

Critically Endangered Arabian leopards *Panthera pardus nimr* in Israel: estimating population parameters using molecular scatology

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Abstract The Critically Endangered Arabian leopard *Panthera pardus nimr* faces severe reduction in population size and is on the brink of extinction. This situation is, to a large extent, a result of human activity. The small populations of this subspecies are restricted to a few areas in the Arabian Peninsula, Israel and Jordan. Information required for conservation of this subspecies, including reliable population estimates and the range of individuals, is currently unavailable. To estimate population size and assign gender to individuals in the population in Israel we used molecular markers in leopard DNA extracted from scats collected in intensive surveys throughout the Judean Desert and the Negev Highlands. This non-invasive mode of sampling, combined with the availability of high-resolution markers (microsatellites) and sex-specific DNA-sequences, was successful in

identifying both individuals and gender. The results indicated the existence of a male and two females in the Judean Desert, and four males and one female in the Negev Highlands. Although the non-invasive procedure we used may underestimate the leopard's true population size, continuous monitoring of population size and sex composition of this small population using scatology is a key component for the management of this species. These data, especially if used in conjunction with similar data from other countries within the subspecies' range, will assist in the establishment of conservation plans for the Arabian leopard.

Keywords DNA, Israel, leopard, microsatellite, non-invasive sampling, *Panthera pardus nimr*, sex-specific markers.

Introduction

Inhabiting diverse habitats across most of Africa and Asia (Nowell & Jackson, 1996) the leopard *Panthera pardus* is one of the most widespread species of large cat. It is not, however, common over substantial portions of its range, mainly because of conflict with humans. Extensive morphological variability within *P. pardus* led to the recognition of numerous subspecies, and taxonomic revisions utilizing morphological and molecular markers resulted in the current total of nine subspecies (Miththapla *et al.*, 1996; Uphyrkina *et al.*, 2001). Some of these are represented by only a single or few populations.

In eastern and southern Africa leopard populations total 100,000–700,000 (Martin & Meulenaer, 1988; Nowell & Jackson, 1996), whereas other populations are on the brink of extinction. The most threatened subspecies is the Amur leopard *P. pardus orientalis* in Russia, with an estimated total population of 25–40 animals (Korkishko & Pikunov, 1994). Leopard populations are in decline

mainly because of habitat loss, poaching and hunting pressure on prey species, and even in protected areas leopards are sometimes shot for trophies or local trade (Korkishko & Pikunov, 1994; Khorozyan, 2001). In many unprotected areas the prey base is depleted and leopards are persecuted when they prey on livestock (Seidensticker *et al.*, 1990). Small leopard populations are also greatly affected by other human-related mortality and infanticide (Ilani, 1990; Korkishko & Pikunov, 1994).

The Arabian leopard *P. pardus nimr* is Critically Endangered (IUCN, 2006; Nowell & Jackson, 1996) and genetically unique (Uphyrkina *et al.*, 2001). This subspecies was formerly widespread in the mountain periphery of the Arabian Peninsula, Sinai Peninsula and in arid areas of Jordan and Israel (Gasperetti *et al.*, 1986; Harrison & Bates, 1991). Known extant populations are confined to the mountains of Dhofar in southern Oman (Spalton *et al.*, 2006), parts of northern Yemen, and the Judean Desert and Negev Highlands in Israel (Fig. 1). A few individuals may survive in the mountains of western Saudi Arabia, in other parts of Yemen, and the Musandam Peninsula of northern Oman. All populations are believed to be small and fragmented (BCEAW, 2004). The estimated total number of individuals is 80–290 but distribution is patchy and many populations are at risk of extirpation (Spalton, 2000; Anon., 2001).

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Received 2 January 2006. Revision requested 26 April 2006.
Accepted 19 May 2006.

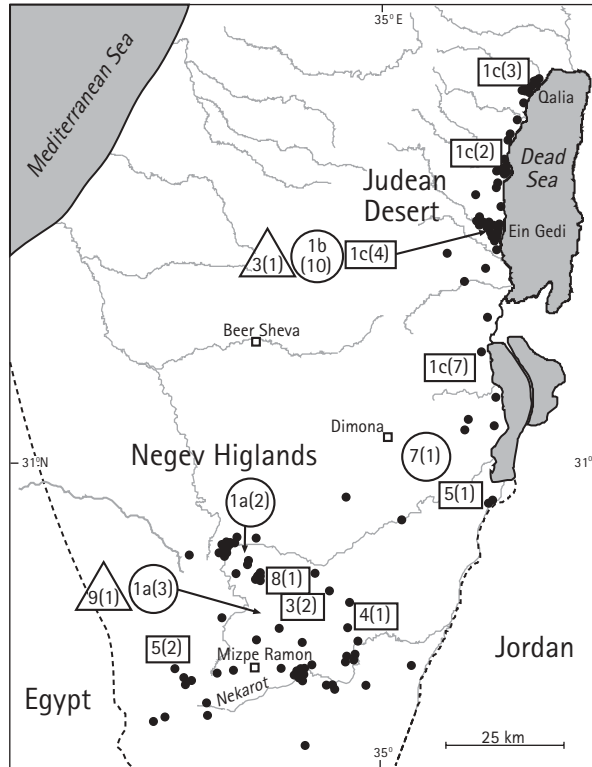


Fig. 1 Localities where Arabian leopards were observed in Israel in 1990–2003 (small filled circles) and genotype assignment (open symbols, with genotype identity, and the number of scat samples in parentheses: rectangles, males; circles, females; triangles, unsexed samples) to 41 of the collected scats (those for which allele assignment is available for all four microsatellite loci; see text for details). Major creeks and towns are indicated.

Arabian leopards in Israel inhabit 3,000 km² of rocky desert, *c.* 85% of which is within protected areas. A 20-year study of the population in the Judean Desert was begun in 1970 (Ilani, 1981, 1990), with nine individuals radio-tracked for up to 16 years (Ilani, 1990; Timna, 2000).

The population of the Arabian leopard has always been small in Israel, with an estimated 15–20 individuals, mostly because of a lack of suitable habitat and limited prey availability (Ilani, 1990). In the 1980s 4–5 males and 4–5 females were known from the Judean Desert and most were radio-collared. However, this population has now been considerably reduced. In 1979 two females were captured and transported to local zoos after repeatedly preying on house pets at Kibbutz Ein Gedi (near Massada). During 1986–1995 two other females died of old age, one was poisoned, one accidentally shot and one male was hit by a bus. In 1999 only a single male was known from the Judean Desert. The population of the Negev Highlands has not been studied as intensively, and only two individuals were radio-collared (a male in 1987 and a female in 1997 that was found dead 18 months

later). However, three surveys were conducted in this area during the 1990s and, based on pug-marks and other signs, the population was estimated to consist of a minimum of 3 males and 3 females, and a maximum of 10 adults (Timna, 2000).

For conservation purposes it is vital to have an estimate of a population's size and to know its composition. We conducted an extensive survey over the entire range of the Arabian leopard in Israel and collected scats for molecular analysis. Here we provide data on population size and the number of males and females, and discuss possible management schemes for ensuring the persistence of this small population.

Methods

Scat sampling

Scats were collected in the Judean Desert and the Negev Highlands in 2001–2002. We systematically searched localities where leopards were seen or their signs observed in the past 14 years (database of the Israel Nature and Parks Authority; Fig. 1). We also repeatedly visited all permanent running creeks, and most springs and temporary water sources in search of scats. Ibx *Ibex nubiana* often visit these locations and some sites support hyrax *Procapra capensis* populations, both of which are chief components in the Arabian leopard's diet (Timna, 2000).

Leopards often use hiking trails and defecate in shallow pits dug at the side of the trail or by large trees or boulders. However, occasionally defecation occurs in atypical locations. Because we could not always find pug-marks by a scat or be sure that a specific scat was from a leopard, we collected all scats similar in size to those of leopard. Scats were placed in individual paper bags and their location was recorded. When necessary scats were dried at 60°C, and all were stored at room temperature until analysed (Farrel *et al.*, 2000).

Molecular identification of individual and sex

Collected scats were used as a source for DNA extraction (for collection advice see Piggott, 2004). We scraped the outer layer of each scat and used *c.* 100 mg of the resulting powder for DNA extraction. DNA was extracted by proteinase K digestion overnight at 50°C and isolated using phenol and chloroform (Ernest *et al.*, 2000) or by a commercial extraction kit (IsoQuick, Orca Research Inc., Bothell, Washington, USA).

To assign species to each scat we amplified a 376 bp fragment of the mitochondrial 16S-rRNA. The reaction profile consisted of an initial denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds,

55°C for 45 seconds and 72°C for 1 minute, and 10 minutes of final elongation at 72°C (for primer sequences see Johnson *et al.*, 1998). Negative control tubes (no DNA template) were set in every Polymerase Chain Reaction (PCR) run to monitor for contamination. This fragment can distinguish between leopard and the other large carnivores at our sampling sites (striped hyaena *Hyaena hyaena*, Arabian wolf *Canis lupus* and caracal *Felis caracal*) and also between domestic cat *Felis domestica* and leopard (Johnson *et al.*, 1998). The latter distinction is crucial because leopards often prey on domestic cats. Amplified mtDNA was sequenced and compared to available sequences using the Basic Local Alignment Search Tool (NCBI, 2006).

To identify individuals we scanned 31 microsatellite markers generated from a genomic library for domestic cat DNA (Menotti-Raymond *et al.*, 1999). Most of these markers are polymorphic in other leopard subspecies (Spong *et al.*, 2000; Uphyrkina *et al.*, 2001). We defined any locus that generated at least two alleles as polymorphic. Amplifications were performed in a total of 25 µl containing 2 µl of DNA extract diluted x100, reaction buffer (Fisher Biotec), 2.5–3.75 mM MgCl₂, 2.5 µg bovine serum albumin, 100 µM of each dNTP, 6–10 pmol of each primer and 0.5–1 units of Taq polymerase enzyme (Fisher Biotec). The reaction profile was the same as for the mitochondrial fragment, except for an annealing temperature of 52–58°C, depending on the primers. PCR products were electrophoresed in an 8% acrylamide gel and visualized by silver staining. A specially detailed marker, encompassing the expected range of allele sizes at 4 bp intervals was used to score allele size. We produced the marker by amplifying fragments of the desired lengths from a commercial plasmid.

Molecular scatology has become increasingly popular and a number of strategies have been proposed to minimize genotyping errors (Fernando *et al.*, 2003). We amplified all homozygotes at least three times to test for allele dropout. To accommodate for false alleles and accurately determine allele size we cloned PCR fragments of homozygote specimens into pGEM-T Easy vector (Promega) and sequenced representatives of observed alleles. The number of unique genotypes was determined with the software *GIMLET 1.3.2* (Valiere, 2002), which takes into account the probabilities of mis-scoring and null alleles.

Because the Y chromosome is male-specific, genes located on it are ideal for sex identification. We used DNA dot-blotting for sex identification because this procedure is not PCR-dependent and is not therefore affected by Taq polymerase inhibitors, which often exist in DNA extracted from faeces. The DNA dot-blotting technique is based on a labelled probe used to detect target DNA by hybridization. The ZFY gene (zinc fingers

on chromosome Y) was chosen as the target DNA. Because of the presence of the homologous ZFX in females (on chromosome X), the probe had to be designed based on a region where ZFX and ZFY display the largest differences. Following an alignment of available sequences of leopard ZFY and ZFX (gi:9623283 & gi: 9623229; NCBI, 2006) a 21 nucleotide segment (P.p.Zfy; TGCATAAAGAAAAAGCCAACA) from the ZFY sequence was found to include 5 mismatch positions with respect to the ZFX sequence (a 3 bp deletion in ZFY and 2 mismatch positions; Pilgrim *et al.*, 2005). A P.p.Zfy oligonucleotide, labelled with Dig-11-dUTP (Roche), was used as a hybridization probe. Three µl of DNA extract (diluted x10) of each sample were loaded onto a positively charged membrane after 10 minutes denaturation at 95°C followed by incubation in ice water. DNA was fixed to the membrane by UV crosslinking (1,200 µJ cm⁻²). Hybridization and detection followed the manufacturer's instructions.

To test our approach for sexing individuals we used DNA extracted from four museum skins of individuals of known sex (Tel Aviv University zoological collections) and scats of captive leopards (Haifa Zoo, Israel). DNA extraction from museum skins followed Iudica *et al.* (2001).

Three other pairs of primers were used as gender non-specific controls to monitor the overall quality of extracted DNA. PCR products of the primers ZfyF/Exon6R (Slattery *et al.*, 2000) and ZF2F/ZFY1R (Spong *et al.*, 2000) were PCR-labelled with DIG and used as probes on whole-DNA dot blots, following manufacturer's (Roche) instructions for labelling and detection. The rps4XF/rps4XR primer set (Murphy *et al.*, 1999) was used in simple PCR reactions.

Population estimation

Rarefaction curves were used to estimate population size (Kohn *et al.*, 1999; Eggert *et al.*, 2003). Assuming that each unique multi-locus genotype represents a single individual, the population size can be estimated by the number of genotypes accumulated as a function of scats scanned. Eggert *et al.* (2003) showed by simulation that the equation used by Kohn *et al.* (1999) grossly overestimates population size, and therefore we used Chessel's equation (Valiere, 2002)

$$y = a - a \left[1 - \left(\frac{1}{a} \right) \right]^x,$$

and Holdridge's equation (Eggert *et al.*, 2003)

$$y = a(1 - e^{-bx})$$

The number of genotypes is given by y , x is the number of scats used, a is the asymptote (i.e. population size

estimate), and b is the slope. The asymptote and the 95% confidence interval are calculated from 1,000 iterations of random sampling of scats (Kohn *et al.*, 1999). The software *GIMLET* (Valiere, 2002) and *R 1.8.0* (Ihaka & Gentleman, 1996) were used for curve fitting and permutations. Because several non-invasive samples could represent a single individual we used *GIMLET* for pooling identical genotypes. To quantify the ability of the molecular markers to distinguish between the different genotypes we used probability of identity (PI) that is computed using allele frequencies. Because the leopard population is small and there has been evidence for incest (Ilani, 1990) we used a less biased equation with a correction for populations that are composed of closely related individuals ($PI_{(sibs)}$; Waits *et al.*, 2001).

We used *Capwire* (LECG, 2005; Miller *et al.*, 2005) to corroborate our rarefaction estimates. *Capwire* uses two capture models, the even capture-probability model (ECM) and the two innate rates model (TIRM). For the ECM every individual is equally likely to be captured on each sampling session with probability of $1 / (\text{population size})$. The TIRM model, which is much more realistic, views the population as a mixture of individuals with distinct capture probabilities. The *Capwire* method is especially suitable in cases such as ours where sampling intensity may be heterogeneous with respect to specific individuals (i.e. some individuals may be represented much more than others in the sampled scats).

Results

Preliminary screening for microsatellite markers

We collected 268 scats from the Negev Highlands and Judean Desert (Fig. 1). Most scats were dry and only a few required oven drying. We obtained amplification product of the mitochondrial 16S from only 112 scats (42%). Fifty-four scats were of Arabian leopard (48.2%), 42 Arabian wolf (37.5%), eight hyaena (7%), five caracal (4.5%), and three of other species. Of the 54 leopard scats, 38 were from the Judean Desert and 16 from the Negev Highlands. There was a significant correlation between sites where leopards were observed in the past 14 years and where scats were collected ($r = 0.61$, $P = 0.008$) but some sites where leopards had often been seen yielded no scats, even though these locations were intensively searched (e.g. Nekarot Wash in the eastern Negev Highlands; Fig. 1).

Out of 31 microsatellite markers tested, six did not amplify and 14 produced unreadable results. Of the remaining 11, seven were monomorphic and only four were polymorphic (Fca453, Fca391, Fca115 and Fca223; Menotti-Raymond *et al.*, 1999). Because missing data may affect genotype assignment in *GIMLET*, we used only the

41 scats for which we had allele assignment for all four loci. *GIMLET* identified seven genotypes, three restricted to the Negev Highlands, one restricted to the Judean Desert, and three from both regions. Genotype 1 was represented by 31 scats. This multi-locus genotype was subsequently assigned to three individuals (1a–c) using additional data (gender and range, see below & Fig. 1). Two other genotypes (3 and 5) were represented by three scats each, and the other four genotypes (4, 7, 8 and 9) were each found in a single scat (Fig. 1).

We examined DNA from skins collected in the Judean Desert in 1964 (N7), 1986 (Tihamat), 1995 (Enigma) and in the Negev Highlands in 1998 (Tzia). Five microsatellite markers were polymorphic (Fca453, Fca391, Fca310, F115 and Fca105; Menotti-Raymond *et al.*, 1999). All loci were heterozygous for N7, whereas all loci but one were homozygous in the more recently obtained skins (Tihamat, F115; Enigma, Fca310; Tzia, F115).

Population size

The mean number of genotypes expected using Holdridge's equation was 12.6 (95% CI 7.5–33.2; range 7.2–33.2). The mean number of genotypes expected by Chessel's equation was 7.2 (95% CI 5.3–8.8; range 4.5–9.4; Fig. 2). The PI values calculated for each of the four microsatellite loci were 0.31–0.66, and $PI_{(sibs)}$ values were 0.56–0.81. The cumulative values, when all four loci are combined, were 0.05 and 0.221 for PI and $PI_{(sibs)}$, respectively. Using the ECM and TIRM models in *Capwire* the population size was estimated as 9 (95% CI width = 0) and 9–11, respectively.

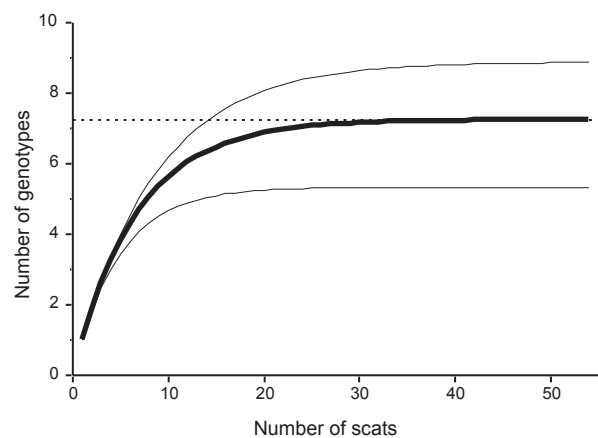


Fig. 2 Rarefaction curve for the number of genotypes as a function of number of scats used. The curves for the mean (thick line) and confidence interval (thin lines) were fitted using Chessel's equation (see text for details). The dashed line indicates the estimated population size (i.e. the asymptote).

Sex determination and individual range

For each leopard DNA sample that did not react with the P.p.Zfy probe we checked whether the negative result was a result of no matching template (i.e. a female leopard) or due to poor extract quality. Only a sample that gave positive results to at least two non gender-specific primer sets and no result with the P.p.Zfy probe was considered to be a female. Of the 54 samples screened, 28 were determined as males (52%), 17 females (31%), and for nine samples we obtained ambiguous results precluding definite sex determination (e.g. failure of both gender-specific and gender non-specific probes to hybridize). Genotype 1 represents one female from the Negev Highlands and a male and a female from the Judean Desert. Genotype 5 is a male that was sampled from the Negev Highlands and the southern Judean Desert. Genotype 3 is a male identified by two samples from the Negev Highlands and one from the Judean Desert (Ein Gedi) but for the latter sample the sex could not be determined. Male 1c roamed much of the Judean Desert whereas a female in that region (1b) stayed mostly around Ein Gedi (Fig. 1). The other female in the Judean Desert was detected by one sample in the south (genotype 7). In the Negev Highlands, we identified six genotypes: four males (3, 4, 5 and 8), one female (1a) and one unsexed genotype (9).

Discussion

The population of Arabian leopards in Israel has been small for many decades (Timna, 2000). Leopards were hunted until the early 1960s when the majority of mammals in Israel became fully protected by law. Although leopards have not been hunted since then, the population has declined due to low breeding success, accidents and management errors (Timna, 2000). This trend is most obvious in the Judean Desert, where only a single male was observed in recent years. Our study has shown, however, that there are at least one female and one male in the Judean Desert, and two females and four males in the Negev Highlands.

As this is a very small population of a threatened subspecies it is important to use the most conservative estimate of population size. In conjunction with independent estimates from surveys (Timna, 2000) we consider Chessel's estimator of population size (7, 95% CI 5–8), which was similar to the estimate generated by *Capwire*, a better reflection of the true number of Arabian leopards than that of Holdridge. These may, however, be underestimates, for two reasons:

1) It is unlikely that we located scats from every single individual in the extensive area searched. For example, we could not find scats along Nekarot Wash, even

though there have been multiple observations of leopards there in recent years. Furthermore, the area was not scanned uniformly; some locations were visited on a number of occasions by various people who collected scats for us, while the more remote sites were only visited by us.

2) The small population of Arabian leopards in Israel has been isolated for many years. Leopards in Sinai are near extinction and the Jordanian population has been reduced to a few individuals (1–8 leopards in the 1970s, and only two known observations in the late 1990s; Timna, 2000). Because of isolation, the leopard population in Israel is highly inbred and three cases of incest have been recorded (Ilani, 1990). Leopards of the Judean Desert appear to be isolated from those in the Negev Highlands. Tracking of radio collared individuals in the Judean Desert revealed large home ranges (mean of 47 and 83 km² for females and males, respectively; Timna 2000) and single movements up to 45 km; yet these individuals have never moved to the Negev Highlands, 80–150 km away. Isolation and inbreeding have the most deleterious effect on genetic heterogeneity. An indication for low genetic variability in our population was expressed by a high probability of identity and only a few polymorphic microsatellite loci from a large set that are highly variable for other leopard populations (Spong *et al.*, 2000). The almost indistinguishable genetic profiles obtained for museum specimens collected 10 years apart also demonstrate the low genetic variability of the population. The suspected level of inbreeding is reflected by the PI and PI_(sibs) values. Both probabilities are much higher than the values considered necessary for individual, non-invasive genotyping (0.001; Waits *et al.*, 2001). Therefore, low genetic variability increases the likelihood that individuals will have an identical genetic profile, making them indistinguishable in our screening procedure (Mills *et al.*, 2000).

The assignment of sex was important because it not only allowed us to distinguish between two individuals in the Judean Desert that had identical genotypes (1b and 1c; Fig. 1) but also provided assurance that there are males and females in both the Judean Desert and Negev Highlands. This is an important piece of information for the conservation and rehabilitation of the leopard population in Israel.

There has been concern that sex identification based on amplification of Y-chromosome specific segments from faecal DNA samples is highly error-prone in carnivores (Murphy *et al.*, 2003). In their analysis of bear *Ursus arctos*, females were erroneously scored as males by this method when fed male prey-items (white-tail deer *Odocoileus*

virginianus), which most probably indicates amplification of prey DNA. The relative importance of such errors is drastically increased by the power of PCR to amplify even minute amounts of DNA, such as prey DNA in faeces. We minimized the risk of contamination by prey DNA using the outer surface of the faeces, and more importantly by verifying the species for each DNA sample according to the sequence of a highly specific marker (16S rDNA). Nevertheless, minute amounts of prey DNA may not be detected by the amplification of 16S rDNA in the presence of much larger amounts of leopard DNA, but may still amplify for Y-specific fragments in lack of competition with the predator's DNA (i.e. in a female leopard). Thus, the use of non-amplification based methods is desirable. In this study we used a hybridization-based method instead, for which we developed a species-specific Y-chromosome hybridization probe, further decreasing the probability of wrong sex assignment.

With respect to the subdivision of genotype 1 we concluded that the same genotype in the Negev Highlands and the Judean Desert must have come from two different females for two reasons. Firstly, sampling in the Judean desert was frequent and therefore scats were always fresh, meaning that the leopard was present close to the time of collection. Secondly, intensive observations over nearly 2 decades (Ilani, 1990) have never documented the movement of individuals between the Negev Highlands and the Judean Desert. In our study, male 5 was identified from scats in the Negev Highlands and the southern Judean Desert (c. 100 km apart; Fig. 1). Another genotype (3) was identified from two samples in the Negev Highlands and one in the Judean Desert. However, because we were unable to sex the sample collected in the Judean Desert, we cannot be certain that all three samples represent the same individual.

Due to the high inbreeding rate, small population size and long duration of the population's isolation, genetic variability of Arabian leopards in Israel is alarmingly low, potentially increasing susceptibility to contagious lethal diseases. One possible remedy is genetic rescue, a translocation of one or more individuals into the population (Ingvarsson, 2001; Keller & Waller, 2002). This is, however, problematic because source populations are not available and captive individuals worldwide are few. Regardless of the exact nature of any rescue operation implemented the expansion of the human population in the Negev Highlands, together with other factors, may hinder any recovery of the leopard. Although the non-invasive procedure we used may underestimate the leopard's true population size, continuous monitoring of population size and sex composition of this small population using scatology is a key component for the management of this species.

Finally, we emphasize the need to conduct similar research in other countries within the current range of the Arabian leopard. The small population sizes throughout the subspecies' range necessitates coordinated efforts if the extinction of this subspecies is to be prevented.

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Biographical sketches

Inbar Perez was a graduate student under the joint supervision of Eli Geffen and Ofer Mokady. The major findings of her MSc dissertation, focused on leopards in Israel, are summarized in this paper. Eli Geffen and Ofer Mokady have been conducting collaborative research at the interface of evolution, ecology and nature conservation for the past 15 years, encompassing various vertebrates and invertebrates.