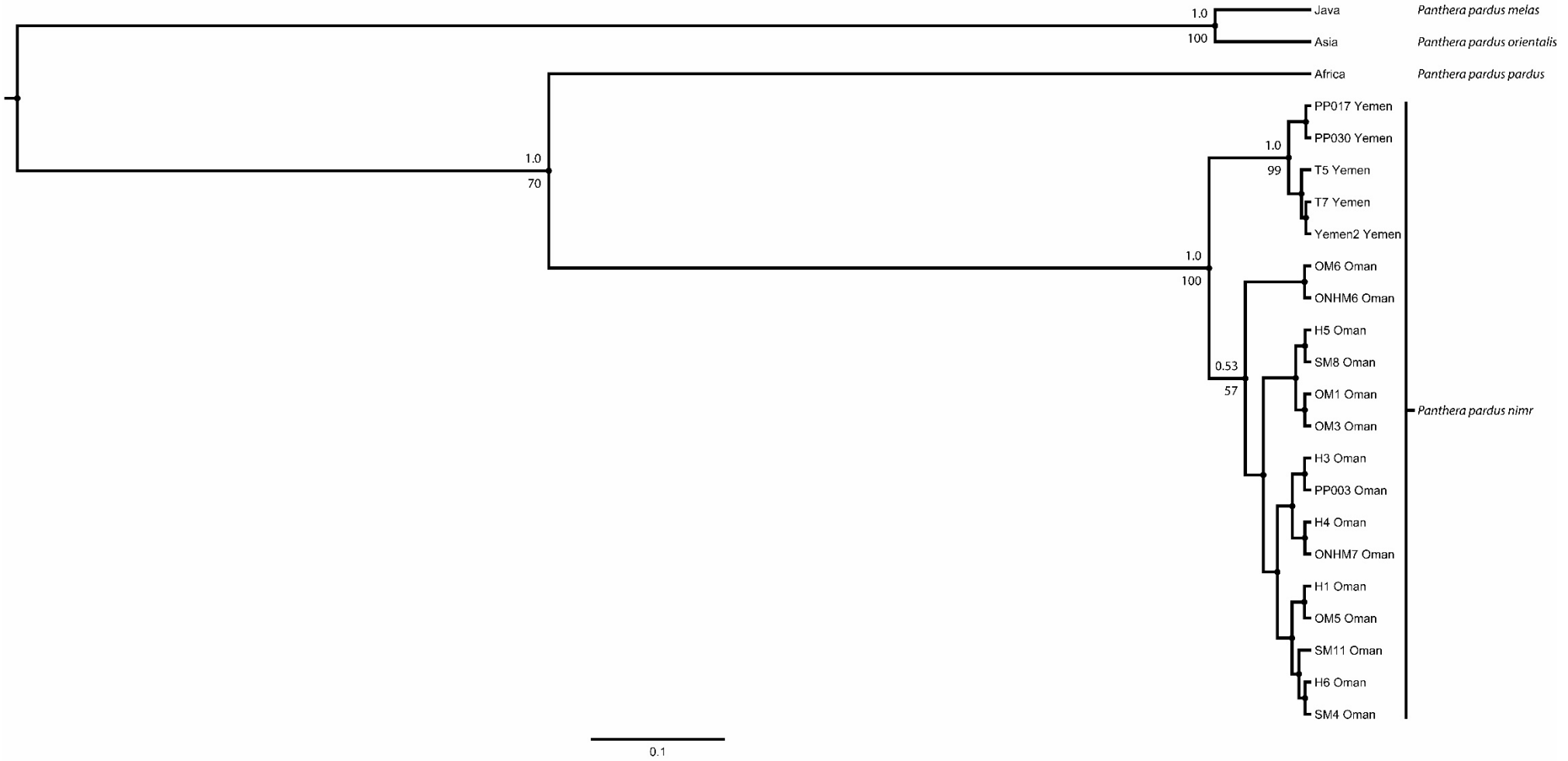

Figure 2.3. Bayesian (BEAST) mtDNA phylogeny. Node support values are shown above (Bayesian posterior probabilities) and below (maximum likelihood bootstrap values) branches. Scale is substitutions per site.

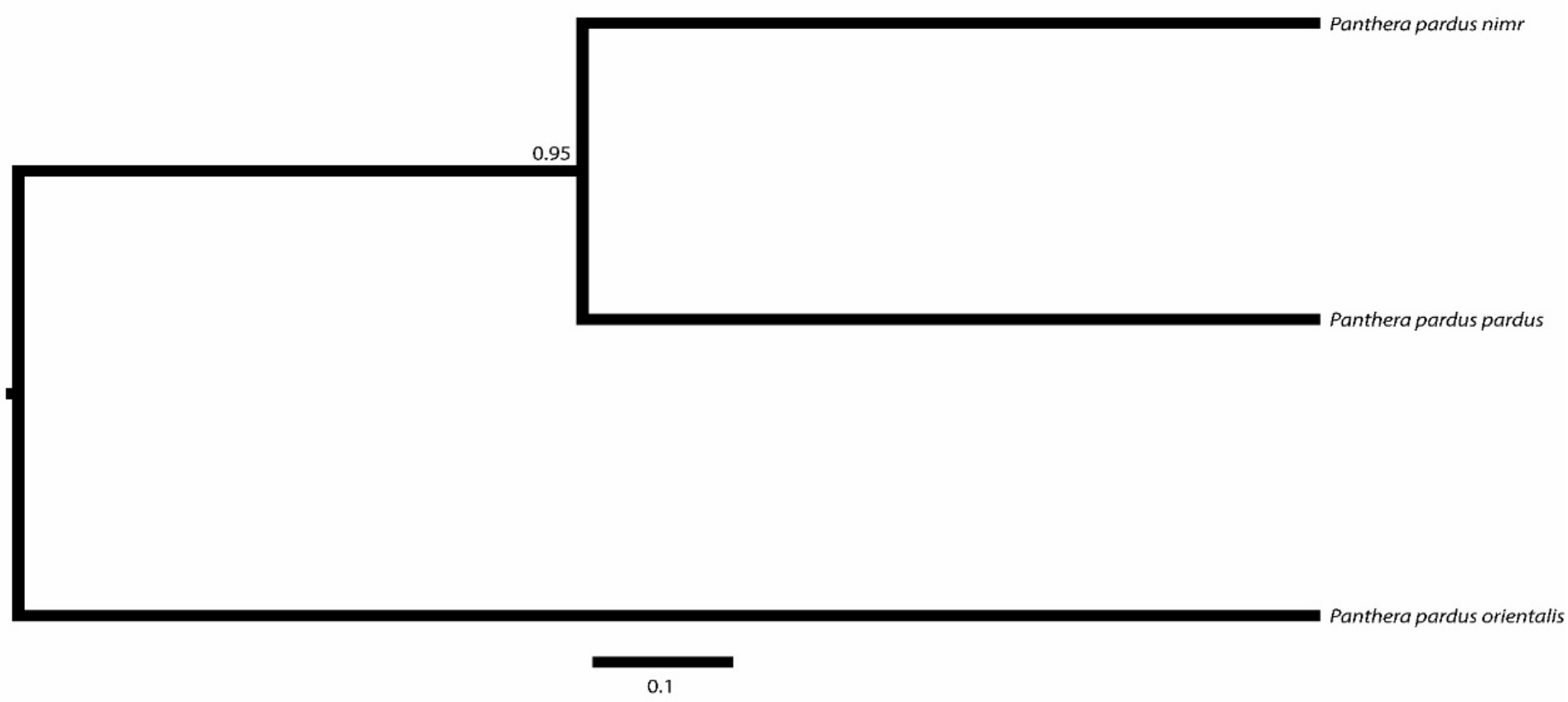

Figure 2.4. Bayesian (*BEAST) inferred mtDNA species tree. Node support value indicates Bayesian posterior probability. Scale is substitutions per site.

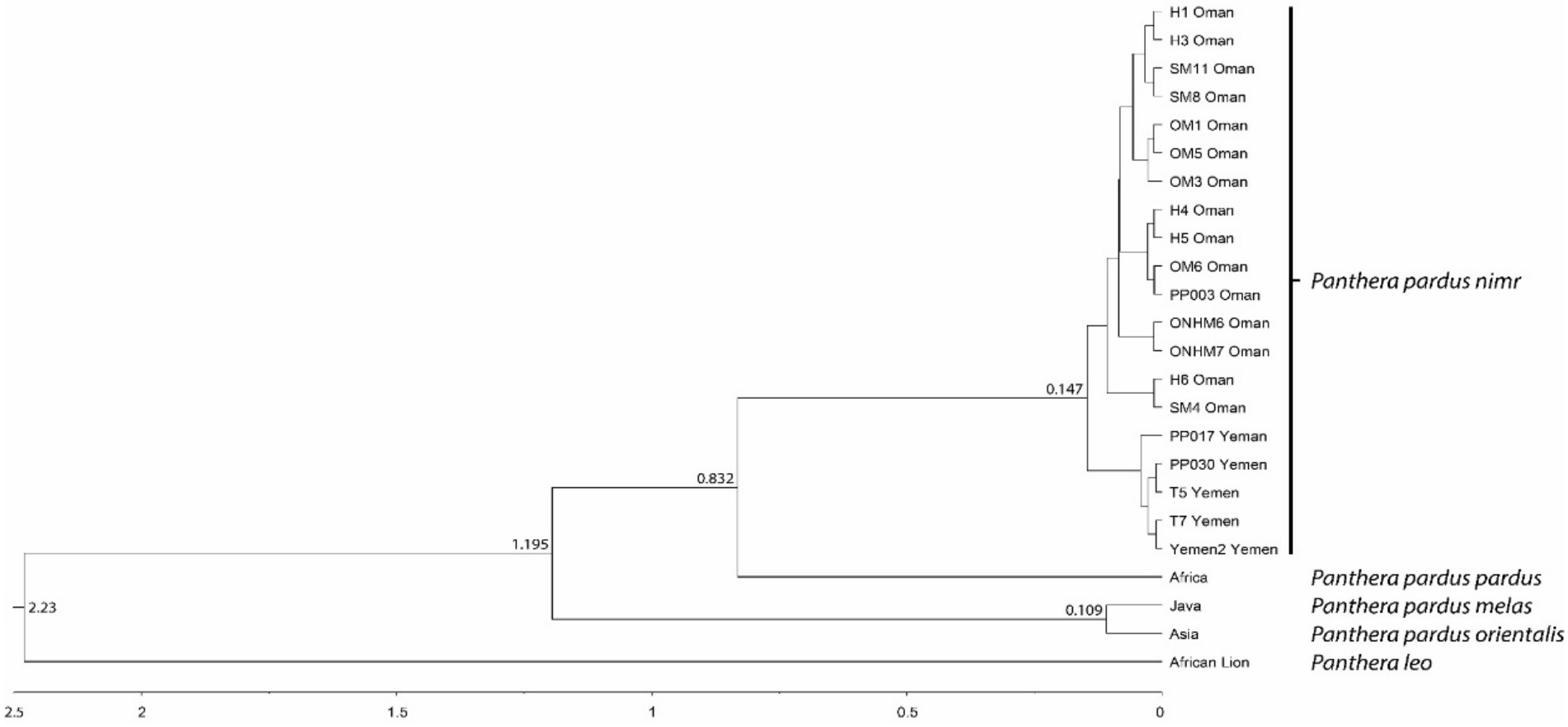

Figure 2.5. Bayesian (BEAST) generated phylogenetic relationships and divergence dating of Panthera pardus spp. using mtDNA. *Panthera leo* is the outgroup. Numbers at nodes indicate estimated origin of TMRCA. Each dated ancestral node receives maximal PP support. Scale is in millions of years.

Figure 2.S1. Maximum likelihood mtDNA phylogeny. Numbers at nodes indicate bootstrap support values. Scale is substitutions per site.

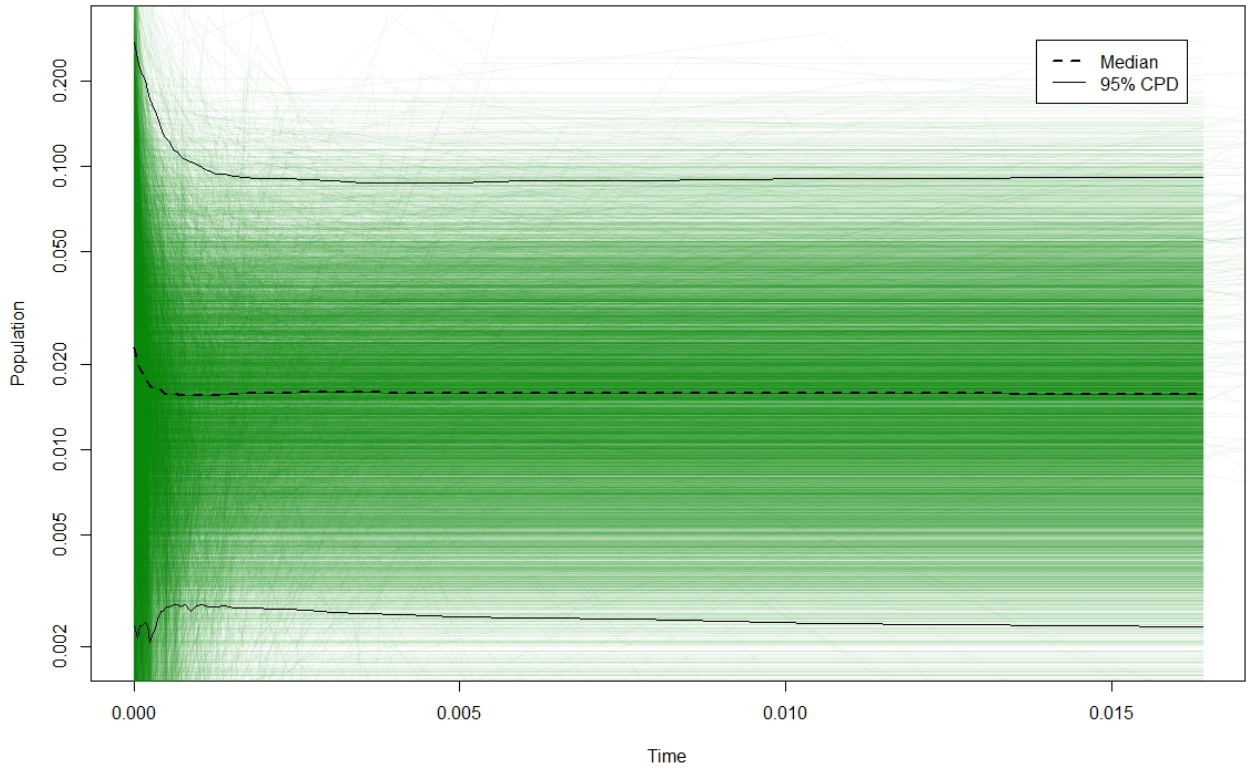

Figure 2.S2. Extended Bayesian Skyline Plot of population size through time for Arabian leopards. The full view of the posterior of all mtDNA samples, summarised by the median and 95% HPD interval, are shown. Time on x-axis in millions of years. Population size on y-axis represents Ne assuming a generation time of 1 year. The 95% HPD interval included 0, preventing rejection of constant population size.

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Camera trap photo of a female Arabian leopard in Jabal Samhan (above) and GPS collaring of a male leopard during the moonson season (below).

3. Assessment of genetic diversity in wild and captive populations of the Critically Endangered Arabian leopard (*Panthera pardus nimr*) and implications for conservation management

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3.1 Abstract

Genetic diversity plays a crucial role in the survival of natural populations, therefore measuring the level of genetic diversity in critically endangered species is very important for their future management and recovery. The Arabian leopard (*Panthera pardus nimr*) has been listed as Critically Endangered since 1996 but very little is known about its genetic status whether in the wild or in captivity. We used a panel of microsatellite DNA markers to survey—for the first time—levels of genetic diversity in wild and captive populations of the Arabian leopard. Our study reveals that the Arabian leopard is genetically impoverished in comparison to other leopard subspecies. However, we found high levels of genetic diversity in the captive population compared to the wild population in the Dhofar mountains; much of the genetic diversity in captivity having originated from wild leopard individuals sourced from Yemen. Genetic diversity levels in the wild Dhofar subpopulations varies across the region. Importantly, this study has revealed minimal loss of genetic diversity across the last 40 years in the Jabal Samhan population, which is surprising given the small size of the population there. One interpretation is that conservation efforts over the four decades, including banning of killing leopards and establishment of the region as a protected area, have served to stabilize the population size. Given the quantity of genetic diversity found in the captive population, we put forward a number of potential scenarios for future management of the Arabian leopard.

Keywords: genetic diversity, endangered species, small population, Arabian leopard, *Panthera pardus nim*.

3.2 Introduction

Genetic diversity plays a crucial role in the survival of natural populations. High levels of genetic diversity are the resource upon which natural selection acts to enable species to evolve and adapt to changing environments (Frankham *et al.,* 2010). By underpinning a species' evolutionary potential, genetic diversity is essential for the long-term viability of wildlife populations (Frankham *et al.*, 2017), particularly those that exist in rapidly changing environments. Such changes include anthropogenic impacts like habitat loss, fragmentation, climate change, and persecution, and are the causes of decline of many threatened taxa. Where the population decline is drastic the species may face a population bottleneck leading to genetic problems commonly associated with small population size. These problems include loss of genetic diversity, increased levels of inbreeding, and subsequent inbreeding depression (such as depression of population- and individual-level fitness; Allendorf *et al.,* 2013, increased mortality; Keller & Waller, 2002, and increased susceptibility to disease; Smallbone *et al.*, 2016). Inbreeding is particularly prominent in small and declining populations and its negative consequences are well documented for a wide range of endangered species, including mammals; prominent examples for feline species include the Florida panther (*Puma concolor couguar*) and the cheetah (*Acinonyx jubatus*) (Terrell *et al.,* 2016).

As a consequence of the genetic problems associated with small population size and the importance of preserving genetic diversity in populations of endangered species, it is vital for conservation managers tasked with recovering these species to determine what levels of genetic diversity exist within them and how that genetic

diversity is distributed across wild and captive populations. Such information can help ensure that conservation management intervention most effectively preserves existing genetic diversity in both wild and captive populations. It is often a challenge to obtain such data for critically endangered species, particularly for highly elusive species whose populations are distributed across remote landscapes, and even more so in cases where populations naturally exist at relatively low densities. In these cases, non-invasive DNA sampling techniques such as those developed to obtain DNA from faecal material (scats) can provide a solution. These techniques have been used successfully across many threatened and endangered species including the Amur leopard (*Panthera pardus orientalis)* (Sugimoto *et al.,* 2014), tiger (*Panthera tigris tigris*) (Thapa *et al.,* 2018) and snow leopard (*Panthera uncia*) (Janečka *et al.,* 2008).

The Critically Endangered Arabian leopard (*Panthera pardus nimr*) is endemic to the Arabian Peninsula. Its pale colour and small body size distinguishes it from all other leopard subspecies (Khorozyan *et al.,* 2006) and phylogenetic analysis using a single sample has suggested that it is a distinct leopard subspecies (Uphyrkina *et al.*, 2001). The Arabian leopard historically occupied a continuous range across Arabia (Figure 3.1) from Palestine in the north, southwards along the Hijaz and Asir Mountains of Saudi Arabia to the mountains of northern Yemen and from the Hadhramaut mountains in southern Yemen to the Dhofar Mountains of southern Oman and Al Hajar mountains of northern Oman and Musandam (Harrison & Bates, 1991; Breitenmoser et al., 2006). It has since disappeared from most of these areas and is today only present in small and fragmented populations in southern Oman, Yemen and Saudi Arabia (Spalton & Al Hikmani, 2014; Islam *et al*., 2018). A small

population existed in the Judean Desert and Negev Highlands until early 2001 but today this population is considered to be extinct (Stein *et al.*, 2016). The most recent global estimate for the Arabian leopard is fewer than 250 leopards (Breitenmoser *et al.*, 2010). However, this estimate is anecdotal.

In Oman a wild population of 44-58 leopards was estimated to inhabit the Dhofar mountains in the south of the country (Spalton & Al Hikmani, 2014). It is distributed across three contiguous mountain massifs known as Jabal Samhan, Jabal Qara, and Jabal Qamar that together form the Dhofar mountains which extend 250 km from Oman's Arabian Sea coastline to the border with Yemen in the west (Figure 3.2). The discovery in 2013 of a small number of individuals in the Nejd, the northern foothills of Jabal Qara, represented a small northward extension of the known range (Al Hikmani *et al*., 2015).

Threats faced by Arabian leopards include illegal killing by livestock owners, prey depletion, loss of prime habitat and also capture for the illegal pet trade (Al Jumaily *et al.* 2006; Spalton *et al.*, 2006; Zafar-ul Islam *et al.,* 2018). However, illegal killing in response to livestock depredation is considered the main cause of decline. For example, over 30 leopards were reported killed in the 1980s by local shepherds in Yemen and the Musandam mountains of northern Oman (Al Jumaily *et al.*, 2006; Spalton *et al.*, 2006).

Conservation initiatives began in the mid-1980s when, following concerns that the Arabian leopard may go extinct, the world's first captive breeding group was established in Oman from four wild leopards caught in Jabal Samhan. In the 1990s additional captive groups were established at centres in Saudi Arabia, UAE and

Yemen; mostly from leopards wild-caught in Yemen. Some of these leopards were bred with the offspring of wild-caught leopards from Oman through a breeding loan agreement between these centres. By 2011, there were nine regional breeding centres with a total captive population of 82 leopards of which 15 were founders (Budd, 2011).

Conservation of the Arabian leopard remains a top priority across the region. As a flagship species, its persistence in the mountains of Arabia is of great environmental and cultural benefit to the Arabian landscape and to the people of Arabia. In Oman the focus of leopard conservation management is to protect the remaining leopard habitat, obtain ecological data about leopards in the wild, and raise awareness locally and nationally of the need to conserve this iconic species. However, ongoing conservation management will also require a better understanding of the genetic diversity of both the remaining wild populations and the growing number of captive populations. For example, some genetic diversity may now only reside in isolated pockets across the leopards' fragmented range or in particular captive populations, paving the way for interventions to be considered which might aid its redistribution. Furthermore, knowledge of the genetic diversity of wild and captive populations is essential for future plans for reintroduction.

In this study, we use microsatellite DNA markers to survey—for the first time—levels of genetic diversity in the wild and captive populations of the Arabian leopard. We use this information to (i) compare levels of genetic diversity in time and space between the different wild subpopulations and interpret observed differences in relation to the species' population size and known demographic history; and (ii) examine the genetic composition of the captive leopard population and put forward a

number of potential scenarios for future management, including the possibility of reintroduction.

3.3 Methods

3.3.1 Sample collection

A total of 477 putative leopard scats were collected from across the Dhofar Governorate through non-invasive scat collection surveys that were carried out between 2010 and 2017 by field teams of the Office for Conservation of the Environment (OCE), Oman. Scats were collected during dedicated presence/absence and capture-recapture (camera-trap) surveys, and also opportunistically (see chapter 5 for detailed methodologies). Additional scats were collected opportunistically by field staff carrying out habitat surveys of overgrazing in Jabal Qamar during the period between December 2016 and April 2017. Collected scats were stored in plastic Ziplock bags at ambient temperature, and each labelled with the date of collection and GPS reference.

Samples from captive leopards were obtained from the Breeding Centre for Endangered Arabian Wildlife in Sharjah, UAE (n=21 individuals), the Prince Saud al-Faisal Wildlife Research Centre in Taif, Saudi Arabia (n=15 individuals); a single leopard from Saudi Arabia (poisoned in 2014) and from the Omani Wild Animals Breeding Centre in Muscat, Oman (n=4 individuals). Ten of these captive samples came from wild-born leopards sourced from Yemen, one from wild leopard from Oman where the remaining samples (n=28) were from captive-born leopards.

Skin and bone samples were obtained from 15 specimens from the Omani Natural Heritage Museum in Muscat (n= 9 individuals; 4 skin, 5 bone) and the Harrison

Institute in Sevenoaks, UK (n=6 individuals; 6 skin,). Fourteen of the museum specimens were wild-killed leopards from Oman, collected between 1976 and 2002. The remaining sample, collected in 1965, originated from a wild-killed leopard from Yemen. The museum samples are useful to compare patterns of historical and contemporary genetic diversity.

Five additional samples of wild-killed (n=2) and wild (GPS collared) leopards (n=3) were obtained from Directorate General of Environment and Climate Affairs in Salalah, Directorate General of Agricultural and Livestock Research in Rumais, Muscat and from Dr Andrew Spalton respectively. A table showing the types and origins of samples is shown in the supplementary information (Table 3.S1).

3.3.2 DNA extraction

Genomic DNA was isolated from scat, skin, blood and bone samples using commercially available DNA extraction kits. The QIAamp Fast DNA stool mini kit (Qiagen, UK) was used to extract DNA from scat samples. Approximately 200 mg of dried scat powder was scraped from the outer surface of each scat into a 2 ml microcentrifuge tube using a sterilised razor blade and subsequent DNA extraction followed the manufacturer's instructions.

DNA was extracted from skin, blood and bone samples using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, UK). The initial digestion approach to DNA extraction varied in terms of the quantity of initial material used for the DNA extraction depending on the type of source material. For blood samples, we used 100 µl of blood mixed with 100 µl of phosphate buffered saline (PBS) and 20 µl of proteinase K. For skin samples, pieces of skin < 25 gm were cut, then finely chopped

using a clean razor blade and placed into a 2 ml microcentrifuge with 300 µl of ATL buffer and 25 µl of proteinase K. The solution was then vortexed and incubated overnight at 56°C on a mechanical mixer. Museum skin samples and any contemporary skin samples that were particularly dry were washed and soaked in PBS for 24 to 48 hours before extraction. Bone samples from which samples were to be subsequently taken were first cleaned with double-distilled water followed by 96% ethanol. The bones were then cleaved, crushed and ground using a cleaned hammer and Sabatier bow saw. The resulting powder (approximately 100 gm) was then placed in a 2 ml microcentrifuge tube with 320 µl of ATL buffer, 40 µl of proteinase K and 40 µl of 0.5 M EDTA and incubated overnight at 56°C.

DNA obtained from scat and skin samples was eluted into 100 µl of elution buffer; 200 µl for blood samples and 50 µl for bone samples respectively. For downstream PCR amplification, DNA from blood and skin samples was further diluted to an appropriate concentration using purified water.

As the procedure of DNA extraction from scat and museum samples can be susceptible to contamination, we used aerosol barrier pipette tips when pipetting and conducted DNA extraction steps inside a pre-sterilized UV fume hood.

3.3.3 Species identification.

A number of carnivore species are found within the study system, including the Arabian wolf (*Canis lupus arabs*), striped hyena (*Hyaena hyaena*) and caracal (*Caracal caracal*), all of which deposit scat that can be misidentified as leopard. To identify and subsequently exclude from further analyses any scats derived from non-target species, mitochondrial DNA (mtDNA) sequencing was utilised for species

identification. We therefore screened all DNA extractions from scat samples by amplifying and sequencing a 200 bp fragment of the NADH5 mitochondrial gene (Forward: ACC TGT TCC AAC TGT TTA TTG GT, Reverse: AAA GAT TTG TTG GAA GTC TCA TGC). This primer set is leopard specific and was designed for this study based on primers from Uphyrkina *et al*. (2001) and optimised using blood samples from Arabian leopards from Oman. We performed PCR amplification in a reaction volumes of 10 µl containing 5 µl MyTag HS Red Mix (Bioline), 1.6 µl dH_2O, 0.2 µl (0.2 µM) of each forward and reverse primer, 1µl BSA (0.01 µg/µL) (Bovine Serum Albumin, New England Biolabs Inc) and 2 µl of DNA. PCR cycling conditions consisted of an initial hot start of 95°C for 8 min followed by 35 cycles of 94°C for 30 s, 50°C for 1 m and 72°C for 1 s, and a final incubation period of 10 min at 72°C. To reduce the risk of contamination between DNA samples, PCR preparation was carried out separately from DNA extractions in different laboratories at the Durrell Institute of Conservation and Ecology (DICE), University of Kent. All PCRs included a negative control (with no template DNA) in each PCR batch to monitor for contamination.

PCR products were initially run out on 2% agarose gels using electrophoresis to check for amplification and to monitor for signs of contamination in the negative controls. PCR products that indicated DNA originating from leopard scat were then purified and sequenced using a 3730X analyser (Macrogen, Amsterdam, Netherlands). The resulting forward and reverse sequences were edited and aligned using Jalview v2 (Waterhouse *et al.,* 2009) and then cross checked with sequences derived from known Arabian leopards from this study. We also cross-checked sequences with the single Arabian leopard NADH5 sequence available on GenBank

(accession number: AY035279). Non-leopard DNA samples were excluded from further analyses.

3.3.4 Microsatellite amplification

A range of microsatellite markers have been developed for felids (Menotti-Raymond *et al.,* 1999; Uphyrkina *et al.,* 2001; Williamson *et al.,* 2002; Mondol *et al.,* 2009) but none had been tested on the leopard population of Oman, Yemen or Saudi Arabia prior to this study. We identified a set of 65 published polymorphic markers, and tested their amplification success and extent of polymorphism in Arabian leopards using DNA from three scat samples genetically confirmed to be from leopards in Dhofar. DNA samples from scats were chosen instead of DNA from blood or skin samples in order to test the utility of the microsatellite markers to amplify degraded DNA given that faecal DNA is frequently of lower quality. Thirty-five markers successfully amplified during initial PCR trials and these markers were then included in the design of seven multiplexes sets (Table 3.S2). Multiplex PCRs were subsequently carried out using fluoro-labelled forward primers to genotype all genetically confirmed leopard samples. Felid-specific PCR primers designed to amplify the amelogenin and zinc-finger regions of y-chromosome (Pilgrim *et al.,* 2005) were used to assign gender to each sample. PCR reactions (10 µl volume) contained 5µl Qiagen multiplex PCR buffer mix (Qiagen Inc.), 0.2 µl (0.2 µM) fluoro-labelled forward primer (Eurofins Genomics), 0.2 µl (0.2 µM) unlabelled reverse primer, 0.5 µl (0.005 µg/µL) BSA, 0.5 µl PCR anti-inhibitor and 3 µl of template DNA. The PCR cycling conditions for all multiplexes consisted of an initial denaturation of 95°C for 15 min, 45 cycles of denaturation (94°C for 30 s), annealing (Ta ranges from 54°C to 58°C for 90 s), extension (72°C for 90 s), and a final

extension of 10 min at 72°C. All PCR products were genotyped using an Applied Biosystems 3730 DNA Analyser and ROX 500 ROX™ size-standard (DBS Genomics, Durham UK).

3.3.5 Genotype data validation

Scats and museum samples are known to yield poor quality DNA which can be prone to genotyping errors, allelic dropout and null alleles. Therefore, to eliminate and reduce the possibility of these errors in the data we genotyped each sample at least three times. Samples that amplified at fewer than four loci were discarded from further analysis. We only accepted a genotype to be true if repeated genotypes matched 100% across all loci at least twice, otherwise the sample was removed from the analysis. Heterozygote genotypes were scored at least twice while homozygotes were scored a minimum of three times (Frantz et *al.,* 2003; Hansen *et al.,* 2008). This approach is considered more cost effective than the multi-tube approach (Aziz *et al.,* 2017). We used Genemapper v3.7 (Applied Biosystems, UK) to identify and score the alleles. Allelic dropout and false alleles were measured using GIMLET v1.3.3 (Valiere, 2002) and scoring errors and null alleles were identified using Microchecker (Van Oosterhout *et al.,* 2004).

3.3.6 Identification of individuals amongst scat samples

As some of the scat samples were likely to be repeat scats (recaptures) from the same leopard, individual identification was required to avoid including repeated samples in the analysis. We thus used all the loci in this study that were observed to be polymorphic (see results) and tested their collective power to discriminate between individuals and distinguish between siblings using samples from known leopards,

including three known siblings from captive Arabian leopards. The program GIMLET was used to determine the probability of identity for siblings (PID [sibs]) and the minimum number of loci needed to distinguish between close relatives. Consensus genotype profiles were subsequently compared in the program Cervus v3.0 (Marshall *et al.,* 1998) to identify 100% matched genotypes with the genotype data set. The sexing locus was also used as an additional means to verify identification of duplicated samples of the same individual. Any matched genotypes were considered to be recaptures of the same individual.

3.3.7 Data analysis

We tested for deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) using the program Genepop (Raymond & Rousset, 1995), and sequential Bonferroni correction (Rice, 1989) was applied for multiple LD tests. Genetic diversity metrics were calculated using GenAlEx v6.5 (Peakall & Smouse, 2006). The R package diveRsity (Keenan *et al.,* 2013) was used to measure allelic richness using the rarefication method which takes account of uneven sample size.

To explore patterns of genetic diversity the genotype data set was partitioned according to region, captive or wild source, and across different time periods (Table 3.1). First, the full dataset was partitioned into two populations comprising (i) all wild samples from Oman ('Wild Oman'), and (ii) comprising samples from wild born leopards from Yemen and captive born leopards from breeding centres in Oman, Sharjah and Taif ('Captive'). Second, the data set was organised to partition the samples of wild born leopards from Yemen ('Wild-born Yemen') in order to compare their genetic diversity with samples of wild leopards from Oman ('Wild

Oman'). This step was considered important because although samples of wild -born Yemen leopard are derived from captive leopards held in Oman, Sharjah and Taif, they are in fact representative of the wild leopard population in Yemen, thereby allowing a comparison between the wild leopard population in Yemen and the wild population in Oman. Third, the data set was partitioned such that the samples from wild leopards from Oman were arranged according to the four sampling regions of this study (Jabal Samhan, Jabal Qara, Jabal Qamar, Nejd; Figure 3.2). Genetic diversity metrics were measured separately for each sampling region/population and comparisons were made between (i) the wild population in Oman and the captive population, (ii) the wild population in Oman and the wild born individuals from Yemen, and (iii) the different regions in Dhofar (Jabal Samhan, Jabal Qara, Jabal Qamar, and Nejd). Lastly, the data set was partitioned to separate the wild samples from the Jabal Samhan mountain region in Oman (Jabal Samhan is considered the last stronghold for leopards in Oman (Spalton & Willis, 1999) and is presumed to have more leopards than other regions in Dhofar) into three temporal periods to compute a time series of level of genetic diversity for the leopard population within this mountain region. Due to the small number of museum samples collected from this region, samples of individual leopards with collection dates between 1976-1979 were included together to represent the '1979' period, and samples from 2001-2006 were included together to represent the '2006' period, while samples of individual leopards from 2012-2017 were included together to represent the '2017' period. For descriptive purposes, the 2017 period is synonymous with use of the term 'contemporary samples'.

Whilst all genetic diversity metrics were calculated, unbiased expected heterozygosity (uHe) was used when making comparisons, as it is appropriate for datasets that are likely to contain samples from close relatives or inbred individuals (DeGiorgio & Rosenberg, 2009). To test for significant differences in genetic diversity between wild and captive populations, regions or time periods, a one-way ANOVA test was performed.

3.3.8 Estimation of temporal changes in effective population size

The dataset for the Jabal Samhan population contained samples that have been collected across a time period of over 40 years, thereby providing an opportunity to examine if the effective population size (N_e) in this mountain region has changed over time. We used a Bayesian method incorporated in the programme tmvp (Beaumont, 2003) which estimates a change in N_e across the time span of the sampling periods. The programme uses the collection date of each sample and allele frequencies to estimate N_e at the time of collection of the oldest sample (N_a= historical) and at the most time of collection of the most recent sample (N_0=contemporary) respectively, while accounting for uneven sample size across the sampling periods and across loci. For the tmvp analysis of the Jabal Samhan population we used a generation time of 4 years (considered to be an appropriate estimate for leopard; Dutta *et al.*, 2013a) with rectangular priors of 0–1000 for historical and contemporary N_e. However, given that the census population size of the Jabal Samhan population is considered to have been less than 200 leopards in the 1970s (based on a recent density estimate of 2.30 leopard/100km^2 and an approximate historical range size in the 1970s of 4000 km^2, we estimated Jabal Samhan to contain only 92 leopards), we re-ran the model using priors of 0–200. The

oldest and most recent samples for Jabal Samhan were from 1977 and 2017 respectively.

3.4 Results

A total of 477 scats sourced from the wild and 60 samples sourced from blood, skin, bone or scat of known Arabian leopards were obtained. DNA was extracted from all samples but five bone samples from museum specimens that did not yield enough DNA for successful amplification of the diagnostic fragment of mtDNA. Species identification analysis confirmed that 161 scats (34%) collected from across the Dhofar Governorate were derived from Arabian leopard. The remaining 316 scats (66%) were likely to have been from caracal, wolf or hyena were therefore discarded from further analysis.

3.4.1 Microsatellite amplification and identification of individuals

Out of the 35 markers applied to the DNA sample set, eight produced suitably scoreable genotypes and were observed to be polymorphic. Ten loci—of which seven were known to be polymorphic in other leopard subspecies (Uphyrkina *et.,* 2001; Mondol *et al.,* 2009; Sugimoto *et al.,* 2014)—were found to be monomorphic in this study (Table 3.S3). The remaining loci either did not amplify consistently across the dataset (4 loci) or failed to amplify (13 loci). One of the sexing markers (zinc-finger locus) failed to amplify. No evidence of false, null alleles or scoring errors were found in the dataset, but we observed a small rate of dropout in the scat samples; mean dropout rate 0.048 (ranges from 0.00 to 0.08).

A total of 109 out of 161 genetically confirmed leopard scats amplified for more than a minimum of five loci. The genotypes of these 109 scats were then analysed alongside genotypes of known leopards to determine the power of seven loci (FCA90, FCA105, FCA126, FCA279, 6HDZ89, 6HDZ635, 6HDZ700), that were observed to be polymorphic in the Dhofar population, to distinguish between any siblings that were contained within the dataset. The accumulative power of these loci to identity siblings increases with increasing the number of loci from PID (sib) 0.44 (one locus) to PID (sib) 0.02 (seven loci). A conservative PID (sib) value of 0.01 was recommended for individual identification from non-invasive samples such as hairs and scats (Waits et al., 2001). However, Woods *et al.* (1999) used a PID (sib) value of < 0.05 to identify brown bears from hair samples. Reaching these values could be difficult for species which occur at small population size and have low allelic diversity.

In this study, we examined the genotypes of known leopards and five loci (FCA279, FCA105, 6HDZ700, 6HDZ635, 6HDZ89) and were able to distinguish between individuals including three siblings. The power of these loci in the scat dataset was PID (sib) 0.05, which corresponds to one in every 20 leopards as a minimum for individual identification. We assumed, based on earlier camera trap work (Spalton *et al.,* 2006), that none of our four studied regions in Dhofar has more than 20 leopards. We therefore used the program Cervus to identify the number of individual leopards within the scat genotype dataset using a minimum of five loci. Following this approach, we identified the presence of 36 individual leopards amongst the 109 scat samples. Ten of these individuals were only identified from scats collected through opportunistic surveys between 2012-2016 and 20 individuals were only identified

from scats collected from predefined survey routes in 2017, whereas six individuals were identified from both surveys. The genotypes of these 36 wild leopards were used in combination with genotypes of 42 leopards, obtained from captive and museum samples, that amplified for a minimum of five loci for final genetic analysis (Table 3.2).

3.4.2 Allelic patterns

A total of 27 alleles were detected across the final dataset, of which 15 alleles were shared between the wild population in Oman and the captive population (including those captive individuals wild born in Yemen). Eight alleles were unique to the wild born Yemen individuals that reside in the captive population. Only two alleles were unique to the wild population in Oman. No unique alleles were found in the museum samples. The number of alleles per locus ranged from 2-5 with a mean of 3.38 alleles per locus (S. E±0.38).

Deviation from Hardy-Weinberg equilibrium was observed in marker 6HDZ700 in Jabal Qamar, marker 6HDZ89 in wild born samples and markers 6HDZ700 and FCA279 in the captive populations. No deviation from the linkage disequilibrium was observed for any pair of loci.

3.4.3 Spatial and temporal patterns of genetic diversity

In this study, the highest level of genetic diversity was observed in the captive Arabian leopard population followed by the wild born leopards from Yemen (Table 3.3). These two populations also had the highest allelic richness. The Oman wild population (all four Oman regions together) had the lowest genetic diversity (uHe

ranges from 0.387 to 0.479) and allelic richness (Ar ranges from 1.89 to 2.13) in this study. However, comparisons of these two genetic parameters (uHe and allelic richness) between the Oman, Yemen and captive populations showed that these differences were statistically nonsignificant. Small but statistically nonsignificant differences were also observed between the Oman leopards of the different regions in Dhofar (Table 3.4).

Temporal genetic diversity and allelic richness were reduced in the Jabal Samhan population from uHe 0.438 in 1979 to uHe 0.387 in 2017 (Table 3.5), yet this reduction was not statistically significant. Only one allele that was observed in the old samples was not found in the contemporary samples indicating it was lost.

3.4.4 Estimation of temporal changes in effective population size

Tmvp analysis showed a proportional reduction of 90% from N_e=853 for the historical population (95% limits 74–1000) to N_e=81 for the contemporary population (95% limits 0–940; priors =0–1000), and a reduction of 67% from N_e=168 for the historical population (95% limits 26–197) to N_e=55 for the contemporary population (95% limits 12–192) when the model parameters were refined (priors=0–200) to more closely reflect biological reality (Figure 3.S1). However, as the 95% higher posterior limits (HPD) surrounding the proportional reductions were very large an overall interpretation of reduction in N_e should be treated with caution.

3.5 Discussion

Our study is the first to provide a temporal and geographic survey of genetic diversity within and between the different mountain populations of the critically endangered Arabian leopard in Oman, and within the captive population across the region. The findings of this study provide a novel opportunity to understand population-level patterns of genetic diversity and to interpret the findings in the context of genetic aspects of small population biology and the ongoing conservation efforts to recover the Arabian leopard.

Based on microsatellite data, we found low level of loci polymorphisms and allelic diversity within the Arabian leopard populations. Ten loci (56%) had only single alleles. The remaining loci (54%) produced an overall of 27 alleles of which eight were unique to the captive and Yemen populations. Although not significant, the captive and Yemen populations have also greater genetic diversity than the Oman population which is considered the last stronghold for the Arabian leopard (Breitenmoser *et al.*, 2010). However, temporal analysis detected only minor loss of genetic diversity in the Oman population during the last 40 years.

3.5.1 Evidence of a long-term bottleneck

Of the 10 loci that we observed to be monomorphic in this study, seven are documented as being polymorphic in other leopard subspecies (Spong *et al.,* 2000; Uphyrkina *et al*., 2001; Mondol *et al.,* 2009; Sugimoto *et al.,* 2014) (Table 3.S3). The loss of alleles from these loci in the Arabian leopard could be due to a recent population crash or as a consequence of random genetic drift. While a population crash can reduce the gene pool, genetic drift changes the distribution of alleles and leads to fixation and loss of alleles (Frankham *et al.,* 2010).

The leopard was historically widespread throughout the Arabian Peninsula (Harrison & Bates, 1991) and its population would had been much larger and inter-connected (Figure 1). However, a large number of leopards (e.g. over 100; for full details see Spalton *et al.* 2006; Qarqz and Baker 2006; Al Jumaily *et al.* 2006) were reported killed in the late 20th century following the introduction of lightweight modern firearms and their use by herders. This activity led to a decline in the leopard population and even local extinction of leopards in northern Oman in 1976, (Spalton *et al.,* 2006), Jordan in 1987 (Qarqz & Baker, 2006) and the UAE in 2001 (Edmonds *et al.*, 2006). Consequently, it can be assumed that the species experienced and perhaps is still experiencing a genetic bottleneck (as a result of population crash), which in turn would explain the loss of genetic diversity.

Despite the small sample size of leopards from Yemen, this population appeared to have several unique alleles that are not found in the Oman population. This is likely to be the result of isolation and restricted gene flow (Allendorf *et al.,* 2013) as the once continuous leopard population of south Arabia has become increasingly fragmented (Breitenmoser *et al.,* 2006). We believe that some of the Yemeni samples were from leopards captured from the Wada'a region in northwest Yemen (Figure 3.S2) that were taken to Yemeni zoos and later to other regional collections (Al Jumaily *et al.* 2006). Wada'a is at least 900 km from Dhofar in Oman and although in the distant past, perhaps in the early 20th Century, there would have been some connectivity of leopard habitat, allowing gene dispersal and flow between southern Oman and northern Yemen, today this is extremely unlikely. The historical range of leopard is currently dominated by human settlements, road networks and

other barriers to leopard movement and thus any remaining population can only be highly fragmented.

In the absence of dispersal, region-specific genetic identity may develop in isolated populations. However, for substantial variation to occur in an isolated population it would not only require isolation for a considerable period of time, but the population would also need to persist at an appropriate effective size to retain those alleles. Populations in southern Oman and northern Yemen may have been isolated for an extended period but the Yemeni population either remained large enough to retain these unique alleles, or interconnectivity with other leopard populations (such as the Asir in Saudi Arabia) was sufficient that allelic diversity was not lost. We obtained a single sample of a poisoned leopard in Saudi Arabia in 2014 that amplified for only three loci, but this individual had alleles that were common to both Oman and Yemen leopard populations. Although this poisoned leopard was the last confirmed record in Saudi Arabia, Islam *et al.* (2018) suggested a small population (~50) could exist. Sampling from this population—if present—would help to clarify whether the detected alleles in Yemeni leopards are unique or shared with leopards found in Saudi Arabia.

If these unique alleles came from a larger population in Yemen, then it would be important to know the current extent of this population. Perhaps this population managed to survive the anthropogenic impacts that extirpated leopards from elsewhere in the region. Yemen has a large human population but over the last 100 years, largely as a result of limited economic development and several extended conflicts, much of the country has remained quite remote and poorly developed in comparison with other countries of the region. In these conditions the leopard may

have been able to survive in greater numbers in remote mountainous areas. There is no reliable information about the current status of wild leopards in Yemen to support this suggestion but sightings and killing of leopards were reported from several areas across the country in recent years, in particular from Dhale, Lahj and Al Marah regions. Thus, knowledge about the number of leopards and the extent of their current genetic diversity in these areas would be essential for future conservation.

The absence of the Yemeni unique alleles in the Oman population could be explained by the lack of dispersal between regional populations but also these alleles could had been lost from the Oman population due to a recent bottleneck or through genetic drift. Given that the historical leopard habitat in Oman was smaller than that of Yemen, the Oman population could be expected to be smaller and thus the negative impact of bottleneck and genetic drift would be greater.

3.5.2 Patterns of genetic diversity

The captive population and the wild born leopards sourced from Yemen showed similar levels of genetic and allelic diversity. This is perhaps not surprising as the captive population mostly originated in northern Yemen. We analysed 33 leopards from the captive population (including eight wild born in Yemen), but as seven founders were not included the captive population may have greater genetic diversity than we detected.

The Yemen population, despite its small sample size, seems to retain the highest level of genetic diversity recorded for the Arabian leopard in the wild (He=0.524, uHe=0.556). This, in fact, is higher than the estimates reported for the Critically Endangered Amur leopard (He=0.43, Sugimoto *et al.,* 2014; He=0.356, Uphyrkina *et*

al., 2001) but lower than estimates reported for Persian (He=0.616, Uphyrkina *et al.,* 2001), Indian (He=0.68, Mondol *et al.,* 2009; He=0.84, Dutta *et al.,* 2013b) and African (He=0.77, Spong *et al.,* 2000; He=0.803, Uphyrkina *et al.,* 2001) leopards. However, the lowest contemporary genetic diversity reported for the Dhofar population in this study (mean He= 0.381, mean uHe=0.423) is similar to that reported for the Amur leopard. The low genetic variability detected in these particular subspecies maybe the result of their small effective population size. The remaining population of these two subspecies was estimated at 84 for the Amur leopard (Vitkalova *et al.,* 2018) and at 44-58 for the Arabian leopards of Dhofar (Spalton & Al Hikmani, 2014).

Empirical evidence shows a causal relationship between heterozygosity and population size (Frankham, 2010). However, our results for levels of genetic diversity found within the different regions of Dhofar do not correlate with the number of leopards detected in these regions. For example, the genotypes of the three and five leopards detected from scats of leopards in the Nejd and Jabal Qara respectively showed them to have slightly higher genetic diversity than the 17 and 11 leopards detected in Samhan and Jabal Qamar respectively. This finding might be a consequence of a small sample size or that there were more leopards in the Nejd and Jabal Qara and that we failed to detect them. Future surveys might reveal if leopards are more abundant in these regions than our initial surveys suggest.

3.5.3 Temporal patterns of genetic diversity in Jabal Samhan population

Our result of small but non-significant temporal loss of genetic diversity in Jabal Samhan across the 41-year time period (a 11% reduction in uHe of 43.8% to 38.7%,

and an observed loss of a single allele) is perhaps an indication that this population has remained at a sufficiently large size such that the effect of random genetic drift and consequent loss of genetic diversity has been minimal. This modest reduction in diversity within the genotype dataset is reflected in a decline in N_e, although the extent of reduction in N_e is somewhat greater (a 67% reduction from N_e =168 to N_e=55). The scenario of a small but relatively stable population size may seem unlikely given the Critically Endangered status of the Arabian leopard and the stochastic effects imposed on a population of such small size as that estimated for the Jabal Samhan population. However, recent conservation measures such as the banning of leopard killing since 1976 and the establishment of the Jabal Samhan Nature Reserve in 1997, together with support from public awareness programs and compensation schemes for livestock herders, are all factors considered to have contributed to the safeguarding of this population. These measures may have helped to mitigate against a continued and drastic decline of the leopard population and in doing so may have slowed any rate of loss of genetic diversity.

3.6 Conservation implications

Maintaining genetic diversity is important to avoid inbreeding, loss of reproductive fitness, loss of evolutionary potential, and ultimately extinction (Frankham *et al.*, 2017). Endangered species which occur at small population sizes are at risk of losing genetic diversity and suffering from inbreeding depression. A number of conservation strategies have been advocated to preserve genetic diversity within endangered taxa and to mitigate problems associated with inbreeding. These strategies include protection of habitat corridors to facilitate gene flow between populations, translocations of individuals from populations with high genetic

diversity to those with low genetic diversity and through reintroduction of individuals from captive populations. In particular, the introduction of individuals which harbour additional or novel genetic diversity into a genetically impoverished population can have the potential to alleviate problems of inbreeding depression through heterosis, commonly known as 'genetic rescue'. Despite concerns surrounding strategies of this kind (e.g. risk of outbreeding depression, loss of local adaption, detrimental consequences of genetic adaption to captivity), this approach has frequently been successful in a number of endangered wildlife populations in increasing levels of genetic diversity and fitness of their natural populations, such as the Florida panther (Hedrick & Fredrickson, 2010; Johnson *et al.*, 2010), Mexican wolves (*Canis lupus baileyi*) (Hedrick & Fredrickson, 2010) and Swedish adders (*Vipera berus*) (Frankham *et al.*, 2017).

In the light of our results, which indicate captive sources of genetic diversity not found in the wild population of Arabian leopards and unevenly distributed levels of genetic diversity in the wild leopards, conservation managers of this Critically Endangered leopard may wish to consider implementing strategies involving genetic rescue to prevent the extinction of Arabia's last big cat. Our findings of the amount of genetic diversity found in Yemen and captive populations, and the number of unique alleles they contain, offer an ideal opportunity for genetic rescue of the Arabian leopard. For example, translocations and crossbreeding management of wild Yemeni leopards, if available, with the Dhofar population of Oman could bring genetic benefits for the genetically impoverished Dhofar population. Whilst literature on reintroduction biology indicates that using wild-sourced animals for reintroduction rather than captive-bred stock leads to a greater probability of

successful establishment following reintroduction (Fischer & Lindenmayer, 2000), the option of wild sourced animals which have additional genetic diversity is not available for the Arabian leopard. Therefore, conservation managers are limited to considering the captive population as the source for enhancing the wild population.

3.6.1 Conservation breeding and reintroduction

The importance of conservation breeding and reintroduction of the Arabian leopard has been highlighted in the "Strategy for the Conservation of the Leopard in the Arabian Peninsula" (Breitenmoser *et al.*, 2010). To achieve the vision of this strategy of a viable and sustainably managed population of Arabian leopard, captive leopards are considered important in order to augment and reintroduce new populations into the leopard's historical range (Breitenmoser *et al.,* 2006). The strategy also emphasises the importance of genetic management of captive leopards to maintain a representative genetic diversity of the wild populations. In order to maintain regional genetic diversity and to provide genetically fit leopards for future reintroductions we put forward two genetic management options for the captive population and highlight the advantages and disadvantages of each option from a genetic perspective.

(a) Integrated captive breeding and reintroduction of introgressed Yemen and Oman leopards

We consider that crossbreeding of leopards from Yemen with those from Oman could reduce any problems associated with inbreeding depression in the captive population (and subsequently increase reproductive fitness), help preserve levels of regional genetic diversity (for example, the unique alleles in Yemen) and provide suitable candidate leopards (i.e. introgressed offspring from Yemen and Oman

genes) for future reintroduction. The reintroduction of reproductively fit individuals will benefit the existing wild populations by increasing their genetic diversity and subsequently their evolutionary potential. Reintroducing a mixture of Yemen and Oman genes would allow the reshaping of the leopard population's gene pool by natural selection and regional adaptation. This approach is however not without risk, with disadvantages including outbreeding depression, genetic swamping and loss of genetic identity. However, the benefits outlined above could help facilitate the recovery of the wild population of the Arabian leopard.

(b) Independent breeding management for Yemen and Oman populations to preserve their genetic integrity.

Phylogenetic analysis show that Yemen and Oman leopards diverged from a common ancestor approximately 65-243 thousands years ago (chapter 2), a duration of independent evolutionary history which may entitle the two lineages to each be managed as an Evolutionary Significant Unit. Consequently, conservation managers may want to manage each population independently to preserve their genetic identity and population uniqueness. Following this management approach would avoid the risks associated with crossbreeding, but this approach could be a missed opportunity to facilitate the potential benefits of genetic rescue.

The Arabian leopard is Critically Endangered, with only a limited number of individuals in captivity (Budd & Leus, 2011) and a small remnant wild population which is already fragmented into several isolated subpopulations (Spalton & Al Hikmani, 2014; Zafar-ul Islam *et al.*, 2018). Given this situation, managing the remnant populations separately is likely to increase their extinction risk due to the inevitable negative consequences of genetic factors associated with small population size. Managers should be encouraged to actively consider the option of captive

breeding and reintroduction of introgressed leopards as an opportunity to boost the recovery of the wild Arabian leopard population by genetic rescue.

3.7 Tables

Table 3.1. Partitioning of Arabian leopard genotype data set for genetic diversity measurements and comparisons between different populations and regions, including temporal comparison in Jabal Samhan population

Date set partitioning and comparisons	Samples
(1) Comparison of genetic diversity between wild leopards from Oman and captive leopards (including wild- born leopards from Yemen)	n=56; Oman vs n=36; captive
(2) Comparison of genetic diversity between wild leopards from Oman and wild- born leopards from Yemen	n =56; Oman vs n=11; Yemen
(3) Comparison of contemporary genetic diversity between regions in Dhofar: Jabal Samhan, Jabal Qara, Jabal Qamar, Nejd	n=17; Jabal Samhan n=5; Jabal Qara n=11; Jabal Qamar n=3; Nejd
(4) Temporal measures of genetic diversity in Jabal Samhan population for three periods: 1976-1979, 2001-2006, 2012-2017	n=4; 1976-1979 period n=5; 2001-2006 period n=17; 2012-2017 period

Table 3.2. Summary of Arabian leopard individuals that screened and genotyped from each population.

Population	Total samples	Samples genotyped for at least 5 loci
Oman	56	45
Yemen	11	8
Saudi Arabia	1	-
Captive-born	28	25

Table 3.3. Measures of genetic diversity in Arabian leopards. Samples are from the wild population (Oman), captive population (Oman, UAE, and Saudi Arabia) and captive leopards wild-born in Yemen.

Population	**N**	**Na**	**Ne**	**uHe**	**Ar**
Wild Oman	45	2.375±0324	1.952±0.258	0.429±0.076	2.35
Captive (including 8 wild-born in Yemen)	33	3.125±0.295	2.436±0.243	0.571±0.042	3.13
Wild-born Yemen (extracted from 33 captive samples)	8	2.875±0.227	2.202±0.189	0.556±0.052	2.88

Sample size: N, No. Alleles: Na, No of Effective alleles: Ne, Unbiased Expected Heterozygosity: uHe. Allelic richness: Ar.

Table 3.4. Contemporary levels of genetic diversity in different regions of Dhofar Governorate.

Population	**N**	**Na**	**Ne**	**uHe**	**Ar**
Jabal Samhan	17	2.250±0313	1.791±0257	0.387±0.076	1.91
Jabal Qara	5	2.000±0.189	1.795± 0.210	0.431±0.038	1.89
Jabal Qamar	11	2.125±0.227	1.762±0.212	0.396±0.074	1.89
Nejd	3	2.125±0.227	1.789±0.201	0.479±0.089	2.13

Sample size: N, No. Alleles: Na, No of Effective alleles: Ne, Unbiased Expected Heterozygosity: uHe. Allelic richness: Ar.

Table 3.5. Time-series of measures of genetic diversity in Jabal Samhan population

Population	**N**	**Na**	**Ne**	**uHe**	**Ar**
Samhan 1979	4	2.125±0.227	1.792±0.207	0.438±0.087	2.13
Samhan 2006	5	2.125±0.227	1.768±0.181	0.433±0.074	2.09
Samhan 2017	17	2.250±0.313	1.791±0.257	0.387±0.076	2.05

Sample size: N, No. Alleles: Na, No of Effective alleles: Ne, Unbiased Expected Heterozygosity: uHe. Allelic richness: Ar.

Table 3.S1. Details of Arabian leopard biological samples collected for genetic diversity study from museums, breeding centres and government departments. MECA=Ministry of Environment and Climate Affairs, MAF=Ministry of Agriculture and Fisheries, OWABC= Oman Wild Animal Breeding Centre, ONHM= Oman Natural History Museum, BCEAW= Breeding Centre for Endangered Arabian Wildlife, PSAWRC= Prince Saud al-Faisal Wildlife Research Center.

Sample ID	Origin	Type	Source (obtained from)
SM4	Samhan/Oman 2002	Blood	Dr Andrew Spalton
SM8	Samhan /Oman 2002	Blood	Dr Andrew Spalton
SM11	Samhan/Oman 2006	Blood	Dr Andrew Spalton
H1	Samhan/Oman 1977	Skin	Harrison Institute, Sevenoaks, UK
H2	Yemen 1965	Skin	Harrison Institute, Sevenoaks, UK
H3	Oman 1976	Skin	Harrison Institute, Sevenoaks, UK
H4	Musandam/Oman1979	Skin	Harrison Institute, Sevenoaks, UK
H5	Samhan/Oman 1977	Skin	Harrison Institute, Sevenoaks, UK
H6	Samhan/Oman 1979	Skin	Harrison Institute, Sevenoaks, UK
OM1	Samhan /Oman 2003	Skin	MECA, Muscat, Oman
OM2	Salalah /Oman 2008	Skin	MAF, Muscat, Oman
OM3	Captive born	Skin	OWABC, Muscat, Oman
OM5	Captive born	Skin	OWABC, Muscat, Oman
OM6	Captive born	Skin	OWABC, Muscat, Oman
ONHM1	Samhan 1994	Bone	ONHM, Muscat, Oman
ONHM2	Samhan 1989	Bone	ONHM, Muscat, Oman
ONHM3	Musandam 1980	Bone	ONHM, Muscat, Oman
ONHM4	Musandam 1980	Bone	ONHM, Muscat, Oman
ONHM5	Samhan 1985	Bone	ONHM, Muscat, Oman
ONHM7	Samhan /Oman1997	Skin	ONHM, Muscat, Oman
ONHM8	Samhan /Oman1989	Skin	ONHM, Muscat, Oman
ONHM9	Oman	Skin	ONHM, Muscat, Oman
ONHM10	Oman	Skin	ONHM, Muscat, Oman
PP003	Samhan 1984	Blood	BCEAW, Sharjah, UAE
PP005	Captive born	Skin	BCEAW, Sharjah, UAE
PP009	Captive born	Skin	BCEAW, Sharjah, UAE
PP011	Captive born	Skin	BCEAW, Sharjah, UAE
PP014	Captive born	Skin	BCEAW, Sharjah, UAE
PP016	Captive born	Skin	BCEAW, Sharjah, UAE
PP017	Wild born in Yemen	Skin	BCEAW, Sharjah, UAE
PP022	Captive born	Skin	BCEAW, Sharjah, UAE
PP026	Captive born	Skin	BCEAW, Sharjah, UAE
PP027	Captive born	Skin	BCEAW, Sharjah, UAE
PP028	Wild born in Yemen	Skin	BCEAW, Sharjah, UAE
PP029	Captive born	Skin	BCEAW, Sharjah, UAE
PP030	Wild born in Yemen	Skin	BCEAW, Sharjah, UAE
PP032	Captive born	Skin	BCEAW, Sharjah, UAE
PP033	Captive born	Skin	BCEAW, Sharjah, UAE
PP037	Captive born	Skin	BCEAW, Sharjah, UAE

Sample ID	Origin	Type	Source (obtained from)
PP041	Captive born	Skin	BCEAW, Sharjah, UAE
PP043	Captive born	Skin	BCEAW, Sharjah, UAE
PP047	Captive born	Skin	BCEAW, Sharjah, UAE
PP052	Captive born	Skin	BCEAW, Sharjah, UAE
PP054	Captive born	Skin	BCEAW, Sharjah, UAE
T1	Captive born	Blood	PSAWRC, Taif, KSA
T2	Captive born	Blood	PSAWRC, Taif, KSA
T3	Captive born	Blood	PSAWRC, Taif, KSA
T4	Captive born	Blood	PSAWRC, Taif, KSA
T5	Wild born Yemen 1995	Blood	PSAWRC, Taif, KSA
T6	Captive born	Blood	PSAWRC, Taif, KSA
T7	Wild born Yemen 1995	Blood	PSAWRC, Taif, KSA
TT1	Wild born Yemen	Blood	PSAWRC, Taif, KSA
TT2	Wild born Yemen	Skin	PSAWRC, Taif, KSA
TT3	Captive born	Skin	PSAWRC, Taif, KSA
TT4	Saudi Arabia	Skin	PSAWRC, Taif, KSA
TH7	Wild born Yemen	Skin	PSAWRC, Taif, KSA
TS8	Captive born	Scat	PSAWRC, Taif, KSA
TS9	Captive born	Scat	PSAWRC, Taif, KSA
TS10	Wild born Yemen	Scat	PSAWRC, Taif, KSA
Yemen 2	Yemen 2014	Blood	OWABC, Muscat, Oman

Table 3.S2. Details of microsatellites multiplexes used in this study.

Multiplex	Number of markers	Temp	FAM	HEX	NED
1	4	58	FCA52	FCA126 6HDZ635	FCA90
2	5	55	FCA193	FCA149	FCA309 FCA77 FCA96
3	7	56	FCA123 FCA391	FCA672 FCA229 FCA220 6HDZ859	ZN
4	4	58	FCA105	6HDZ700	FCA205 6HDZ610
5	6	54	FCA304 FCA26	FCA441 AM	FCA310 FCA45
6	7	58	FCA075 FCA453 6HDZ817	FCA628 FCA224 6HDZ7	6HDZ317
7	4	58	F41	6HDZ64 6HDZ89	FCA279

Locus AM was also amplified separately at a lower annealing temperature (Ta 52 C).

Table 3.S3. Characteristics of the microsatellite markers amplified in the Arabian leopard and their characteristic in other leopard subspecies.

Locus/Subspecies	This study	Amur leopard	Indian leopard	African leopard
F41	Polymorphic		Polymorphic	
FCA90	Polymorphic	Polymorphic	Polymorphic	
FCA105	Polymorphic	Polymorphic		Polymorphic
FCA126	Polymorphic		Polymorphic	Polymorphic
FCA279	Polymorphic		Polymorphic	
6HDZ89	Polymorphic			
6HDZ635	Polymorphic			
6HDZ700	Polymorphic			
F52	Monomorphic		Polymorphic	
FCA075	Monomorphic	Polymorphic		Monomorphic
FCA453	Monomorphic		Polymorphic	Polymorphic
6HDZ817	Monomorphic			
FCA628	Monomorphic		Polymorphic	Polymorphic
FCA224	Monomorphic	Polymorphic		Monomorphic
FCA309	Monomorphic		Polymorphic	
6HDZ610	Monomorphic			
FCA310	Monomorphic		Polymorphic	Polymorphic
6HDZ64	Monomorphic			

3.8 Figures

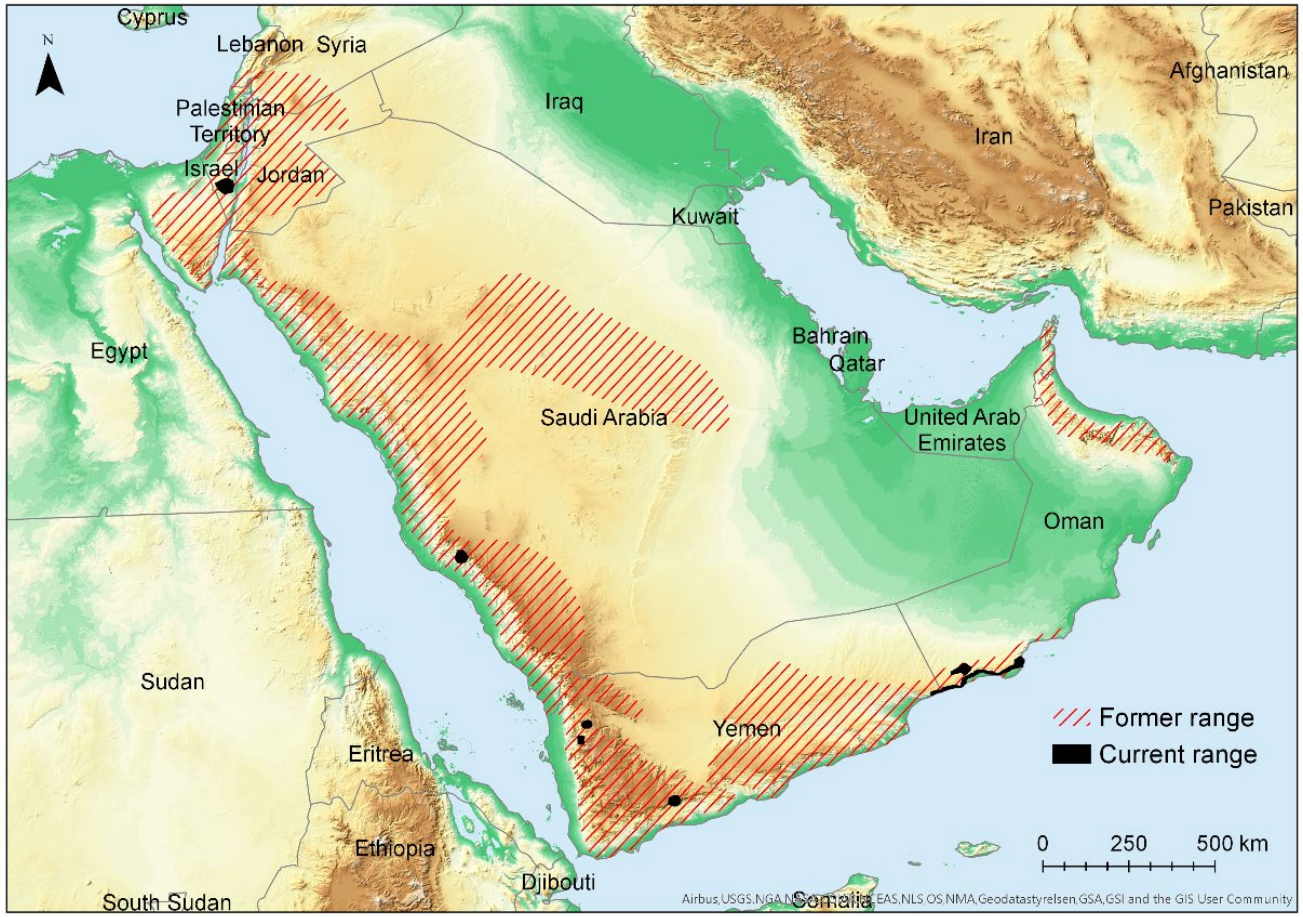

Figure 3.1. Historical and current distribution of Arabian leopard in the Arabian Peninsula (Spalton & Al Hikmani, 2014).

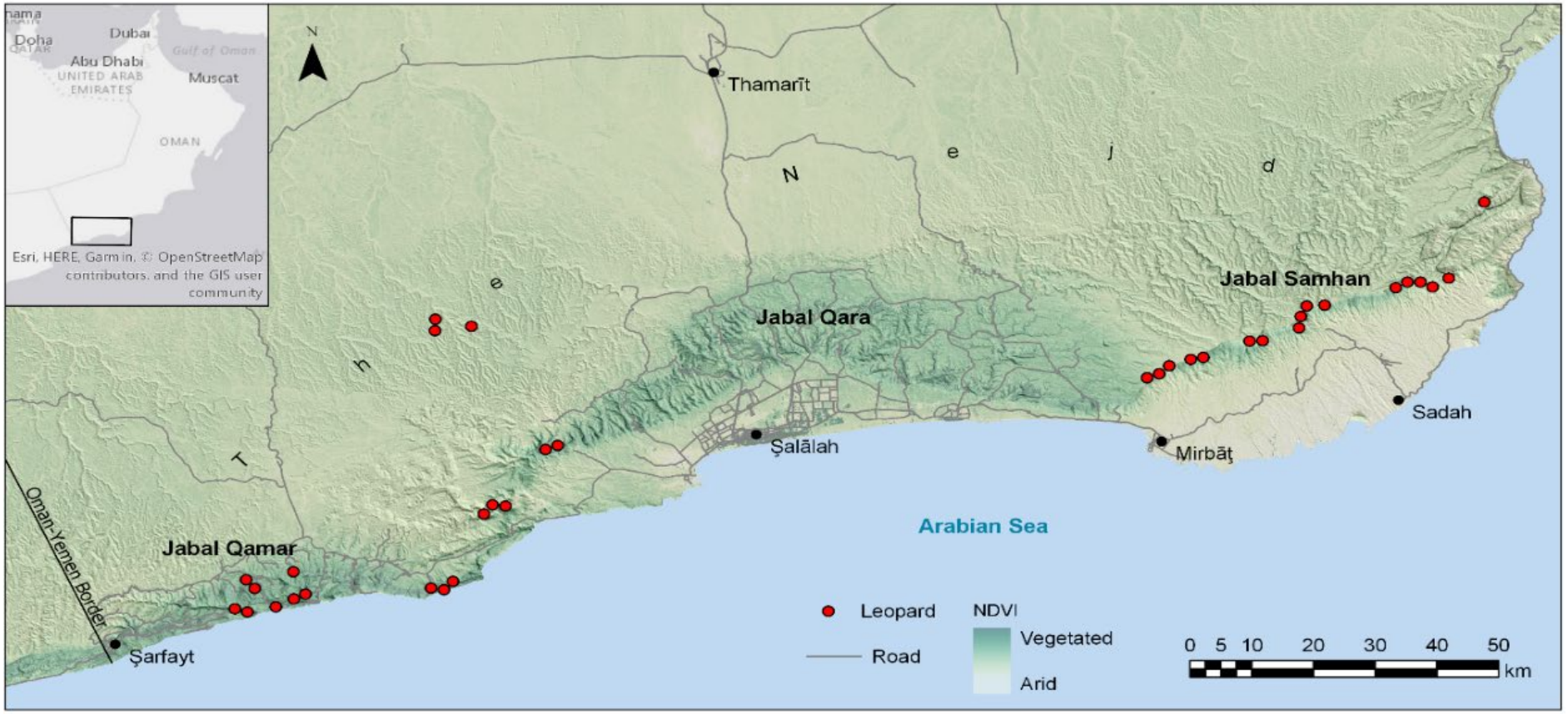

Figure 3.2. The location of the study regions in Dhofar and the spatial distribution of 36 leopards identified via genetic analysis in this study. The inset map shows the position of Dhofar within Oman.

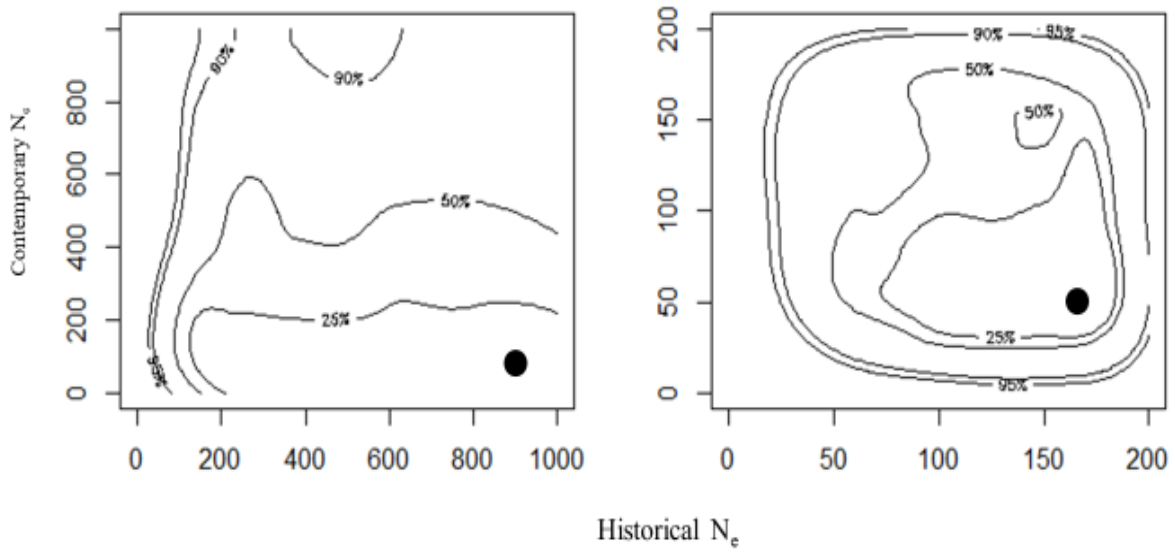

Figure 3.S1. Tmvp estimates of historical and contemporary effective population sizes (Ne) for Jabal Samhan population following the methods of Beaumont (2003). Left: a rectangular prior of 0–1000; Right: refined rectangular priors of 0–200. The single black circle is the joint mode; circles indicate density limit of posterior distribution 25–95%.

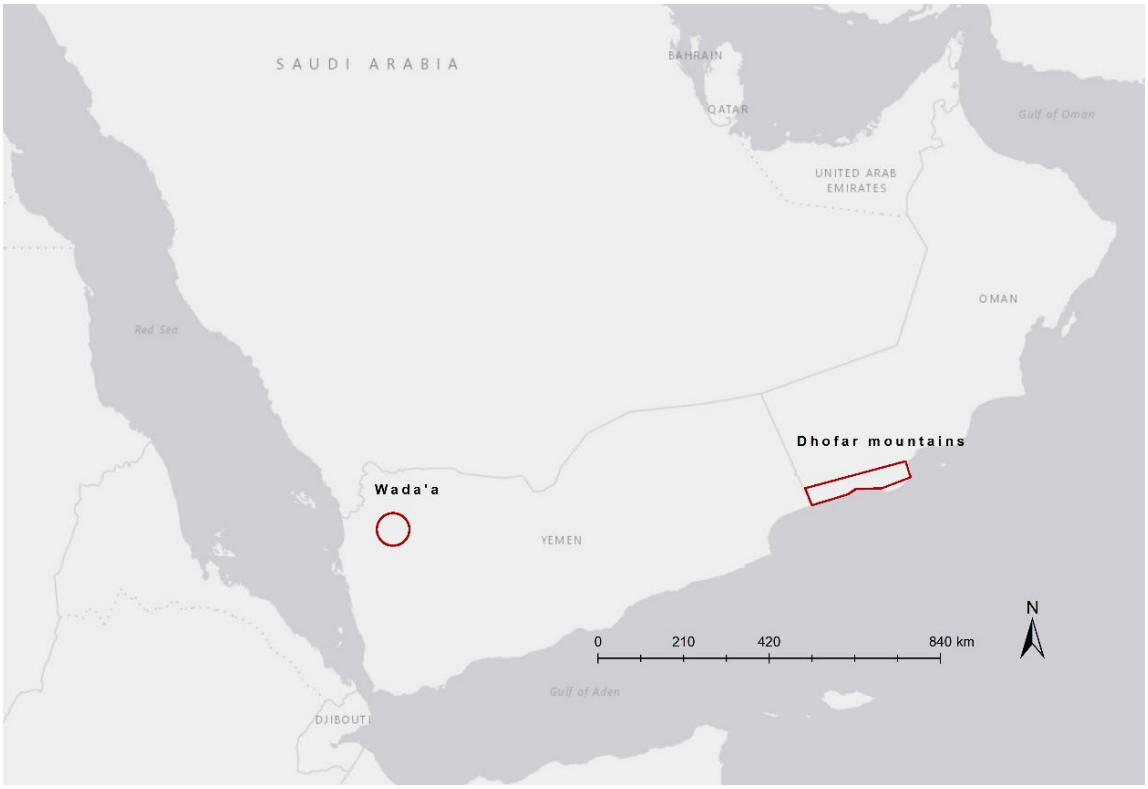

Figure 3.S2. The location of the Dhofar mountains in Oman, home to the Arabian leopard, and the Wada'a region in northwest Yemen from where the wild born 'Yemen' leopards are thought to have been sourced for the captive population (Al Jumaily et al., 2006).

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Leopard habiat in Dhofar mountains; the arid Jabal Samhan (above) and the clould forest of Jabal Qamar (below).

4. Fine-scale population genetic structure and gene flow in the remnant wild population of the Critically Endangered Arabian leopard in Oman

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4.1 Abstract

The Arabian leopard (*Panthera pardus nimr*) is a Critically Endangered species which like many other carnivores it is threatened by habitat loss and fragmentation across its range due to anthropogenic factors. The Arabian leopard has been eliminated from 98% of its historic range in Arabia making the Dhofar mountains of southern Oman its last stronghold in the wild. However, the Dhofar mountains are today under heavy human and livestock pressure which are considered to have affected the spatial distribution, gene flow and connectivity of the remnant leopard population in Dhofar. To investigate these claims, we conduct the first a fine-scale genetic structure and gene flow surveys of leopards in the Dhofar mountains using non-invasively collected faecal (scats) samples. We genotyped 36 leopards samples, collected between 2012 and 2017 across the leopard range in Dhofar, at 7 polymorphic microsatellite markers. Our study detects a signature of fine-scale genetic structure and low levels of gene flow within the leopards of Dhofar which could be imputed to recent human development in the Dhofar mountains (e.g. roads, settlements, livestock number). Although STRUCTURE analyses revealed only two clusters, GENLAND analyses indicated the Dhofar population as being clustered into three subpopulations; Jabal Qamar (cluster 1), Jabal Qara and Nejd (cluster 2) and Jabal Samhan (cluster 3). F_{ST} analyses showed significant levels of population differentiation between these three clusters. Given the threat status of the Arabian leopard, we suggest there is a need for urgent identification and protection of potential corridors to enable dispersal and therefore gene flow between the leopard populations in the Dhofar mountains. We also suggest consideration is given to creating and establishing wildlife crossings along the main roads in Dhofar. Such

approach will be beneficial to the leopard as well as other terrestrial species of the region that live alongside the leopard.

Keywords: habitat fragmentation, gene flow, population structure, Arabian leopard, Dhofar mountains.

4.2 Introduction

Habitat destruction and fragmentation is one of the major threats to terrestrial mammals, especially large carnivores (Tigas *et al.,* 2002; Crooks *et al.*, 2017). The effects of habitat fragmentation cause populations to decline but can also limit gene flow and affect the genetic structure of their natural populations through loss of genetic diversity, inbreeding and genetic drift (Frankham *et al.*, 2017). The impact of these factors is especially problematic for endangered species which are often found in small populations.

To address the negative consequences of habitat fragmentation, it is important to maintain connectivity between fragmented populations. Doing so can slow the rate of loss of genetic diversity through dispersal and gene flow, and thus preserve genetic diversity within the existing connected populations of an endangered species (Frankham *et al.*, 2017). Connectivity also reduces levels of inbreeding that may accumulate in isolated populations. Reducing levels of inbreeding has the benefit of minimising inbreeding depression and therefore maximising population fitness parameters such as reproductive output and survival (Frankham *et al.,* 2010; Allendorf *et al.,* 2013).

It is very challenging to measure or estimate the extent to which populations of highly elusive, critically endangered species are connected because they are so difficult to observe. This problem can mean that the detrimental impacts (both ecological and genetic) of habitat loss, fragmentation and resulting isolation often go unnoticed.

Conservation biologists can more effectively manage isolated populations of threatened taxa if they have information about which populations are in fact connected and which populations are genetically isolated. Resources can then be targeted towards creating or restoring habitat corridors between those populations that have become isolated in order to reconnect them. Empirical evidence shows that the creation and preservation of corridors can link fragmented populations and promote genetic diversity and geneflow. For example, the creation of corridors has linked isolated populations of African elephants (*Loxodonta africana*) in Kenya (Green *et al.*, 2018), and allowed the movement of cougar (*Puma concolor*) (Gloyne & Clevenger, 2001) and wolves in Canada (Shepherd & Whittington, 2006).

The Arabian leopard (*Panthera pardus nimr)* is an example of an endangered species whose population has become fragmented as a consequence of habitat loss (Al Jumaily *et al.*, 2006; Spalton *et al.*, 2006; Breitenmoser *et al.*, 2010; Zafar-ul Islam *et al.*, 2018). The Dhofar mountains, located in southern Oman, comprise a chain of three contiguous mountain massifs that span approximately 250 km (Figure 4.1), and are considered the last stronghold for the Arabian leopard (Breitenmoser *et al.*, 2010). Two of these mountains, Jabal Qamar and Jabal Qara, are strongly influenced by the annual monsoon (mid-June to mid-September) and as a result a unique cloud forest, rich in endemic flora (Patzelt, 2015) is found on the plateaus and south-facing aspects. The third mountain, Jabal Samhan, has the highest elevation (1,760 meters above sea level [a.s.l]) but is hardly affected by the monsoon and is consequently mostly hyper-arid with sparse desert vegetation. The northern foothills of these mountains are known as the Nejd, and although leopards have been observed in this

region (Al Hikmani et *al.,* 2015), the Nejd is considered to be on the periphery of core leopard habitat (Mazzolli *et al.*, 2017).

Changes in the extent of the human footprint on the Dhofar mountains may have impacted upon the ability of leopards to move freely within and between regions. Prior to 1970 the mountains were mostly undeveloped and the level of human impact on the environment across the Dhofar region was considered low (Shaw Reade *et al.*, 1980). However, since then the region has seen rapid development and human settlements have been built throughout Jabal Qara, on the northern plateau and coast of Jabal Qamar, and along the foothills of Jabal Samhan (Figure 4.1). In the 1970s a major road was constructed from Salalah, the regional capital of Dhofar, north to Thumrait and the capital Muscat. Later many more roads were built including from Salalah west to Jabal Qamar and east to Taqah and Tawi Ater (Figure 4.1). This rapid development was accompanied by a dramatic increase in the human population, rising from an estimated 20,000 in the 1970s (Shaw Reade *et al.*, 1980) to 434,952 in 2016 (Oman National Centre for Statistics and Information, 2017). At the same time, livestock numbers increased from approximately 68,000 in the early 1970s to 632,265 in 2013 (MAF, 2013).

The rapid development of the region, and of the mountains in particular, is likely to have had a negative impact on the levels of connectivity between wildlife populations. Roads may fragment habitat, limiting leopard movement that may in turn lead to population isolation. Carnivore species such as the Felidae are regarded to be particularly sensitive to roads and there is evidence that roads have a negative influence on their home range and genetic structure (Poessel *et al.*, 2014; Ceia-Hasse *et al.*, 2017).

In this study we used non-invasive molecular methods to identify spatial patterns of genetic structure for the Critically Endangered Arabian leopard in order to (i) relate these patterns to physical and human barriers to dispersal, and (ii) estimate gene-flow between different populations across the Dhofar mountains. We interpret our findings in a way that can facilitate their incorporation into the design of appropriate conservation management strategies for the Arabian leopard.

4.3 Methods

4.3.1 Sample collection

Non-invasive scat sampling for DNA is one of the most widely used methods to study rare and elusive species such as big cats. DNA extracted from faecal material (scats) can be used to estimate population abundance, levels of genetic diversity and degree of population genetic structure. Given the highly elusive nature of the Arabian leopard we therefore used scat samples that were collected from wild leopards across the Dhofar mountains, including the northern Nejd region, as a source for DNA in order to examine spatial patterns of genetic structure across the wild population. This approach enabled us to understand the structure of the Dhofar population, whether it is fragmented and to what extent patterns of genetic structure may be due to recent development. Scats were collected during dedicated presence/absence and capture-recapture (camera-trap) surveys, and also opportunistically (see chapter 5 for detailed methodologies). Additional scats were collected opportunistically by field staff carrying out habitat surveys of overgrazing in Jabal Qamar during the period between December 2016 and April 2017. Scat samples were stored dry in Ziplock plastic bags, and each labelled with date of

collection and GPS location. After transport from Oman to the UK scat samples were stored at ambient room temperature.

4.3.2 DNA extraction and genotpying

Procedures for DNA extraction, species identification, and individual and sex identification of scats are described in full in chapter 3. We used the QIAamp Fast DNA stool mini kit (Qiagen, UK) to extract DNA from scat samples, and screened all extractions by amplifying and sequencing a 200 bp fragment of the NADH5 mitochondrial gene (Forward: ACC TGT TCC AAC TGT TTA TTG GT, Reverse: AAA GAT TTG TTG GAA GTC TCA TGC). This primer set is leopard specific and was designed for this study based on primers from Uphyrkina *et al*. (2001) and optimised using blood samples from Arabian leopards from Oman (see chapter 3 for details of PCR reactions and cycling conditions).

PCR products that indicated DNA originating from leopard scat were then purified and sequenced using a 3730X analyser (Macrogen, Amsterdam, Netherlands). The resulting forward and reverse sequences were edited and aligned using Jalview v2 (Waterhouse *et al.,* 2009) and then cross checked with sequences derived from known captive Arabian leopards. We also cross-checked all edited sequences with the single Arabian leopard NADH5 sequence available on GenBank (accession number: AY035279). These procedures are important for further confirmation of the identity of each sample prior to downstream genotyping analysis using microsatellite markers. Non-leopard DNA samples were excluded from further analyses.

Genetically confirmed leopard scats were genotyped using 37 microsatellite markers (Table 3.S2), but as only seven markers were polymorphic for the Dhofar

population, we used a final set of seven markers (FCA90, FCA105, FCA126, FCA279, 6HDZ89, 6HDZ635, 6HDZ700) for individual identification and subsequent genetic analysis. Felid-specific PCR primers designed to amplify the amelogenin regions of y-chromosome (Pilgrim *et al.,* 2005) were used to assign gender to each sample.

Each sample was genotyped at least three times to reduce the possibility of genotyping errors, allelic dropout and null alleles. We accepted a genotype to be true if repeated assays matched 100% across all loci at least twice, otherwise the sample was removed from the analysis. Heterozygote genotypes were scored at least twice while homozygotes were scored a minimum of three times (Frantz et *al.,* 2003; Hansen *et al.,* 2008). This approach is considered more cost effective than the multi-tube approach (Aziz *et al.,* 2017). We used Genemapper v3.7 (Applied Biosystems, UK) to identify and score the alleles. Allelic dropout and false alleles were measured using GIMLET v1.3.3 (Valiere, 2002) and scoring errors and null alleles were identified using Microchecker (Van Oosterhout *et al.,* 2004)

4.3.3 Genetic analysis

(a) Spatial patterns of population differentiation

GenAlEx v6.5 (Peakall & Smouse, 2006) was used to quantify the extent of spatial genetic differentiation (F_{ST}) between populations of the Arabian leopard and to test for isolation by distance (IBD) using the Mantel test (Mantel, 1967). Mantel tests were performed on genetic and geographic pairwise distance matrices derived from genotypes of scat samples and their geographic sample locations. We tested for IBD both in the whole data set and between regions. To test for statistical significance of

any correlation coefficient (*r*) between genetic and geographic distance, we ran the test with 9,999 permutations, with instances of significant correlation ($P \leq 0.05$) considered to be evidence of IBD.

(b) Spatial patterns of genetic structure

We used two Bayesian cluster analyses to infer fine scale population structure of the Dhofar leopards, STRUCTURE 2.3.4 (Pritchard *et al.,* 2000) and GENELAND (Guillot *et al.,* 2005) programs. STRUCTURE uses Markov Chain Monte Carlo (MCMC) algorithms to infer the most likely number of genetic clusters (K) within the data set. We performed the analysis using the no-admixture model with correlated allele frequencies and including prior information such as region and population ID. The no-admixture model is considered suitable for detecting subtle structure in potentially isolated populations where there is little opportunity for gene flow (Pritchard *et al.,* 2010) whilst prior information is important to aid clustering (Falush *et al.,* 2003). The analysis was performed for 10 independent runs specifying a value of K ranging from one to five, each run comprising 500,000 MCMC iterations with a burn-in of 50,000.

Structure Selector (Li & Liu, 2018) was used to identify the most likely number of clusters using the Puechmaille (2016) method. This method is based on the count of the number of different clusters to which at least one of the user-defined populations belongs. The Puechmaille method accounts for uneven sample size and is considered more accurate at detecting the correct population structure than the delta K and posterior probability methods proposed by Evanno *et al.* (2005) and Pritchard *et al.* (2010) respectively. Finally, we used Structure Selector (Li & Liu, 2018) to plot

clusters, and CLUMPP (Jakobsson & Rosenberg, 2007) to estimate individual assignment from the 10 independent runs. We assigned individuals to a single cluster only when they had a membership coefficient (q) higher than 0.5 while individuals with membership coefficient of q = < 0.5 were considered to be admixture.

GENELAND is a spatial structure analysis which uses individual multi-locus genotypes together with their geographic locations to infer the number of populations and identify any genetic discontinuity within these populations (Guillot *et al.,* 2005a). This analysis is considered to provide superior estimates of the number of clusters as it takes account of the geographic location of each sample. We applied GENELAND analysis to examine signals of genetic structure within the dataset following two steps as per Guillot *et al.* (2005b). We first ran 10 independent runs with 500,000 MCMC iterations and a burn-in of 100 under the spatial model and specifying uncorrelated allele frequency assuming unknown K. We then repeated the analysis using the same parameters but treating the number of clusters as known, using a previously determined number from step one (K=3). This latter step helps to obtain a map of the distribution of each cluster and accurate individual assignment.

(c) Patterns of gene flow

The program BayesAss 1.3 (Wilson & Rannala, 2003) was used to estimate rates of recent immigration (gene flow) among the Dhofar populations. BayesAss uses a Bayesian method, assumes linkage equilibrium, and relaxes the assumption that the populations are in Hardy–Weinberg or migration-drift equilibriums. We assumed contemporary gene flow to be over the last five generations (i.e. 20-25 years) based on a generation time of 4-5 years for leopard (Dutta *et al.*, 2013). The analysis was

performed using five independent runs with different randomly generated starting seeds to ensure consistency between runs. We adjusted the delta values and used 50,000,000 iterations with a burn in of 5,000,000 and sampling every 2000th iteration. To test the reliability of our data we compared our migration rate estimates with the mean and 95% confidence intervals (CI) expected for uninformative data that is provided by BayesAss.

4.4 Results

From a total of 161 leopard scats collected (and genetically verified to be leopard), 109 produced genotypes for a minimum of 5 loci (Table 4.1). Based on these genotypes we identified the presence of 36 individual leopards of which 17 were identified from scats collected from Jabal Samhan, 5 from Jabal Qara, 11 from Jabal Qamar and 3 from the Nejd. Genotypes of these 36 leopards were used to infer spatial patterns of genetic structure for the Dhofar population and to estimate levels of contemporary gene flow between leopard populations across the different regions.

4.4.1 Spatial patterns of population differentiation

All analysed populations showed a significant level of population differentiation (Figure 4.2); the Nejd region was excluded due to small sample size (n=3). The highest level of differentiation was observed between the leopards of Jabal Qara and Jabal Qamar (F_{ST}=0.108, P=0.013) followed by Jabal Samhan and Jabal Qamar (F_{ST}=0.094, P< 0.001), whilst the lowest level of differentiation was observed between Jabal Samhan and Jabal Qara leopards (F_{ST}=0.070, P=0.050).

Mantel tests for isolation by distance showed a weak but significant relationship between genetic and geographic distance for the whole data set (r=0.124, P=0.002) and between Samhan and Jabal Qamar (r=0.203, P=0.002) (Table 3). We did not detect any significant IBD relationship between genetic and geographic distance between Jabal Samhan and Jabal Qara or Jabal Qara and Jabal Qamar (Table 4.2).

4.4.2 Spatial patterns of genetic structure

STRUCTURE analysis using the Puechmaille (2016) method indicated the possibility of two genetic clusters (Figure 4.3). Cluster 1 predominantly included all leopards from Jabal Samhan, Jabal Qara and the Nejd with estimated probability (q) of population membership ranging from 69% - 72%. Cluster 2 predominantly comprised leopards of Jabal Qamar. The estimated probability of population membership to cluster 2 was 61%.

Spatial analysis via GENELAND indicated the presence of three genetic clusters within Dhofar (Figure 4.4). Cluster 1 corresponds to Jabal Qamar, cluster 2 to the leopards of Jabal Qara and the Nejd, while cluster 3 corresponds to the leopards of Jabal Samhan. The probability of Jabal Qamar leopards belonging to cluster 1 was 94%- 99%, while Jabal Qara and the Nejd had a probabilitiy of 94%- 99% belonging to cluster 2. The leopards of Jabal Samhan had a 97%- 100% probability belonging to cluster3.

4.4.3 Spatial patterns of gene flow

Estimates of contemporary rates of migration (Nm) between Dhofar populations were generally low (mean Nm of 0.026 - 0.081; Figure 4.5). Significant geneflow

(Nm>0.1) was detected between Jabal Qara and the Nejd (0.100 and 0.105). The lowest levels of migration were from Jabal Qara to Jabal Qamar (0.026), and from the Nejd to Jabal Qamar (0.028).

4.5 Discussion

Rapid development and huge increases in livestock numbers in the Dhofar mountains are considered to have had negative impacts on the natural habitat of this regional landscape (Miller & Morris, 1988; Ghazanfar, 1998). Given that large carnivores are sensitive to human development and disturbance (Woodroffe, 2000; Smith *et al.,* 2015), these impacts are expected to affect the distribution and connectivity of the Critically Endangered Arabian leopard population across these mountains. We used genetic data from wild leopards obtained via non-invasive scat surveys to investigative if recent growth in the human footprint across the Dhofar region has affected the genetic structure of the leopard population.

Despite the limited sample size, especially from Jabal Qara (5 individuals), and the relatively low number of polymorphic loci, our study detected signs of genetic differentiation and spatial structure between the leopards of Jabal Samhan, Jabal Qara and Jabal Qamar. The differentiation appears most pronounced between the leopards of Jabal Qara and Jabal Qamar where we also detected low levels of contemporary gene flow. Both STRUCTURE and GENELAND analyses cluster the leopards of Jabal Qara and Jabal Qamar as separate clusters. However, GENELAND grouped those of the Nejd with Jabal Qara and identified Jabal Samhan as a third cluster while STRUCTURE grouped Jabal Qara leopards with both the Nejd and also Jabal Samhan. The clustering by STRUCTURE of Jabal Qara and the Nejd with

Jabal Samhan is probably due to the limited sample size from these first two regions (Jabal Qara =5 leopards; Nejd =3 leopards). The analyses by GENELAND incorporates spatial information and is considered more robust in instances where geneflow is low or when there is relatively weak genetic structure (Basto *et al.*, 2016). Thus, the clustering results produced by GENELAND analyses are likely to be most representative of the true genetic structure for the leopard populations across Dhofar.

An interesting finding of this study is the pronounced differences between the leopards of Jabal Qara and Jabal Qamar which are supported by both Bayesian analyses. Jabal Qara and Jabal Qamar are geographically relatively closer to each other and the leopards of these regions would be expected to show lower difference unless there exists some barrier between the two populations. We are unaware of any substantial geographical barrier between Jabal Qara and Jabal Qamar but consider that human disturbance, especially the main Salalah-Sarfait road that runs through Jabal Qamar and also settlements and large numbers of livestock in this area, may restrict leopard movement between these two regions.

Roads are known to limit species movement and cause population subdivision (Lesbarrères *et al.*, 2006; Forman & Deblinger, 2010). For example, Riley *et al.*(2006) found that populations of two carnivore species (bobcats and coyotes) that occur alongside a freeway were genetically differentiated despite moderate levels of gene flow between their respective populations. Human activities have also been found to affect habitat use and behaviour of Asiatic leopard (*Panthera pardus*) in Thailand (Ngoprasert *et al.,* 2007). It is therefore likely that for the Jabal Qamar and Jabal Qara leopard populations, the Salalah-Sarfait road in combination with

settlements and livestock herds have restricted leopard movement and induced the genetic differentiation observed between them. This interpretation is further supported by data from a GPS collared leopard from Jabal Qamar that did not cross the Salalah-Sarfait road while it was tracked (December 2003-February 2004 and June- July 2005) (Spalton & Al Hikmani, 2014).

The Dhofar conflict in 1965-1975 (Hughes, 2009), including the construction of the 50 km 'Hornbeam' defence line in 1973, at the western end of Jabal Qara (Figure 4.S1; Tusa, 1988) might had also played a role in isolating leopard populations. Built of barbed-wire and mines the Hornbeam line was designed to prevent rebels from crossing from Jabal Qamar to Jabal Qara and getting close to the town of Salalah. Together with the wider conflict it may also have restricted the movement of leopards, preventing dispersal and isolating populations resulting in genetic drift and subsequent differentiation. The defence line was dismantled in the late 1970s. Additional surveys with large sample sizes are needed to determine if the observed genetic differentiation is indeed the result of this defence line or because of road construction and human disturbance.

Jabal Qara has the most roads and livestock of Dhofar's mountains and thus leopard populations may be more impacted than elsewhere. However, our study included scats from just five different leopards at the western end of the mountain and thus we were unable to investigate local scale impacts on this heavily used mountain region. Future surveys should focus on this region and investigate if the leopard populations of western and eastern Jabal Qara are still connected or are in fact now isolated. Our F_{ST} results indicate that there are at least some differences between the leopards of western Jabal Qara and Jabal Samhan, and our GENELAND analyses cluster the

leopards of these two mountains into two distinct clusters. These results together with those from Jabal Qamar indicate fragmentation and subdivision of the leopard population in Dhofar.

The only significant connectivity that our study revealed was between the leopards of western Jabal Qara and the Nejd. We found evidence for significant geneflow between these two regions and our GENELAND analyses grouped the leopards of Jabal Qara and the Nejd into one distinct cluster. This result is unsurprising given that the leopards found in the Nejd are thought to disperse from western Jabal Qara. In contrast to the eastern and central parts of Jabal Qara which are separated from the Nejd by 20-30km of monsoon rangeland that is heavily used by people and their livestock, the mountains in western Jabal Qara are very narrow and the distance to the dry north wadis of the Nejd is very small. In these conditions, leopard movement and geneflow between the Nejd and western Jabal Qara is conceivable.

4.6 Conservation implications

Despite the low number of loci that were found to be polymorphic (10 of 18 loci tested [56%] were monomorphic; chapter 3) and low levels of genetic diversity in the Arabian leopard (chapter 3), our marker set was able to detect significant spatial genetic structure within the leopard population of Dhofar. Although we might underestimate the true genetic structure in this landscape due to the small number of polymorphic loci, our results provide important insight into how habitat fragmentation, restricted geneflow and isolation has led to genetic structure and differentiation among these Critically Endangered leopards.

Our study finds evidence for population subdivision and restriction of gene flow between the leopards of western, central and eastern Dhofar. Given the threat status of the Arabian leopard, we recommend urgent identification and protection of potential corridors to enable dispersal and therefore gene flow between the leopard populations in the Dhofar mountains. For example, conservation authorities should consider implementing conservation measures to ensure the continuity of leopard movement between western Jabal Qara and the Nejd. Although based on limited sample size the leopard populations in these two regions are shown to have significant gene flow between them and also exhibit relatively higher genetic diversity in comparison to other regions in Dhofar; i.e. Jabal Samhan, Jabal Qamar (chapter 3). We also suggest consideration is given to creating and establishing wildlife crossings such as underpasses or overpasses along the main roads in Jabal Qara. Wildlife crossing structures have been shown to enable dispersal among several taxa including bear species (Sawaya *et al.,* 2014), wolves (Shepherd & Whittington, 2006) and cougar *Puma concolor* (Gloyne & Clevenger, 2001). The creation and protection of habitat connectivity through such corridors could conceivably promote dispersal and geneflow across existing barriers to leopard dispersal and this will help to reduce inbreeding and subsequent inbreeding depression, preserve genetic diversity and subsequently maximise population persistence. Such initiatives will also be beneficial to other terrestrial species of the region that live alongside the Arabian leopard.

4.7 Tables

Table 4.1. Summary of scat samples collected, screened and genotyped from each of the sample regions in Dhofar, Oman between 12th January 2012 and 6th April 2017.

Regions	Samples collected from the field	Samples genetically verified to be leopard	Samples genotyped for at least 5 loci	No. of individual leopards
Jabal Samhan	191	113	70	17
Jabal Qara	95	11	9	5
Jabal Qamar	161	32	26	11
Nejd	30	5	4	3
Overall	477	161	109	36

Table 4.2. Results of Mantel IBD tests in the Arabian leopard for the whole data set and between regions based on genetic and geographic pairwise distance. Bold numbers indicate evidence for IBD.

	Dhofar	**Samhan vs Qara**	**Samhan vs Qamar**	**Qara vs Qamar**
Mantel correlation coefficient	0.124 (**0.002**)	0.173 (0.075)	0.203 (**0.002**)	0.045 (0.307)

4.8 Figures

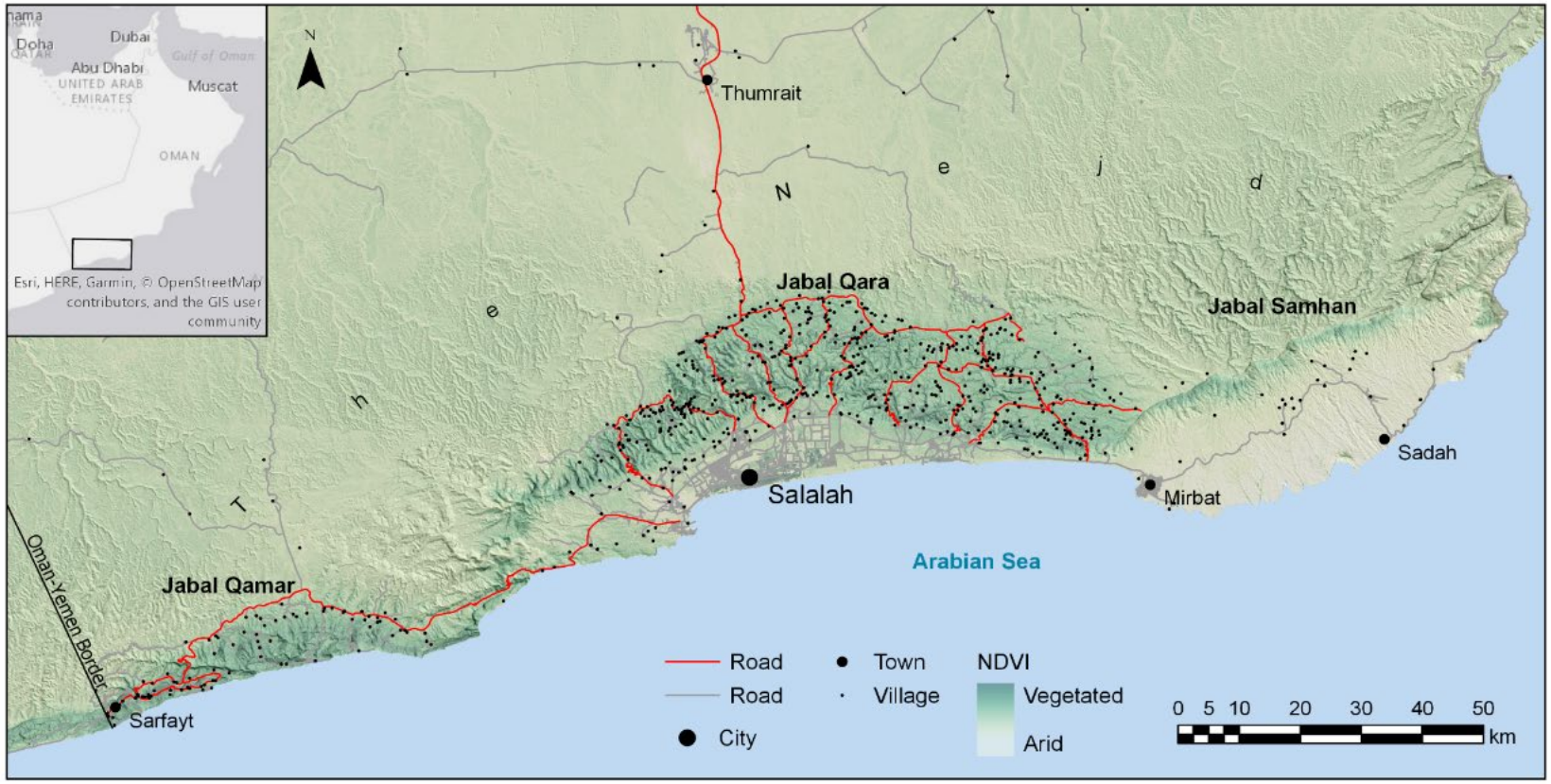

Figure 4.1. The location of the study regions in Dhofar. Roads in red indicate potential barriers to leopard movement. The inset map shows the position of Dhofar within Oman.

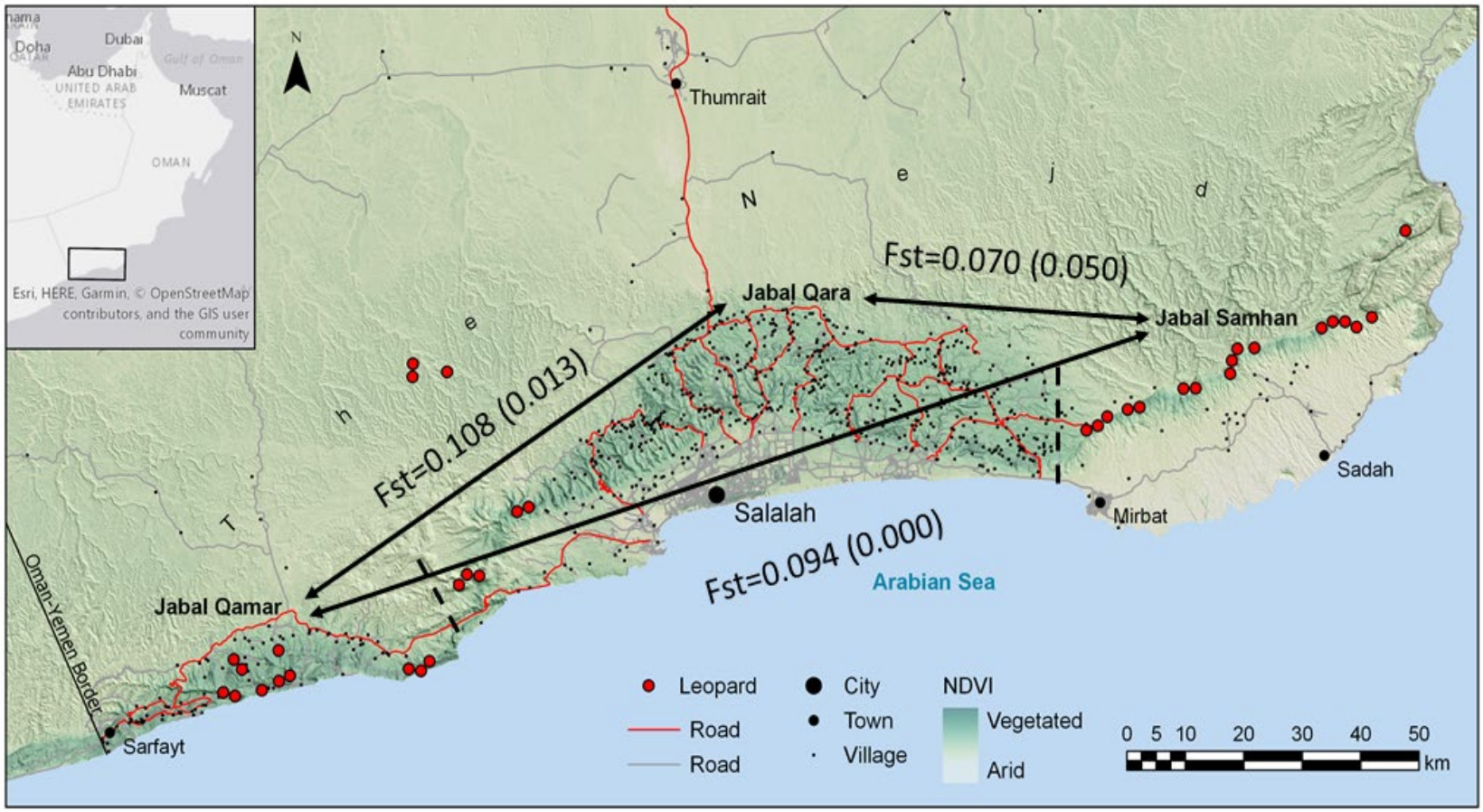

Figure 4.2. Estimates of genetic differentiation (FST) between the main populations of Arabian leopards of Dhofar. Numbers in brackets indicate P values. Dash lines delineate between the geographically distinct regions.

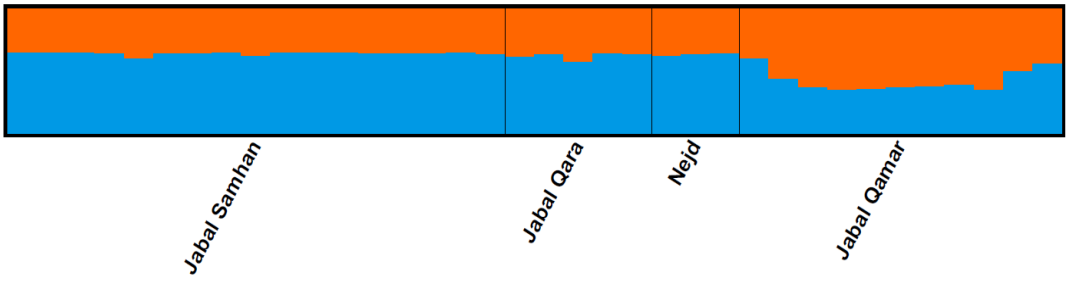

Figure 4.3. Genetic structure of leopard population from STRUCTURE at K = 2 using Puechmaille (2016) method for 36 individuals typed at 7 microsatellite loci.

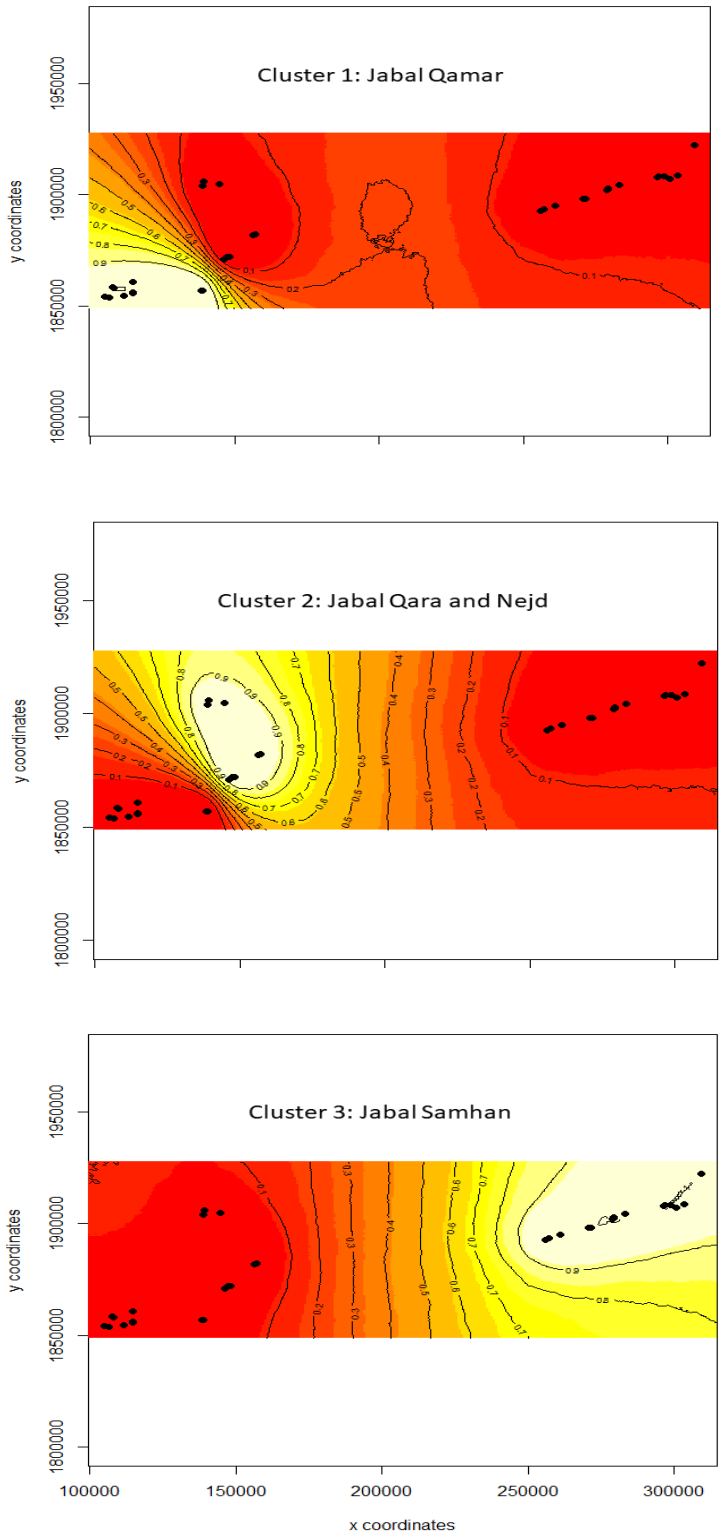

Figure 4.4. Maps of GENELAND individual assignments for 36 individuals typed at 7 microsatellite loci. Membership values are in yellow and the level curves illustrate the spatial changes in assignment values.

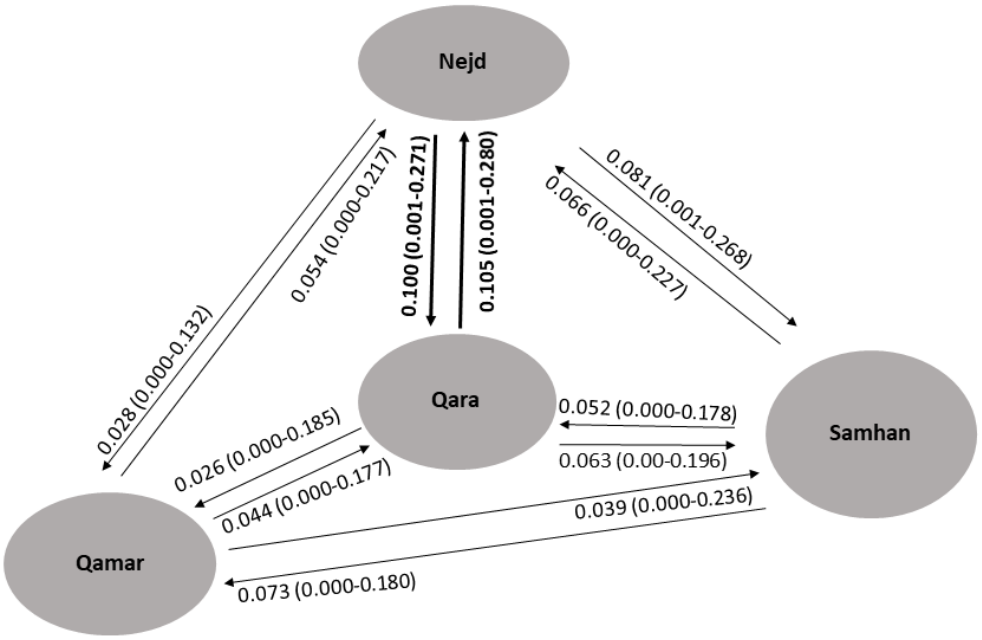

Figure 4.5. Patterns of contemporary gene flow in the wild population of Arabian leopards across the Dhofar region based on migration rates estimated in BayesAss (Wilson & Rannala, 2003). Arrows indicate the direction of migration and numbers above rows indicate migrations rates. Numbers in brackets indicate 95% confidence intervals. Bold numbers indicate significant gene flow.

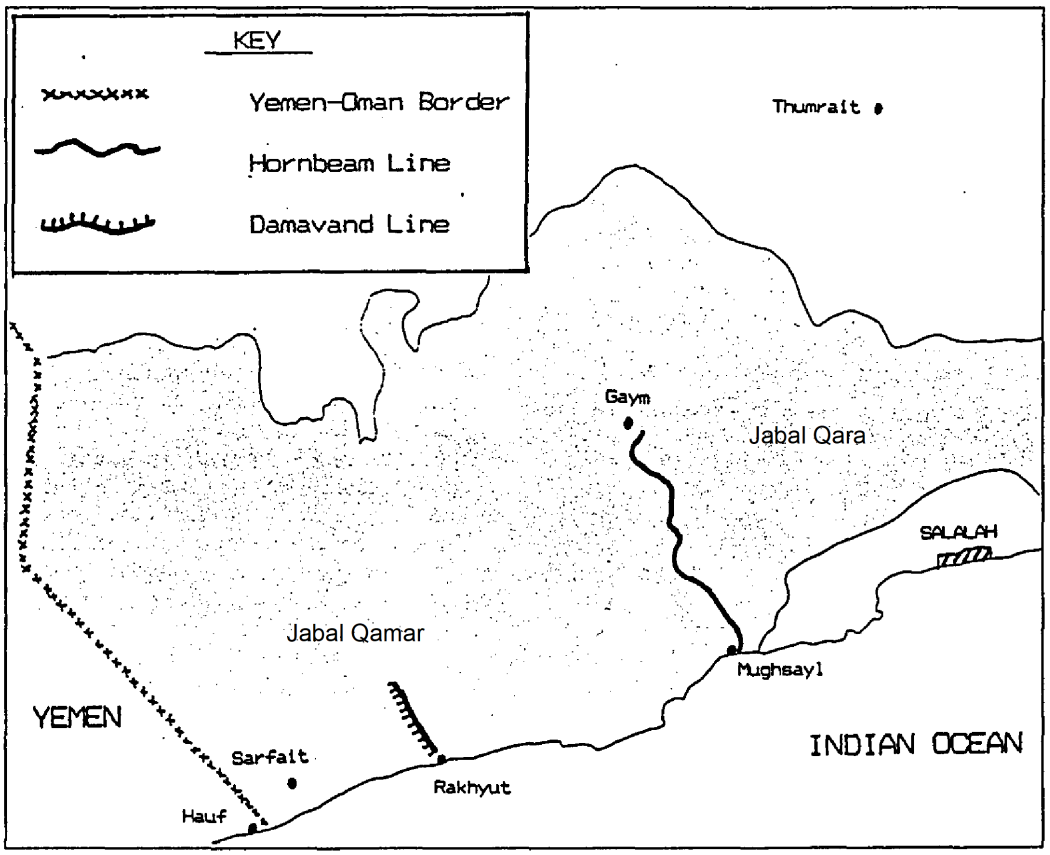

Figure 4.S1. The position of the Hornbeam defence line in Jabal Qara, extracted from Tuas (1988).

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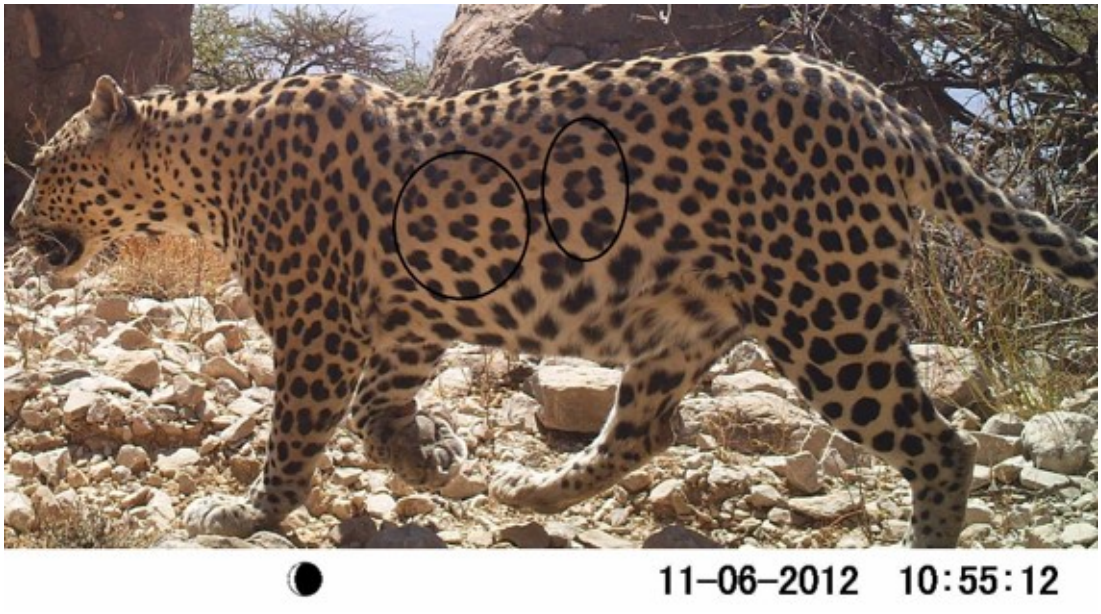

Example of individually identifiable leopards based on their spot patterns. Top and bottom photos show the same individual male leopard that recoded in different location in Jabal Samhan at diffrenet date, almost one year later.

5. Applying a spatially explicit capture recapture approach to DNA surveys to estimate population size and density for the Critically Endangered Arabian leopard

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5.1 Abstract

Population size and density estimates are crucial parameters for conservation managers tasked with recovering and managing endangered carnivores. The Critically Endangered Arabian leopard of Oman's Dhofar mountains is an example where a detailed understanding of the population is lacking but is needed in order to develop effective conservation management. In this study we use scat genetic sampling within a spatial explicit capture recapture (SECR) approach to estimate population size and density of the Arabian leopard of Dhofar. Given the difficulties associated with individual identification from non-invasive scat sampling we also use camera trapping to authenticate the use of genetic sampling for monitoring of the Arabian leopard. Our study show that non-invasive DNA sampling can provide estimates of density that are comparable with estimates derived from camera traps. Using data from DNA sampling we estimate an overall density of 2.30 ±0.53 S.E leopard/100 $km^{2.}$ and a population estimate of 51 leopards (95% CI: 32-79) in the Dhofar mountains. This study demonstrates the reliability of non-invasive DNA surveys to provide robust estimates of key population parameters even in genetically impoverished species such as the Arabian leopard, and this method can be used for future monitoring of the population alongside the existing monitoring techniques such as camera trapping.

Keywords: Population density, genetic sampling, camera trapping, Arabian leopard, Dhofar mountains.

5.2 Introduction

Monitoring of large carnivores that are elusive and exist at low densities present a challenge for conservation managers (Farhadinia *et al.*, 2018). Given their behaviour and frequently large home ranges, such species are notoriously difficult to locate and study directly. Alongside this, large carnivores play a vital role in their ecosystems as a top predator (Miller *et al.*, 2001) and are often considered flagship species (Caro & O'Doherty, 1999). Obtaining robust estimates of population size and density for these ecologically important species remains crucially important for those managers tasked with recovering and managing such populations.

The need to produce reliable estimates of population size and density for carnivore populations becomes increasingly important when they negatively impact on human use of landscapes. For example, in cases where human-wildlife conflicts exist, such as livestock predation, there is a need for wildlife managers to understand when and where changes in carnivore density occur so that effective policies can be put in place to mitigate the conflict (Treves & Karanth, 2003). Conservation interventions to manage human-wildlife conflict aim to allow the carnivore population to recover from persecution. However, without robust methods that are capable of producing reliable estimates of density and population size of such elusive species, this cannot be effectively measured.

The Arabian leopard (*Panthera pardus nimr*) is Critically Endangered, with a global population estimated to be fewer than 250 individuals (Breitenmoser *et al.,* 2006), of which the largest wild population exists in the Dhofar mountains of Oman (Spalton & Al Hikmani, 2014). The elusive nature of Arabian leopards and their distribution

across these vast and mountainous landscapes mean that reliable estimates of density and population size are an essential prerequisite to guide appropriate actions to protect and recover the population. The Arabian leopard is threatened by a combination of anthropogenic factors including a reduction of prime habitat due to human development and urbanization, loss of prey species as a result of illegal hunting, and subsequent human persecution of leopards in response to depredation of livestock (Al Jumaily *et al.*, 2006; Spalton *et al.*, 2006a; Zafar-ul Islam *et al.*, 2018). In Oman, some of these threats are being addressed through the establishment of protected areas, formation of wildlife ranger units, and a program to compensate local communities for livestock depredation; the main cause of leopard persecution in the Dhofar mountains (Spalton & Al Hikmani, 2006). To make informed decisions concerning priority areas for protection, daily programs for ranger patrols, and when and where to implement compensation programs for livestock loss, conservation managers require a solid understanding of leopard population size and density. Subsequent measurement of the efficacy of such interventions are also of considerable importance, and are reliant on the quality and informativeness of the underlying data.

The deployment of camera traps and collection of scat samples for genetic analysis have become widely used, non-invasive methods for estimating density and population size of wild animal populations. These approaches have been applied to a number of elusive, threatened species including tigers *Panthera tigris* (Kalle *et al.*, 2011; Aziz *et al.*, 2017), jaguars *Panthera onca* (Sollmann *et al.*, 2013) and leopards *Panthera spp*. (Kalle *et al.*, 2011; Vitkalova *et al.*, 2018). Traditional approaches have used capture recapture models that assume a closed population to derive

estimates of density. These approaches used *ad hoc* methods to calculate effective sampling area (ESA) such as mean distances travelled by animals captured in multiple camera traps (Karanth & Nichols, 1998; Trolle *et al.*, 2007). This approach has been criticized when estimating ESA (Borchers & Efford, 2008). Recently Spatial Explicit Capture-Recapture (SECR) modelling has provided the opportunity to compute more robust estimates (Borchers & Efford, 2008) and use state and observational models to estimate ESA and subsequently density. Whilst SECR methodology is considered more reliable it requires multiple recaptures to deliver impartial estimates. This requirement can be a challenging task for field teams monitoring elusive and widely distributed species with large home ranges, such as the Arabian leopard. However, multiple methods such as camera trapping and genetic sampling can be used in combination to increase detection probability for more robust estimates of density, and to authenticate density estimates derived from single methods.

In this study we use genetic sampling and camera trapping methods to estimate density in the Critically Endangered Arabian Leopard. Given the difficulties associated with individual identification from non-invasive scat sampling we use camera trapping to authenticate the use of genetic sampling for monitoring of the Arabian leopard. We use i) SECR to model capture-recapture data for the Arabian leopard from molecular scatology and ii) camera-trap surveys in the Dhofar mountains of southern Oman. We produce estimates of leopard density derived separately from camera-trap and DNA data sets and use this information to derive an estimate of the size of the Arabian leopard population. Finally, we compare our estimate of the current number of leopards to previous estimates of population size

and interpret this information in light of current conservation management actions to restore the leopard population in the Dhofar mountains.

5.3 Methods

5.3.1 Study species and site

The Dhofar mountains, located in southern Oman, comprise a chain of three contiguous mountain massifs that span approximately 250 km (Figure 5.1), and are considered the last stronghold for the Arabian leopard (Breitenmoser *et al.*, 2010). Two of these mountains, Jabal Qamar and Jabal Qara, are strongly influenced by the annual monsoon (mid-June to mid-September), and a unique cloud forest, rich in endemic flora (Patzelt, 2015), is found on their plateaux and south-facing aspects. The third, Jabal Samhan, has the highest elevation (1,760 meters above sea level [a.s.l]) but is mostly hyper-arid with sparse desert vegetation, being largely unaffected by the annual monsoon. The northern foothills of these mountains are known as the Nejd, and although leopards have been observed in this region (Al Hikmani *et al.,* 2015), it is considered to be on the periphery of core leopard habitat (Mazzolli, 2009).

The Dhofar mountains support a diverse mammal fauna in addition to the leopard, including other carnivores such as wolf (*Canis lupus*), hyena (*Hyaena hyaena*), caracal (*Caracal caracal*) and wildcat (*Felis silvestris*) (Spalton & Al Hikmani, 2014). The principle prey species of leopard include Nubian ibex (*Capra nubiana*), Arabian gazelle (*Gazella arabica*), rock hyrax (*Procavia capensis*), porcupine (*Hystrix indica*) and small rodents such as the Arabian spiny mouse (*Acomys dimidiatus*). However, ibex and gazelle are not found on the woody slopes and

plateau grasslands of Jabal Qara and Qamar, and leopards may rely more heavily on small mammals, birds and reptiles in these areas.

The mountain people of Dhofar have settlements scattered throughout Jabal Qamar, Jabal Qara, and along the foothills of Jabal Samhan. Most own livestock (camels, cattle, goats), the numbers of which have increased dramatically over the last 40 or so years, and present an alternative food source for the leopard, especially where principle prey species are absent or few in number. Livestock owners are intolerant to losses, leading to the persecution and killing of leopards. However, it is anticipated that recent conservation interventions may change retaliatory behaviour, permitting leopard populations to recover.

5.3.2 Field methods

We selected four sampling areas (Figure 5.2a-c) in which to survey for leopard scats; three in the main mountains and one in the Nejd region to the north of the mountains. To maximize our sample size (leopard 'captures') we conducted surveys along predefined search routes in each sampling area (Figure 5.2a-c). Routes were chosen to optimize detection based on information from previous camera trap and scat surveys. Surveys were conducted between 5th January and 13th April 2017. In total we surveyed 43 routes (88 km) in Jabal Samhan, 26 routes in Jabal Qamar (75 km), 16 routes in Jabal Qara (46 km), and 17 routes in the Nejd (49 km). Each mountain route was surveyed between two and four times, with routes in the Nejd surveyed twice. Surveys were carried out by field teams of the Office for Conservation of the Environment (OCE), Oman. Collected scats were stored in plastic Ziplock bags at

ambient temperature, and each labelled with the date of collection and GPS reference.

In our Jabal Samhan study area, on the routes surveyed for scats, we set up 42 camera trap (Bushnell 119456 model) stations. At each station a single camera was placed on either side of the route to photograph both the left and right flanks of a leopard for individual identification. Stations were spaced approximately two kilometres apart to allow individual leopards to pass by more than one station and thus maximize the probability of detecting them. Cameras operated on a 24-hour basis and were programmed to take three photographs (with a one second delay between pictures) when triggered. Rangers visited stations every two weeks to check cameras and replace SD cards and batteries.

Due to logistical and time constraints we were unable to deploy camera traps simultaneously with scat surveys in Jabal Qara and Jabal Qamar. No camera trap information were obtained from the Nejd. Instead we used data from the same sampling regions in Jabal Qara (13 camera trap stations, Bushnell 119435 model) and Jabal Qamar (20 camera stations, Bushnell 119435 model) that were collected between 20th September and 15th December 2013, and 10th April and 20th July 2014 (Figure 2b, c). However, neither of these two localities produced enough captures for independent density estimates (a minimum of 10 recaptures is needed for application of SECR). However, we did use this data to determine the minimum number of leopards in each region and to assist our interpretation of leopard identification from scat surveys. The deployment of camera-traps in Jabal Qamar was identical to those deployed in Jabal Samhan. Camera-traps in Jabal Qara were originally deployed to

determine leopard presence and absence and were more distantly spaced (3-10 km apart).

5.3.3 Individual leopard identification

Procedures for individual leopard identification from scats are described in full in chapter 3. We used a set of seven polymorphic microsatellite loci to identify individual leopards from scats samples.

Individual leopards were identified by eye from photographs based on unique rosette patterns on the flanks such as the shape, size, and formation of the spots plus other distinctive markings (Spalton *et al.*, 2006b). Genitalia were used to determine sex and reference photographs from previous surveys were used to aid identification. Blurred photographs that did not show enough of an individual were not utilized for idenfication purposes. Identifciation checking was done by the main researcher. Each individual was given a unique identifition number.

5.3.4 Density estimation

We used a maximum likelihood SECR approach (Borchers & Efford, 2008), implemented in R package SECR v3.1.3 to estimate leopard density. Two input files per data set were provided to run the analysis, comprising a capture history of individual leopards and their spatial detections. For the camera trap dataset, the spatial locations were the coordinates of camera traps, while for the scat dataset the survey areas were divided into a grid of 1 km x 1 km cells and the center point of these cells were used as fixed traps. Camera trap data were grouped into eight

occasions (each occasion consisting of 8 days) while scat data were assigned to two (Jabal Qamar) and three occasions (Jabal Samhan).

For SERC analysis we assumed a Poisson distribution of leopards and used the half-normal detection model while detectors type was specified as proximity. We used the buffer function in SECR to infer the appropriate buffer for our datasets. As the inferred buffers included large areas of non-leopard habitat (open desert water surfaces, settlements) we used ArcGIS v10.5 to create a map of potential leopard habitat based on leopard distribution (Figure 5.S1) from Spalton and Al Hikmani (2014). Including non-habitat for density estimates is considered to bias results (Efford, 2011) therefore excluding non-habitat is deemed more appropriate as it is likely to be a closer fit to reality. We ran the analysis based on maps of potential habitat using constant detection probability (lambda0~1) and spatial movement models (sigma~1), and also individual heterogeneity (2–class finite mixture: lambda0~h2 sigma~1, lambda0~1 sigma~h2, lambda0~h2 sigma~h2; see Efford, 2019 for details and descriptions of used models) models for each data set. Sex was included as a covariate in all models. Models were ranked based on the Akaike information criterion (AIC) using AICwt and delta AICc corrected for small sample sizes (Burnham & Anderson, 2002). Models with delta ≤ 2 were considered to have more support. As no single model had a weight (AICwt) of 0.9 (see table 5.S1) final density was computed using the model averaging function in SECR.

We only estimated density for Jabal Samhan and Jabal Qamar due to low DNA captures of leopards in the other regions. The overall density derived from the scat dataset was then extrapolated to estimate leopard population size for the whole of the Dhofar mountains where suitable habitat exists. We estimated leopard core habitat in

Dhofar to be 2,213 km^2 (Figure 5.S1) and then extrapolated the density estimate derived for Jabal Samhan and Jabal Qamar to produce a figure for the full extent of habitat. Although the leopard was recorded recently in northern central wadis of the Nejd, we considered this region as an outpost of margin habitat and excluded it from our overall extrapolation to avoid an overestimation of population size.

5.4 Results

5.4.1 Genetic analyses

A total of 270 scats were collected from across the different mountains of Dhofar including the Nejd region to the north. Analysis of mitochondrial DNA confirmed that 106 (39%) of the samples were leopard. The remaining 164 (61%) are likely to have been from other carnivores such as caracal, lynx, and Arabian wolf, and were discarded from further analysis. Extracted genomic DNA from a total of 69 leopard scats (65%) amplified for more than four loci and these genotypes were then used to identify the number of individual leopards represented within the scat samples. Using these genotype profiles, we identified a total of 26 individually identifiable leopards, comprising 13 from Jabal Samhan, 4 from Jabal Qara, 7 from Jabal Qamar and 2 from the Nejd (Table 5.1). These 26 leopards comprised 14 males and 12 females.

5.4.2 Analysis of camera trap data

Camera traps in Jabal Samhan accumulated 2,665 trap days and recorded leopards at 21 out of 41 stations. A total of 397 photographs captured the presence of leopards (Table 5.1), of which 306 (77%) were suitable for individual identification. From these images we identified 11 individual leopards (7 male, 4 female). The remaining

photographs (23%) were taken at night when light levels rendered them unusable for purposes of individual identification.

A total of 60 photographs of leopards were obtained from Jabal Qara, and 85 from Jabal Qamar cameras (Table 5.1). From these we identified 5 individuals (3 male and 2 female) in Jabal Qara, and 6 individuals (3 male and 3 female) in Jabal Qamar. Leopards were recorded in 5 of the 13 camera trap stations in Jabal Qara, and in 12 of the 20 camera trap stations in Jabal Qamar.

5.4.3 Density estimation

SECR provided support for more than one model when data were analysised independently per region. However, when all regions genetic data set were combined to estimate overall density, the constant detection probability and spatial movement model had strong support (Table 5.S1). Based on the models ranked highest using AIC (Table 5.S1), analysis of the genetic data sets using SECR yielded an overall leopard density estimate of 2.30 ±0.53 S.E leopard/100 km^2 (Table 5.2), with the highest density of leopards estimated in Jabal Qamar (3.60 ±1.57 S.E).

The camera trap data produced a slightly higher estimate of leopards in Jabal Samhan (2.65±1.07 S.E leopard/100km2) compared to the estimate for that region using the genetic data (2.03 ±0.58 S.E).

Baseline detection probability for camera trap surveys was higher for females (0.052 ±0.019 S.E) than males (0.011 ±0.006 S.E) in Jabal Samhan. However, both sexes showed similar detection probability using the genetic analysis (Table 5.2). In Jabal Qamar, males (0.097 ±0.064) had a slightly higher detection probability than females

(0.077 ±0.049). There was little variation between males and females in terms of their spatial movement within populations, but signification variation was observed between different populations. Higher spatial movement estimates were recorded in the Jabal Samhan population (7.56-8.64 km) in comparison to the Jabal Qamar population (2.85-2.99 km). Based on an overall genetic density estimate of 2.30 ±0.53 S.E leopard/100 km^2, we estimated the Dhofar mountains to support approximately 51 leopards (95% CI: 32-79).

5.5 Discussion

Conservation management of endangered species requires reliable information in order to effectively conserve them and prevent their extinction (Borah *et al.*, 2014). The Critically Endangered Arabian leopard of Oman's Dhofar mountains is an example where a detailed understanding of the population is lacking but is needed in order to design effective conservation management. We used two non-invasive survey techniques (i.e. camera traps and genetic sampling from scats), and applied a spatially explicit capture recapture approach to provide the first robust estimates of population density and overall population size for the region.

5.5.1 Evaluation of leopard survey techniques

Our findings from Jabal Samhan show that non-invasive DNA sampling can provide estimates of density that are comparable with estimates derived from camera trap data. We find that both survey techniques can identify a similar number of individual leopards in each mountain region (Table 5.1). However, camera traps overestimate density in Jabal Samhan in comparison to genetic sampling despite both techniques recording similar number of leopards (e.g. camera traps=11; genetic sampling=13).

Yet, the estimate from genetic sampling in this region has higher precision as the coefficient of variation (CV; SE/density) was lower (28%) in comparison to that from camera traps (40%). The variation in density estimates between the two methods might have been due to the lower number of leopard detections/captures obtained from camera traps (e.g. camera trap=27 detections, genetic sampling=46 detections; Figure 5.S2). Our scat surveys are likely to have covered more ground in terms of their ability to 'capture' leopard presence beyond the fixed locations of camera traps, and in doing so it is likely that the scat sampling approach obtained more data than camera traps which only record passing animals. Therefore, our density estimates from the genetic sampling are probably more representative of the true density of leopards in Jabal Samhan.

5.5.2 Patterns of spatial movement

Likewise, the density estimates, the genetic sampling provided more precise estimates of detection probability and spatial movement than the camera trapping method in Jabal Samhan. Yet, both techniques show that male and female leopards have similar spatial movement patterns in Jabal Samhan, and scatology found this to also hold true for Jabal Qamar. However, the leopards of Jabal Samhan exhibit larger spatial movement patterns than the leopards of Jabal Qamar, indicating interpopulation variation. Home range and movement patterns are often larger for males than females in most territorial carnivores but Marker & Dickman (2005) did not find significant difference in range size between male and female leopards in Namibian farmlands. The only estimate of home range for the Arabian leopard is derived from a study in which GPS collars were fitted to two individuals, a male from Jabal Samhan and a female from Jabal Qamar (Spalton & Al Hikmani, 2014).

This study estimated home range to be 168 km^2 for male leopards and 64 km^2 for female leopards, with average daily movement to be 8.5 km and 3 km respectively. Our study did not find evidence for difference in spatial movement between male and female leopards, but we did detect differences between populations, with high levels of spatial movement within Jabal Samhan. If this interpopulation variation is a true reflection of differences in spatial movement then it may be explained by differences in habitat. Jabal Samhan comprises semi- to hyper-arid habitat and leopards of this region may need to travel large distances to find food and water in comparison to Jabal Qamar. Jabal Qamar is located in the monsoon zone, and though both Nubian ibex and Arabian gazelle are absent, the greater primary productivity of the monsoon forests is likely to support greater numbers of small prey.

5.5.3 Density and population size estimation

Our study shows that Jabal Qamar harbours a higher leopard density than Jabal Samhan. The density of any large carnivore is considered to be associated with the density of its preferred prey species (Karanth *et al.*, 2004; Hayward *et al.,* 2007), and this relationship has been found to be associated with rainfall and vegetation productivity (East, 1984). Although no large ungulates are found in the woody slopes and plateau grasslands of Jabal Qamar (Spalton & Al Hikmani, 2014), a number of small prey species such as rock hyrax, porcupine and small rodents are found within this region (Spalton & Al Hikmani, 2014) and though we do not know the density of these prey species, they are likely to be abundant (pers. obs). In addition, livestock including camels and cattle, are abundant in Jabal Qamar and are known to provide an alternative food source for the leopard; several cases of leopard livestock depredation have been confirmed by camera traps in this locality. A diet of

plentiful small prey species supplemented with livestock may allow for a higher leopard density in Jabal Qamar than in the elevated arid region of Jabal Samhan.

The overall density estimate of 2.30 leopards/ 100 km^2 for the Dhofar mountains is low when compared to other leopard species and among the lowest density estimates reported for *Panthera pardus* in Asia. Vitkalova *et al.* (2018) reported a density estimate of 1.4 leopards/100 km^2 for the Critically Endangered Amur leopard *Panthera pardus orientalis* in Russia and China, while Thapa *et al.* (2014) reported estimates of 3.78 leopards/100 km^2 for the Indian leopard *Panthera pardus fusca* in Nepal. Both the Arabian and Amur leopards are considered to exist at small population sizes and their low-density estimates are likely to be indications of their low numbers (Jacobson *et al.*, 2016).

The Arabian leopard is believed to have been more widespread in the past, occurring throughout the Dhofar mountains including the Nejd region to the north (Spalton & Al Hikmani, 2014). Today leopards are mostly confined to the southern part of Jabal Samhan and the escarpment and south-facing slopes of Jabal Qara and Jabal Qamar (Spalton & Al Hikmani, 2014). They were recently observed to have returned to the Nejd region (Al Hikmani *et al.,* 2015) but this area is unlikely to be part of their core habitat (Mazzolli, 2009). The first camera trap surveys of the Dhofar population between 1997-2000 recorded the presence of 17 individual leopards in Jabal Samhan and between 9-11 leopards in the western Dhofar mountains of Jabal Qara and Jabal Qamar between 2001-2004 (Spalton *et al.*, 2006a, b). Using these camera trap records with data from two individual GPS collared leopards, the Dhofar population was estimated at 44-58 leopards (Spalton & Al Hikmani, 2014). Although this previous study used different methodology to estimate population size, our estimate

of 51 leopards (95% CI: 32-79) in the Dhofar mountains aligns closely with the previous estimate of 44-58 leopards in Dhofar which was based on camera trap data from 1997-2000 and two months of GPS collar data from 2002, 2004 and 2005. Similarly, the 26 leopards we identified from scats are comparable to the number reported by Spalton *et al.* (2006a, b) in the same region (Jabal Samhan:17; Jabal Qara-Qamar: 9-11). If these results are a true reflection of population size, they suggest that instead of population decline, Dhofar leopards may have remained relatively stable for at least the last two decades. Given that there is evidence of increasing anthropogenic pressure on leopards and their habitat (e.g. general development, overgrazing, and direct persecution) it is all the more remarkable that the population has remained stable. It is tempting to conclude that this might be the result of conservation efforts; Jabal Samhan Nature Reserve was established in 1997, the Arabian Leopard Survey began in 1997 and since that time there has been increasing attention given to the leopard that has included a program for compensation for livestock loss. Hopefully conservation has made a difference, but the population remains extremely small and fragmented and thus highly vulnerable.

Our estimate of 51 leopards is also somewhat surprising given the expectations of small population biology which would suggest that the population is at high risk of extinction (Soulé & Wilcox, 1980). A census population size of 51 is likely to have an effective population size (N_e) that is smaller than the N_e of 50 individuals that has been proposed as the minimum size to avoid the deleterious effects of inbreeding depression in domestic animals (Soulé & Wilcox, 1980). Our estimate of 51 is also smaller than N_e of at least 100 that has been suggested for wild populations in stressful environments (such as in degraded or suboptimal habitats) which can be

expected to exhibit more pronounced effects of inbreeding depression compared to domestic or captive populations (Frankham *et al.,* 2014). Inbreeding depression can cause reduced levels of productivity, reduced survival and reduced lifetime reproductive success and ultimately extinction. The small size of Dhofar's Arabian leopard population may mean that it is highly susceptible to the negative effects of inbreeding depression. Against this backdrop therefore, conservation efforts must be directed as a priority to recover the population of this unique leopard of Arabia. In the meantime, the Arabian leopard must remain listed as Critically Endangered as the estimated global population is still less than 250 leopards (Breitenmoser *et al.*, 2010), none of the remaining known subpopulations contain more than 50 individuals (Zafar-ul Islam *et al.*, 2018; this study), and there is continuous decline in other regions outside Oman (e.g. Yemen and Saudi Arabia) (Al Jumaily *et al.*, 2006; Judas *et al.*, 2006).

5.6 Conservation implications

Genetic monitoring of low density, elusive large carnivore species can yield highly valuable results if the challenges associated with non-invasive DNA studies can be overcome. Scats generally produce low quality DNA compared to blood or tissue samples and comparatively low yield, meaning that DNA extractions from such samples are susceptible to problems of PCR amplification such as dropout and false alleles (Taberlet, 1996; Waits *et al.,* 2001). Consequently, the use of scat DNA to identify individual leopards is not always straightforward. Furthermore, identification of individuals becomes even more challenging when the concerned species has low levels of genetic diversity as is the case for the Arabian leopard. This additional challenge is because identification of individuals within a genetically

impoverished population requires a suite of microsatellite loci with sufficient power (number of alleles) to distinguish between individuals and siblings (Waits *et al.,* 2001). Despite these difficulties, our suite of seven polymorphic loci were shown to be able to identify individual Arabian leopards from scat samples. This result was supported by the camera trap data which recorded similar numbers of individual leopards in each of the three mountain regions surveyed. Although additional polymorphic loci can increase the power of identification, the current set of loci presented in our study can be used for future genetic monitoring of the Dhofar population alongside the existing monitoring techniques such as camera trapping. Genetic monitoring can provide additional information about the population that cannot be obtained via camera trapping such as levels of genetic diversity, and extent of population structure and gene flow. Additionally, the density and population size estimates provided by this study provide crucially important baseline information for future monitoring of the status of the Dhofar leopards, and to assess to what extent current longstanding conservation interventions such as the establishment of protected areas, deployment of wildlife ranger units and compensation for livestock depredation are assisting the population to recover. It is hoped that future conservation will continue to make a positive difference to the species recovery, but the population remains extremely small and fragmented and thus highly vulnerable.

5.7 Tables

Table 5.1. Summary of Arabian leopard photographs/scat samples obtained from each of the sampling regions in Dhofar. Numbers in parentheses indicate the number of leopard photographs/scat samples that were used for individual identification.

Region	Method	Sampling duration (days)	No. of leopard photographs/scats	No. of individuals detected
Jabal Samhan	Camera traps	65 days	397 photographs (305)	11
	Scat sampling	35 days	76 scats (46)	13
Jabal Qara	Camera traps	84 days	60 photographs (42)	5
	Scat sampling	25 days	9 scats (8)	4
Jabal Qamar	Camera trap	100 days	85 photographs (74)	6
	Scat sampling	28 days	18 scats (13)	7
Nejd	Scat sampling	24 days	3 scats (2)	2

Table 5.2. Arabian leopard density parameters estimates with spatially explicit capture-recapture (SECR) based on top ranked models.

Area-Method	Sex	No. of individual detected	Effective sampling area (km^2)	Density per 100 km^2 / (S.E)	Probability of detection / (S. E)	Spatial distance moved / (S. E)
Jabal Samhan — Camera	F	4	733	2.65 (1.07)	0.052 (0.019)	7.56 (1.65)
	M	7			0.011 (0.006)	7.61 (1.80)
Jabal Samhan — Scat	F	5	733	2.03 (0.58)	0.059 (0.015)	8.64 (1.46)
	M	8			0.056 (0.013)	8.56 (1.42)
Jabal Qamar — Scat	F	3	298	3.60 (1.57)	0.077 (0.049)	2.85 (0.83)
	M	4			0.097 (0.064)	2.99 (0.91)
Overall based on scat dataset (Jabal Samhan & Jabal Qamar)		20	1031	2.30 (0.53)	0.050 (0.010)	7.98 (1.14)

Table 5.S1. Model selection results from Arabian leopard density estimates using photographic and genetic capture-recapture data from Dhofar in the program SECR using half normal detection function. lambda0 is the capture probability at home range center. Sigma is the spatial scale parameter of capture function. *h*2 is the 2-class finite mixture probability for heterogeneity. dAIC is Akaike's information criterion adjusted for small sample size. AICwt represents Akaike weight. Bold indicates the model that fit the data and has good support. When there is support for more than one model, densitiy is estimated using the model averaging function in SECR (see text for details).

Region	Model	No parameters	dAIC	AICwt
Samhan_camera2017				
Model_Samhan_camera_2	**lambda0~h2 sigma~1**	**5**	**0**	**0.5086**
Model_Samhan_camera_3	**lambda0~h2 sigma~h2**	**6**	**0.316**	**0.4342**
Model_Samhan_camera_0	lambda0~1 sigma~1	4	7.241	0.0136
Model_Samhan_camera_1	lambda0~1 sigma~h2	5	8.204	0.0084
Samhan _Scat2017				
Model_Samhan_scat_0	**lambda0~1 sigma~1**	**4**	**0**	**0.5084**
Model_Samhan_scat_2	**lambda0~h2 sigma~1**	**5**	**1.682**	**0.2193**
Model_Samhan_scat_1	**lambda0~1 sigma~h2**	**5**	**1.961**	**0.1907**
Model_Samhan_scat_3	lambda0~h2 sigma~h2	6	3.658	0.0816
Qamar_scat2017				
Model_Qamar_scat_0	**lambda0~1 sigma~1**	**4**	**0**	**0.4349**
Model_Qamar_scat_2	**lambda0~h2 sigma~1**	**5**	**0.934**	**0.2726**
Model_Qamar_scat_1	**lambda0~1 sigma~h2**	**5**	**1.65**	**0.1906**
Model_Qamar_scat_3	lambda0~h2 sigma~h2	6	2.901	0.102
Overall scats (Samhan and Qamar)				
Model_scat_Dhofar_0	**lambda0~1 sigma~1**	**4**	**0**	**0.7354**
Model_scat_Dhofar_2	lambda0~h2 sigma~1	5	3.502	0.1277
Model_scat_Dhofar_1	lambda0~1 sigma~h2	5	3.609	0.121
Model_scat_Dhofar_3	lambda0~h2 sigma~h2	6	7.659	0.016

5.8 Figures

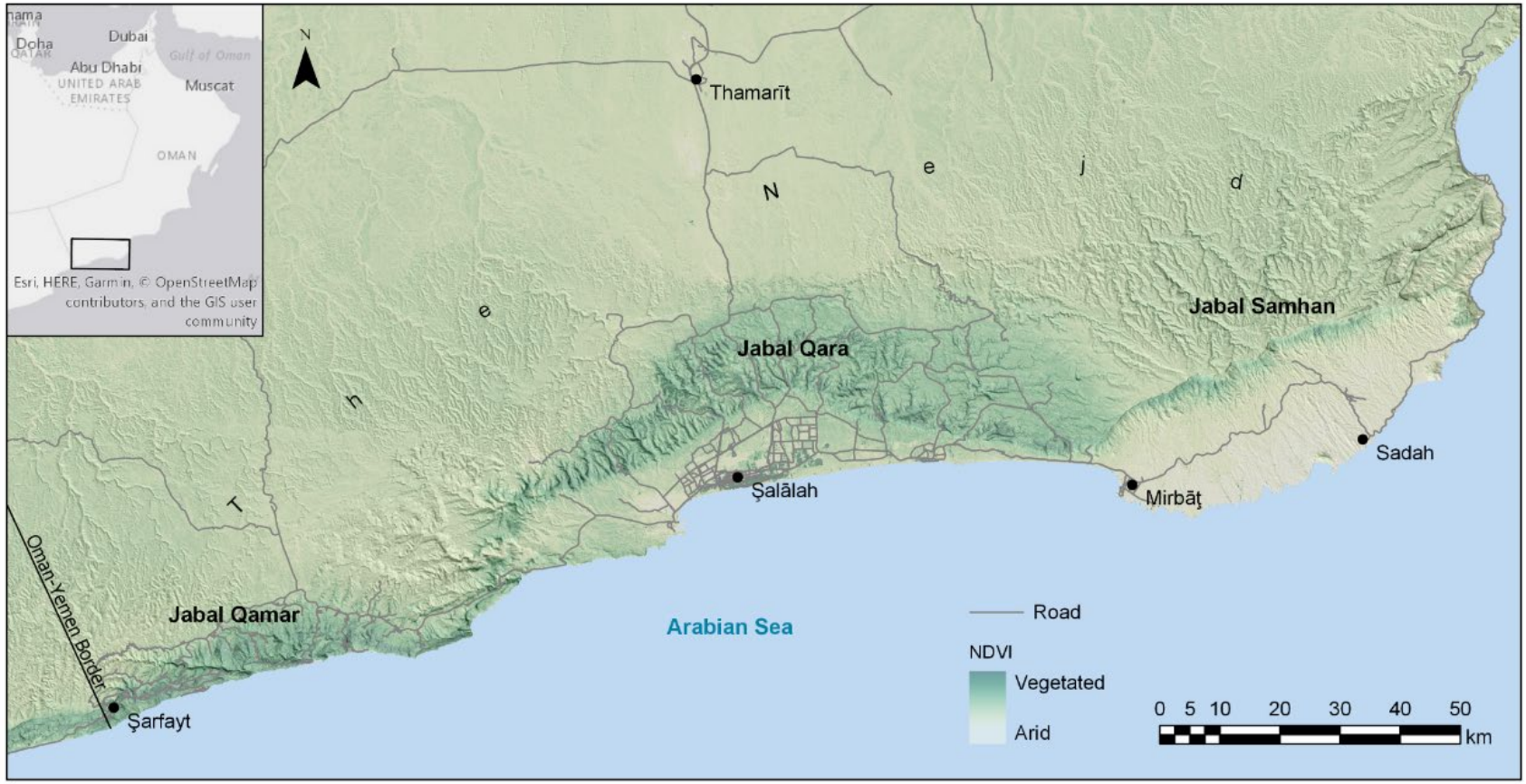

Figure 5.1. The location of the Dhofar mountains and the Nejd region. The inset map shows the position of Dhofar within Oman.

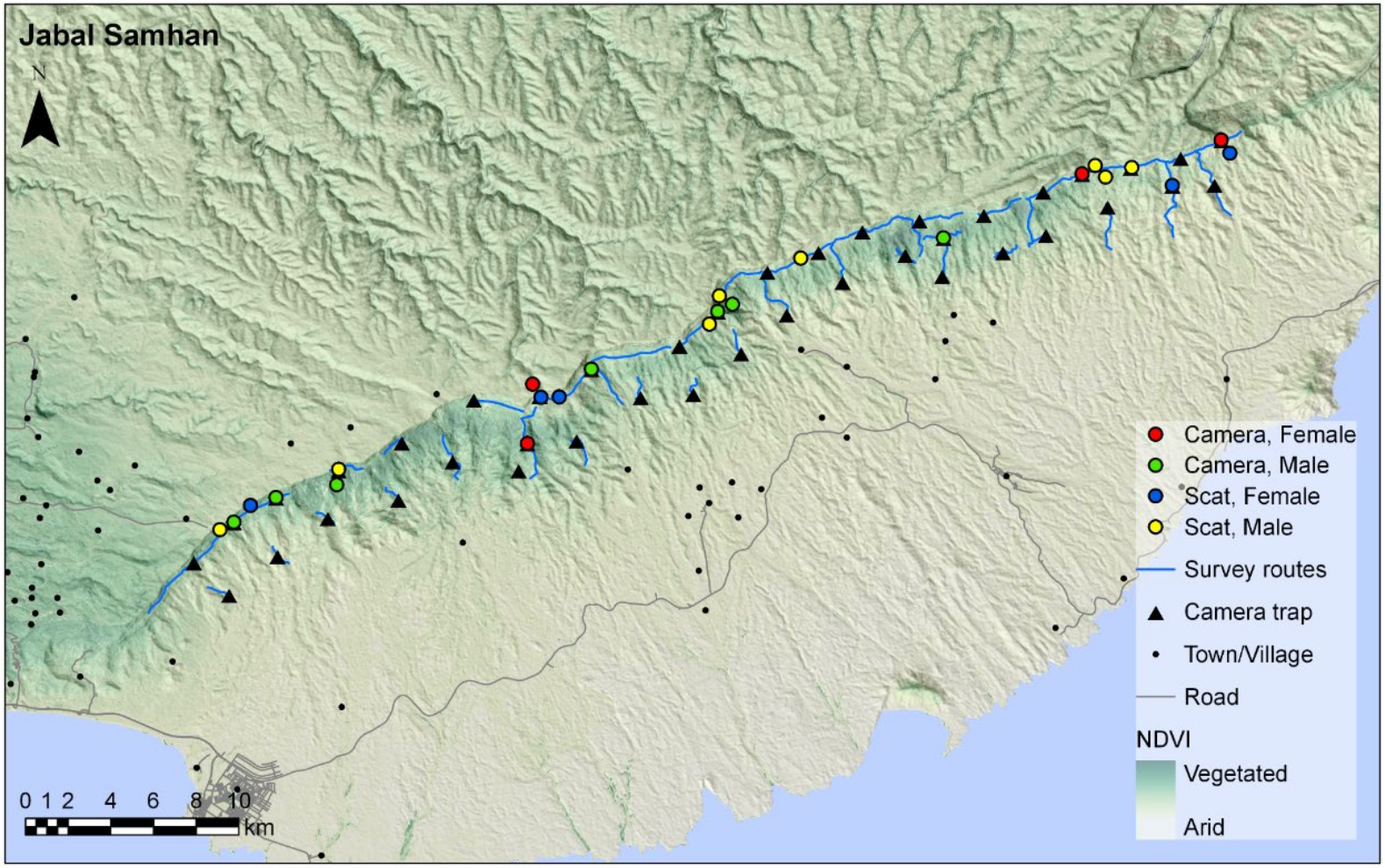

Figure 5.2a. The location of scat survey routes, camera traps and individual leopards derived from both survey techniques in Jabal Samhan.

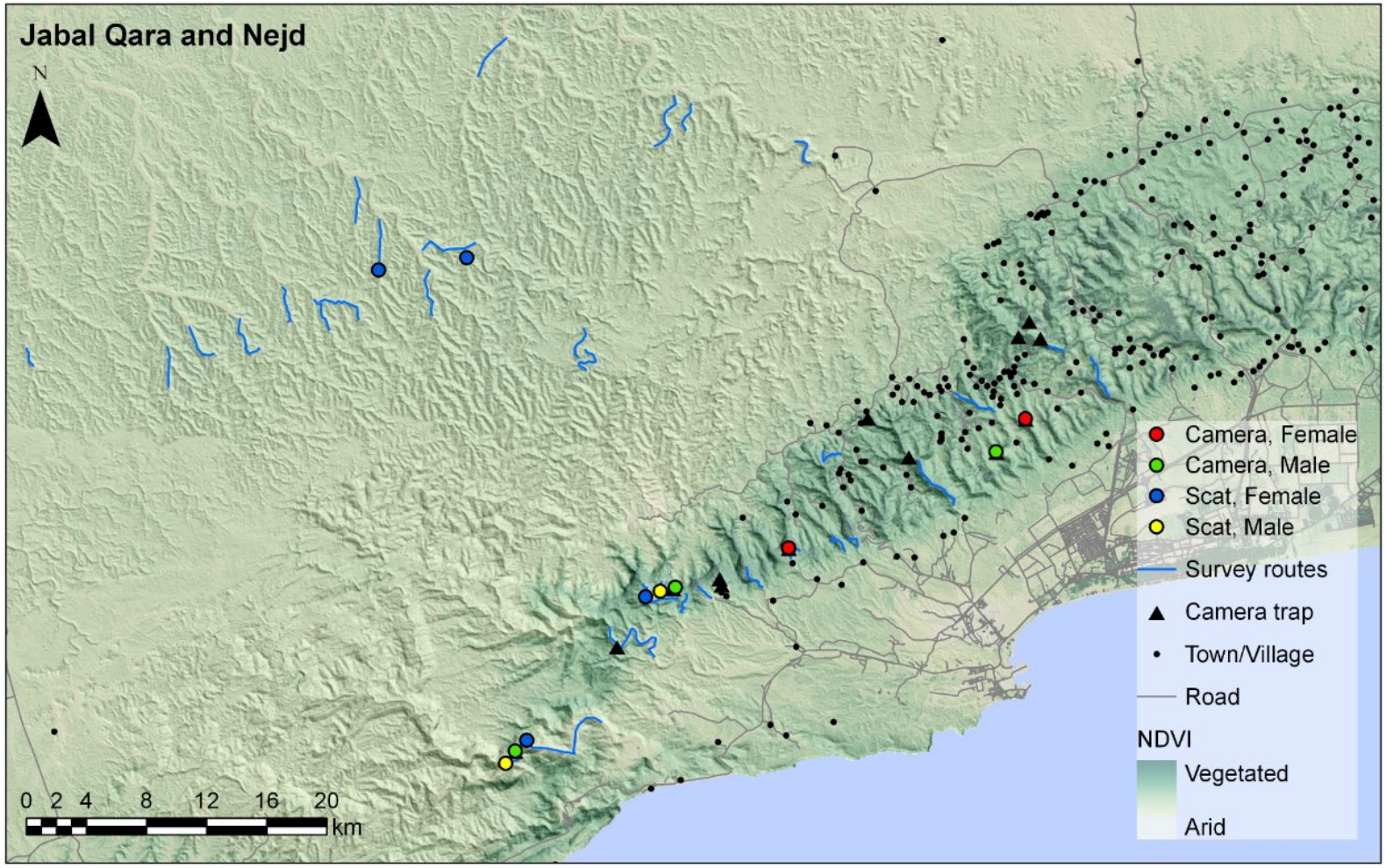

Figure 5.2b. The location of scat survey routes, camera traps and individual leopards derived from both survey techniques in Jabal Qara. The survey routes in the north represent the Nejd study region.

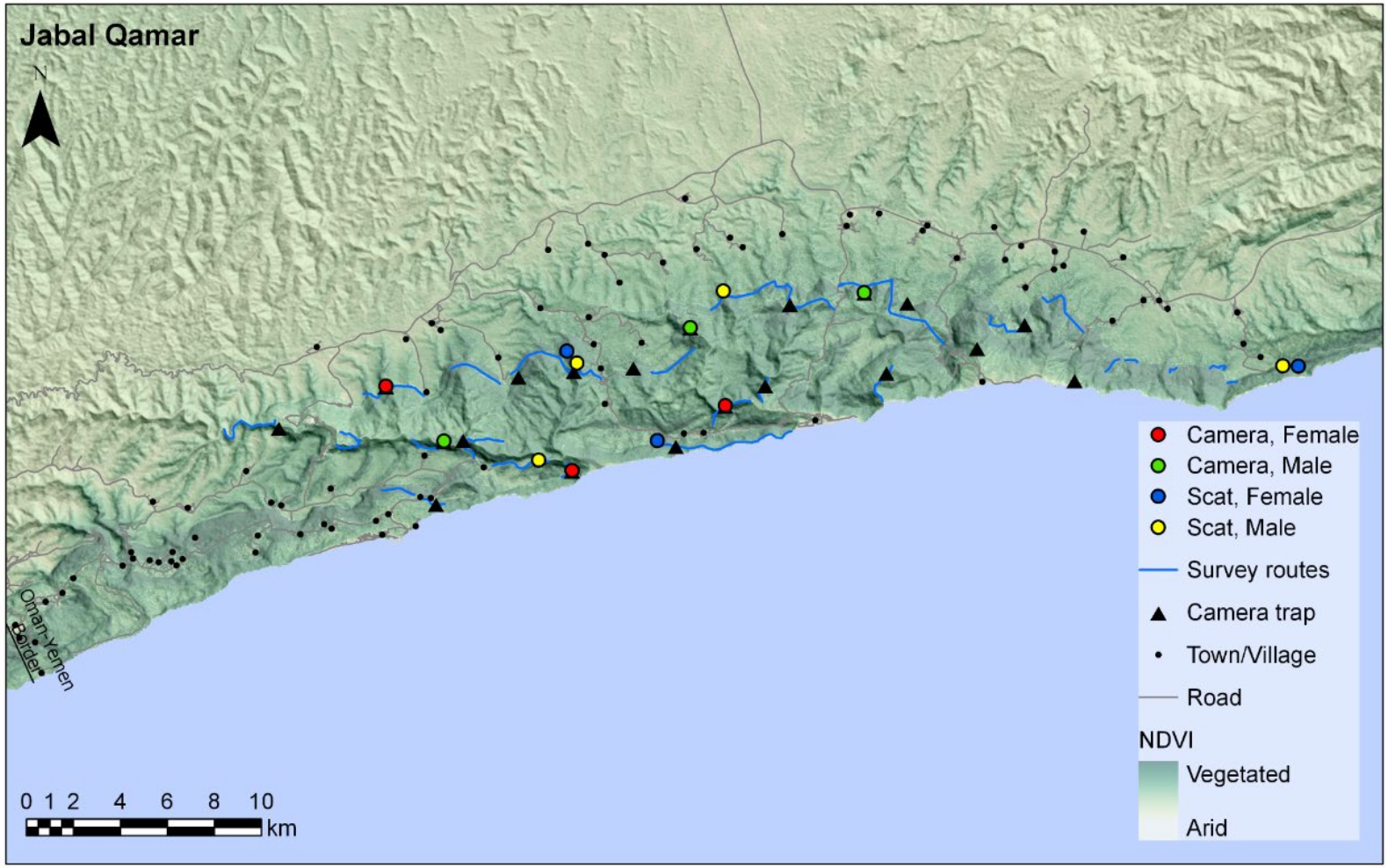

Figure 5.2c. The location of scat survey routes, camera traps and individual leopards derived from both survey techniques in Jabal Qamar.

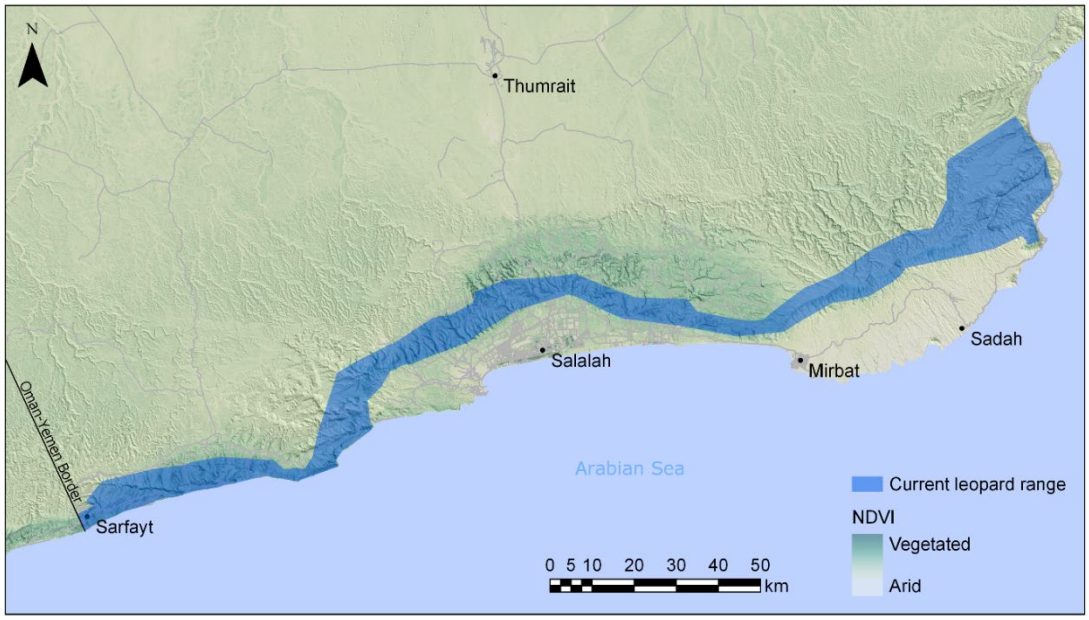

Figure 5.S1. Map of potential habitat of the Arabian leopard in the Dhofar mountains based on leopard presence data from camera traps and GPS collars (Spalton & Al Hikmani, 2014).

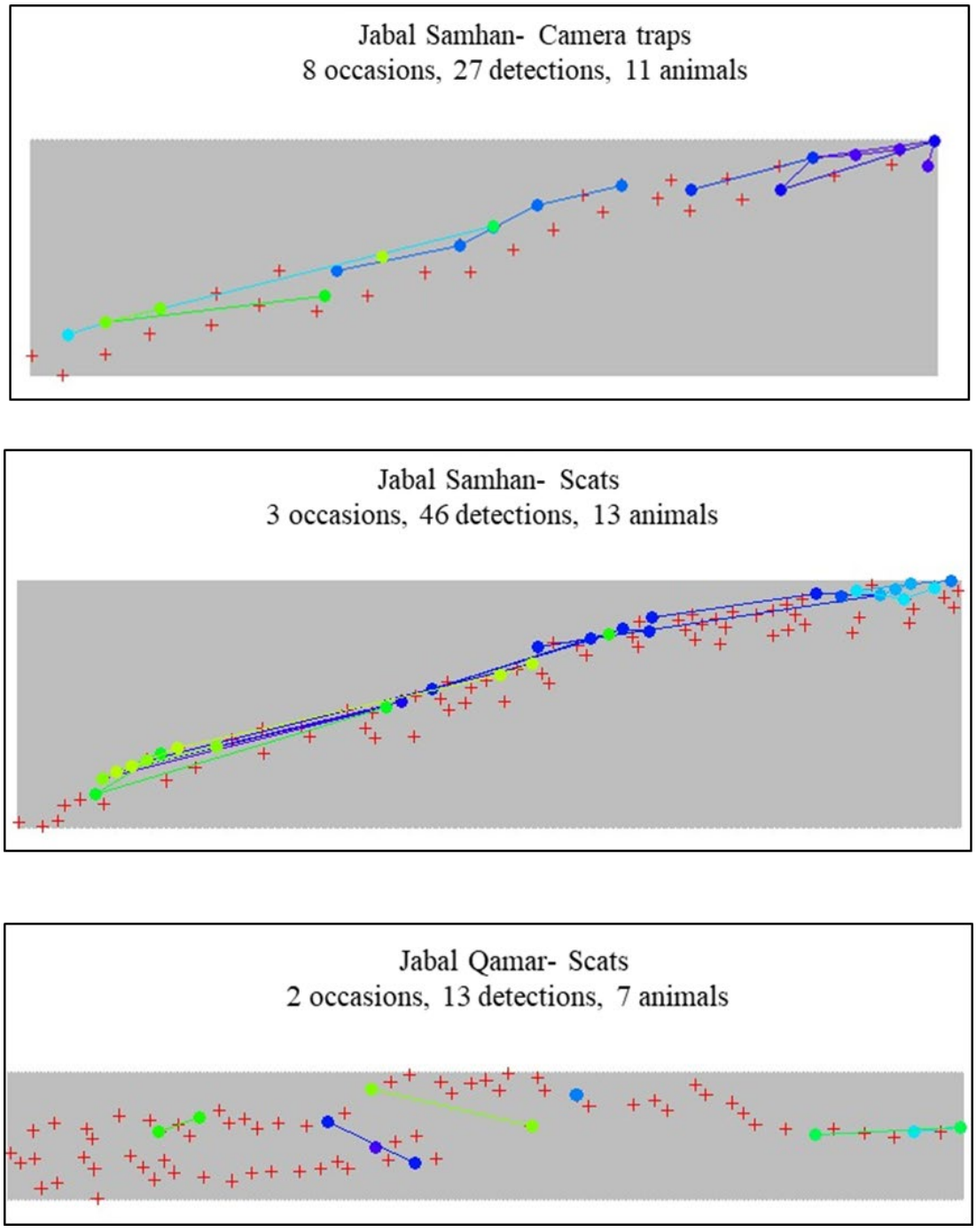

Figure 5.S2. Detection history of leopard generated in SECR modelling. The red crosses are camera trap/scat sites and coloured circles are individual leopards. Locations of symbols relate to geographic position of cameras/scats.

5.9 References

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Although the Arabian leopard population is considered to be declinging in Arabia, it is stable in Dhofar and once in a while images of cubs are recorded by camera traps.

6. General discussion

The Arabian leopard is the last remaining big cat to be found in the region. It is recognised as a flagship species and its persistence in the mountains of Arabia is of great environmental and cultural benefit to the Arabian landscape and its people. Leopard conservation is therefore a top priority in Oman and across the Arabian Peninsula (Spalton & Al Hikmani, 2014). However, not only does the Arabian leopard remain Critically Endangered and increasingly under threat (Breitenmoser *et al.*, 2010; Spalton & Al Hikmani, 2014; Jacobson *et al.*, 2016), information on its evolutionary history, population genetic makeup, and past and present demographics remain largely unknown. The aim of this Ph.D. was to fill these knowledge gaps by providing the missing information on their phylogenetic history, genetic diversity, population structure and population density. In investigating these crucial research areas, this study has generated a number of vitally important, original findings that will be incorporated directly into conservation management strategies by biodiversity managers and organizations responsible for the conservation of the Arabian leopard.

6.1 Uncovering the evolutionary history and genetic status of the Critically Endangered Arabian leopard

The Arabian leopard was described taxonomically more than 180 years ago, and is currently recognised as a tentative subspecies by the IUCN Red List of Threatened Species (Stein *et al.*, 2016). Kitchener *et al.* (2017) reassessed the taxonomy of all Felidae and implied that the Arabian leopard could be consubspecific with African leopards but should be retained as a separate management unit if its proven so.

Generating a comprehensive mitochondrial DNA sequence database that included sequence data from wild Arabian leopard populations across the Arabian Peninsula provided evidence that the Arabian leopard is evolutionarily distinct from other leopard subspecies. The genetic data aligns with morphological studies which found that the leopards of Arabia are distinct from Asian subspecies (Khorozyan *et al.*, 2006). Given this accumulative evidence, the Arabian leopard should be treated as a distinct subspecies and managed appropriately to conserve its unique evolutionary pathway.

Although there is no documented evidence of morphological differentiation within Arabian leopards, genetic data has uncovered the presence of two phylogenetically distinct lineages in Arabia; the first corresponding to western Arabia (Yemen) and the second to eastern Arabia (Oman). The distinctiveness of these two lineages is supported by both mitochondrial evidence (chapter 2) and microsatellite DNA analyses (see chapter 3). However, the nucleotide divergence between the two populations is very low (0.2%- 0.6%) and could be a result of region-specific historical phylogenetic diversity, or isolation due to recent habitat fragmentation and lack of gene flow (genetic drift) between Oman and Yemen populations. The cause of this fragmentation may be due to anthropogenic impacts, as the historical range of the Arabian leopard has been significantly reduced and is now largely dominated by human settlements and road networks. Such differentiation may also be attributed to natural climatic differences. Arabia experienced dramatic increase in rainfall during the last interglacial period (74-130 kya) with the creation of lakes and large water bodies (Parker, 2010). Although this wet period may have created ideal habitat for leopards to migrate and colonize north-western and eastern Arabia, rivers and larger water bodies have been found to influence the spatial distribution and gene flow of

mammal species (Goodman & Ganzhorn, 2004; Cullingham *et al.*, 2009; Basto *et al.*, 2016; Brunke *et al.*, 2019); these conditions may therefore have also established physical barriers to leopard movement. Another possible cause of population differentiation could result from historical biogeography followed by recent anthropogenic drivers further reinforcing region-specific genetic identity.

Understanding patterns of genetic diversity among wild and captive individuals is essential to inform long term conservation management of Critically Endangered species, such as the Arabian leopard. For example, some genetic diversity may now only reside in isolated leopard populations or in particular captive populations, paving the way for interventions to be considered which might aid its redistribution.

Due to inherent difficulties in obtaining DNA samples from such elusive animals, conserving the genetic health of the remaining Arabian leopards has not been included in conservation action plans to date. In this study, high levels of genetic diversity and unique alleles were discovered in wild and captive Arabian leopards of Yemen origin, compared to the wild leopards of the Dhofar mountains of Oman, an area considered to be their last stronghold. Although the wild leopard populations in some regions within the Dhofar mountains have slightly higher genetic diversity than in other parts of these mountains, the leopards of Dhofar appear to be genetically impoverished in comparison to Yemen leopards and to other species of big cat. For example, 10 of 18 loci that amplified successfully in this study are observed to be monomorphic for the Arabian leopard; in contrast these 10 loci are documented as polymorphic in other big cats including other leopards (see chapter 3, Spong *et al.,* 2000; Uphyrkina *et al*., 2001; Mondol *et al.,* 2009; Sugimoto *et al.,* 2014). The discovery that the leopards in Oman are genetically impoverished highlights the

urgent need to develop genetic management and restoration strategies to recover lost genetic diversity, and ensure this Critically Endangered taxon does not go extinct. Current data on levels of genetic diversity provide an important baseline for future genetic monitoring of the population as well as for guiding any future genetic management of the captive and wild leopards.

Although no detailed analysis has yet been done to fully investigate the threats facing the Arabian leopard, habitat fragmentation due to human activities and development are considered to be major (Al-Jumaily *et al.*, 2006; Judas *et al.*, 2006; Spalton *et al.*, 2006; Zafar-ul Islam *et al.*, 2018). Using a suite of microsatellite DNA markers, this study revealed a signal of fine scale genetic structure within the leopard population of Dhofar likely due to recent human development (e.g. roads, settlements, livestock number) in this region. Human associated land use and roads have been found to influence the genetic structure of African leopards due to population fragmentation and the limitation of gene flow between fragmented areas (Mcmanus *et al.*, 2015). Conservation organizations in Oman should therefore work closely with development organisations to ensure development plans do not impact the spatial distribution of leopards, nor minimise their ability to disperse between different regions of the Dhofar mountains. The Dhofar population is already very small (~51 animals) and fragmentated and any further fragmentation will reduce existing levels of genetic diversity and increase the rate of accumulation of inbreeding. Such effects will intensify the elevated risks faced by the Arabian leopard, bringing it closer toward extinction.

The innovative combined use of two non-invasive techniques, scat genetic sampling and camera trapping, has provided, for the first time, robust estimates of density and

population size for the leopards of Dhofar. The current estimated population size of just 51 individual leopards is in alignment with previous estimates derived from camera traps and GPS collars (Spalton & Al Hikmani, 2014) and therefore demonstrates the reliability of genetic sampling for population size and density monitoring of the Arabian leopard. This small population size in the Dhofar mountains appears to have remained stable for at least the last two decades, conceivably due to conservation efforts and the attention being given to leopards in this region. However, as the extinction risk is high, the population still requires intensive monitoring and assessment. Such population size estimates provide crucially important baseline information for future monitoring and to assess to what extent current longstanding conservation interventions such as the establishment of protected areas, deployment of wildlife ranger units, and compensation for livestock depredation are assisting the Dhofar population to recover.

6.2 Should the Arabian leopards of Oman and Yemen be considered as one or two Evolutionary Significant Units (ESUs)?

The evolutionary divergence and distinctiveness of the Arabian leopards of Oman and Yemen is intriguing given the absence of any major geographical barrier separating these two populations. In fact, the Oman and Yemen populations were expected to have been a single contiguous population until perhaps as recently as the early 20th century. However, phylogenetic divergence occurred between the Yemen and Oman leopard populations 65,000-243,000 years ago. Consequently, we need to consider whether these two populations should be managed as a single or separate ESUs. Literature in conservation biology suggest that populations with a history of reproductive isolation, and reciprocally monophyletic phylogenetic divergence can

be treated as ESUs (i.e. for long term management purpose) or as Management Units(MU) (i.e. for short term management purpose) and manged separately to maintain their evolutionary history and adaptive differences (Ryder, 1986; Moritz, 1994; Funk *et al*., 2012). However, Crandall *et al.* (2000) reviewed previous criteria for ESUs and suggest that ecological data (ecological exchangeability) such as population life history traits, ecological requirements, morphology, and demographic characteristics should also be used alongside data on genetic distinctiveness and gene flow (genetic exchangeability) to delineate conservation units and in turn inform management practices.

Following the approach of Crandall *et al.* (2000) I used a set of simple ecological and genetic characteristics /criteria to determine the conservation management status of the Arabian leopards of Oman and Yemen (Table 6.1). Both populations were considered to be ecologically and genetically exchangeable historically, at least 65,000- 243,000 years ago. However, I used recent genetic evidence from this study along with published ecological information and personal observations to determine recent population differences and distinctives between the leopards of Oman and Yemen.

Although both mitochondrial and microsatellite analyses show genetic differentiation between the leopards of Oman and Yemen and strongly indicate the existence of two reciprocally monophyletic linages, the genetic differentiation between the two populations is considered to be low (0.2%-0.6%). This low genetic differentiation does not provide conclusive evidence to reject genetic exchangeability, and drawing conclusions based on a monophyletic phylogenetic tree alone may not be sufficient for conservation management of this critically endangered species.

There are no studies to have addressed the ecological and environmental differences between the leopards of Oman and Yemen. However, the leopards of these two regions are considered to be morphologically the same and use similar habitats (Harrison & Bates, 1991). Captive individual leopards from Yemen have been observed to have darker coloured coats, but it is not known whether this phenotypic difference is evolutionary or is a result of being raised in captivity, differences in diet, or the effects of senescence. Nevertheless, leopards from both wild populations in Yemen and Oman have been mixed and bred in captivity and they produce fertile offspring (Edmonds *et al.*, 2006; Budd, 2011; Budd & Leus, 2011). Although this does show some ecological exchangeability between the two populations, further studies with more samples from both countries are required to better understand the relationship between the leopards of Oman and Yemen. Unstudied leopards may still be present in the south and east of Yemen (Al Jumaily *et al.*, 2006), and therefore, additional sampling and assessment of these animals would provide further insight into the ecological difference, genetic structure and evolutionary history of the Yemeni populations. Moreover, future studies should use a genomic approach which includes both adaptive and neutral loci instead of the standard population genetic analysis using microsatellite. Genomic data is considered more appropriate and powerful to delineate ESUs and define conservation units than neutral microsatellite (Funk *et al.*, 2012; Carroll et *al.*, 2018). In contrast, microsatellite data is regarded better for delineating conservation units below ESU level such as MUs (Funk *et al*., 2012). However, while waiting for results from such studies and given the lack of obvious morphological differences, and low-levels of genetic differentiation, the leopard populations of Oman and Yemen should be considered as one ESU but managed as two separate MUs until further studies using genomic approach

(adaptive and neutral loci) find conclusive evidence for these populations to be managed as separate ESUs.The global Arabian leopard population is very small (a total wild and captive population of <350 individuals) is Critically Endangered (Breitenmoser *et al.*, 2010; Stein *et al.*, 2016), and fragmented. Therefore, the management of the leopards of Oman and Yemen as one single Evolutionary Significant Unit would seem to be beneficial for their long-term conservation. This approach could provide an opportunity to address the genetic impoverishment of the Arabian leopard in Oman by using Yemen-sourced individuals currently found in the captive population in further captive breeding as well as in reintroduction to the wild. The genetic rescue of the Arabian leopard in Oman, including rejuvenating its genetic diversity and evolutionary potential, could have profound conservation benefits for its long-term survival.

6.3 Opportunities for genetic rescue of the Arabian leopard

Genetic rescue is a management tool that has been demonstrated in several other species systems to elevate the genetic diversity, and reproductive fitness of genetically impoverished populations through the introduction of genetically diverse outbred individuals into small and isolated populations (Frankham *et al.*, 2010, 2017). This approach has been used effectively for the recovery of several species including Florida panthers (Johnson *et al.*, 2010), adders (Madsen *et al.*, 1999), bighorn sheep (Hogg *et al.*, 2006) and wolves (Vilà *et al.*, 2003). The detection of unique genetic diversity within captive leopards originated from Yemen, may provide a source for genetic rescue of the Critically Endangered Arabian leopard in the wild. Given the finding of a mean percentage sequence divergence between Yemen and Oman leopards of just 0.2%-0.6%, this relatively low level of

differenation could suggest that introgression of Yemen genes into the Oman population maybe unlikely to cause problems associated with outbreeding depression. The risks associated with outbreeding are likely outweighed by the advantages of genetic rescue. The expectation is that the unique alleles, if reintroduced from captivity into the genetically impoverished wild population, could increase Arabian leopard genetic diversity. This in turn should increase individual-level and population-level reproductive fitness and recruitment, and subsequently increase levels of population growth and persistence. This genetic information is highly informative for Arabian leopard breeding institutions to develop an integrated programme of captive breeding of Yemen and Oman leopards. Introgressed leopards and their progeny can then be used for future reintroduction and to reinforce existing wild populations if deemed appropriate. Future genetic moinotoring can then use genomic DNA and Next generation sequencing (NGS) to identify adaptive markers that responsible for fitnees and adaptation thus minimize the risk of outbreeding depression (Flanagan *et al.,* 2017).

6.4 Habitat connectivity and management considerations for the Dhofar population

Oman's first priority should be to safeguard existing leopard populations by continuing and strengthening current programs for survey, compensation and enforcement of wildlife laws. However, given that habitat fragmentation is considered one of the major threats to the Arabian leopard and in light of the genetic structure found within the leopard population of Dhofar, conservation managers should consider the establishment of habitat corridors that link the Dhofar population and prevent its further fragmentation. The creation of habitat corridors will facilitate

leopard movement and consequently gene flow, and should reduce the likelihood of local extinction as well as the negative impacts associated with genetic isolation such as genetic drift and inbreeding.

The establishment of corridors would require the protection and safeguarding of critical habitat. The most critical areas are the south facing slopes and escarpments of Jabal Qara and Jabal Qamar that form a narrow strip, rarely more than 20 km wide, that runs from Wadi Henna in the east to Sarfayt and the border with Yemen in the west (Figure 6.1). This includes the belt of drylands close to Mughsayl that separate Jabal Qara from Jabal Qamar. Specific action, over and above the protection of these areas should include the incorporation of these corridors in the Oman National Spatial Strategy currently under development, within which is provision for special planning zones in areas of biodiversity importance. This approach would ensure any development would not negatively influence leopard populations in these areas.

As the Dhofar mountains are already crossed by a number of roads consideration should be given to constructing wildlife crossings, underpasses or overpasses, to allow animals to safely cross these barriers. This would include the Salalah to Sarfayt road that links Jabal Qara with Jabal Qamar.

Our detection of the highest density of leopards in Jabal Qamar demonstrates there is an urgent need for the declaration of Wadi Sayq protected area to help safeguard leopards of western Dhofar. However, declaration of the reserve is insufficient to guarantee the safety of leopards and the reserve will need to be actively managed with the participation of local communities.

Since the Nejd is less populated by humans and consequently there is less human-wildlife conflict, and the vast area appears to support healthy populations of large prey species (namely Nubian ibex and Arabian gazelle) the repopulation by leopards of this area should be encouraged. This might be done by increasing ranger patrolling and ensuring that corridors to the main leopard areas remain open. Although the current situation in Yemen means that wildlife conservation is not a high priority it is hoped that the situation will improve , and that cross-border conservation will be possible in the future. Thus, Oman should keep in mind the need to maintain leopard populations and habitat up to the Yemen border, and perhaps also consider structures that would allow leopards and other wildlife to move across the border fence.

Ultimately conservation of Oman's and the region's last viable population of leopards will require actions that go beyond surveys and studies. The continued strengthening of law enforcement is critical and the recently established program of compensation is very welcome. However, long term sustainability of leopards and leopard conservation requires participation of local communities. This should not simply be through employment as rangers (thought this is very positive) but local people need to be involved in decision making, and most of all in schemes that bring revenues. One such initiative would be to develop programs related to tourism, which is a sector currently being championed by the Oman government. Local people could be encouraged to develop simple guesthouses, make and sell local handicrafts to tourists, and to work as guides in leopard areas. If local people derive benefits from having leopards in their area, they are more likely to contribute meaningfully to their conservation, and therefore less likely to persecute them. Finally, it should not be forgotten that one of the greatest threats to leopard habitat is

the continued degradation of the mountains as a consequence of overgrazing. Measures need to be sought to reduce the number of livestock that are not just a threat to the region's biodiversity but are an economic burden for most local people.

This PhD thesis is the most comprehensive assessment to date of genetic architecture, evolutionary history, current population size and connectivity for the remnant population of the Critically Endangered Arabian leopard, and has provided an important basis for future conservation management recommendations.

6.5 Tables

Table 6.1. Conservation management status of Arabian leopards of Oman and Yemen.

Criteria	Historical	Recent
Reproductive status	The leopard population of Arabia was considered a single continuous population.	No evidence of recent interbreeding between wild populations, especially those from western and northern Yemen and the Dhofar population of Oman, but captive leopards from both wild populations in Yemen and Oman have been mixed and bred in captivity producing fertile offspring.
Morphology	Presumably similar	Similar although leopards from Yemen that reside in captivity show darker coloured coats.
Habitat	Presumably similar	Similar
Body size	Presumably similar	Similar
Population differentiation at neutral microsatellite loci	Presumably no differentiation as populations were considered connected historically.	Microsatellite analyses show significant population differentiation (F_{ST} =0.108) between Oman and Yemen populations.
Population differentiation at mtDNA loci	Presumably no differentiation as populations were considered connected historically.	Low levels of genetic differentiation between populations (0.02% - 0,06%)
Phylogenetic analysis	Presumably one linage before populations split at 65,000 to 243,000 years ago.	Strong to moderate support for two independent reciprocally monophyletic linages.
Management recommendation: Given that leopards from both populations have produced fertile offspring in captivity and the lack of obvious morphological differences, and low levels of genetic differentiation, the leopard populations of Oman and Yemen should be considered as one ESU but managed as two separate MUs until further studies using genomic approach (adaptive loci) find conclusive evidence for these populations to be managed as separate ESUs.		

6.6 Figures

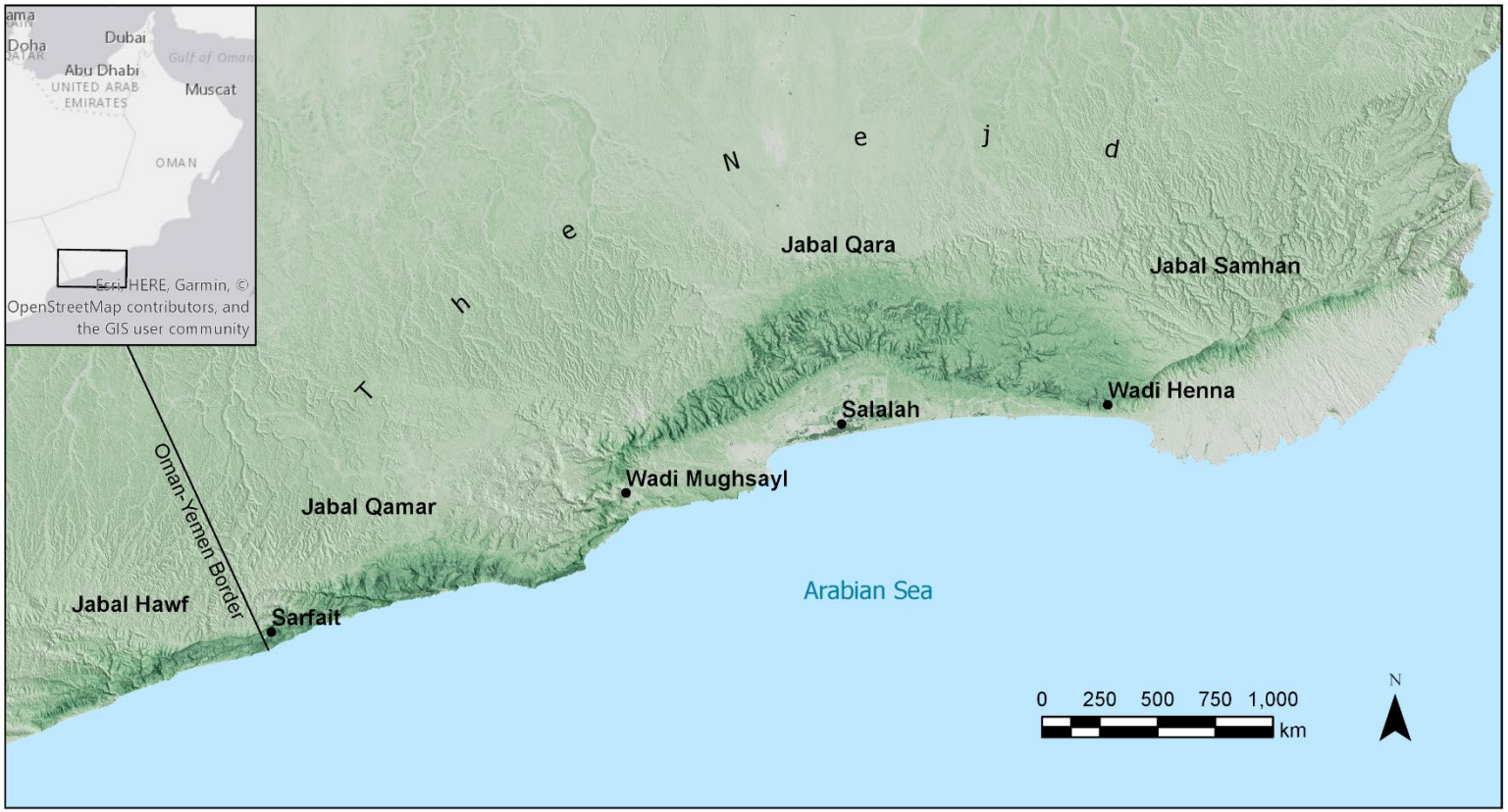

Figure 6.1. Map of the main mountains /Jabals of Dhofar including wadi Henna and Mughsayl.

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