Short Communication

Genetic analysis of scat reveals leopard *Panthera pardus* and cheetah *Acinonyx jubatus* in southern Algeria

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Abstract Assessing the biodiversity of a region, particularly when it includes rare, cryptic and nocturnal species, is notoriously difficult but nevertheless of great importance, especially in regions that have received little prior research interest. Species in these regions are often only perceived to be present through the identification of animal signs such as scat or hair. Here we show how molecular techniques can be used to sequence mitochondrial DNA extracted from the scat of essentially unknown carnivore species and thus assess the species present in a remote desert region in southern Algeria. In doing so we present a reliable and reproducible method for determining carnivore biodiversity through non-invasive sampling. We also identify two species of large carnivore in an area where one, the cheetah *Acinonyx jubatus*, is known to exist but at very low densities, and another, the leopard *Panthera pardus*, has never previously been observed.

Keywords *Acinonyx jubatus*, Algeria, biodiversity assessment, cheetah, leopard, mitochondrial DNA, *Panthera pardus*, scat.

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Non-invasive genetic sampling (i.e. the extraction of DNA from hair and faeces) is being utilized increasingly to estimate abundance, identify behavioural parameters and understand population structure in species that are rare, threatened or cryptic (Piggott & Taylor, 2003). It is particularly helpful where survey methods such as transects are infeasible or impossible. There are many studies documenting the use of molecular techniques, often in combination with field-based surveys, which aid our understanding of the genetic processes underpinning threatened carnivore populations (Wayne & Morin, 2004; Hedmark & Ellegren, 2007). Samples collected non-invasively present a cheap, safe and efficient way of obtaining genetic information about a species. However, whilst techniques have been developed to identify scats of closely related species and to verify the accuracy of identification (Davison et al., 2002; Waits & Paetkau, 2005; Barea-Azcon et al., 2007), they have rarely been used to identify the species present in an area by sequencing mitochondrial DNA from essentially unknown scat samples. Here, we present an attempt to use these reliable technologies to assess rare carnivore distributions, confirming the presence of one threatened felid species and reporting an extension in the range of another.

The Ahaggar of southern Algeria is a mountainous desert region in the central Sahara where logistic and economic constraints have contributed to a lack of wildlife surveys. Consequently, little information is available on large carnivore presence in the area. Historical records (Fig. 1a) and, more recently, the discovery of pelts and the capture of live animals by Tuareg nomads, confirm that cheetah *Acinonyx jubatus* still inhabit the Ahaggar. Although formerly recorded further north in Algeria, these observations represent the present northern limit of known cheetah range in the Sahara (Hamdine et al., 2003; FB, unpubl. data). The leopard *Panthera pardus* was formerly known in Algeria from the Mediterranean coastal region as far south as the Tell Atlas but has been considered extinct in northern Algeria for at least 50 years and has never been observed or documented in the Algerian central Sahara (Fig. 1b; Kowalski & Rzebik-Kowalska, 1991; Kingdon, 1997). Nevertheless, in the Ahaggar some local people report large spotted cats they believe to differ from cheetah (KDS, pers. obs.). Verifying

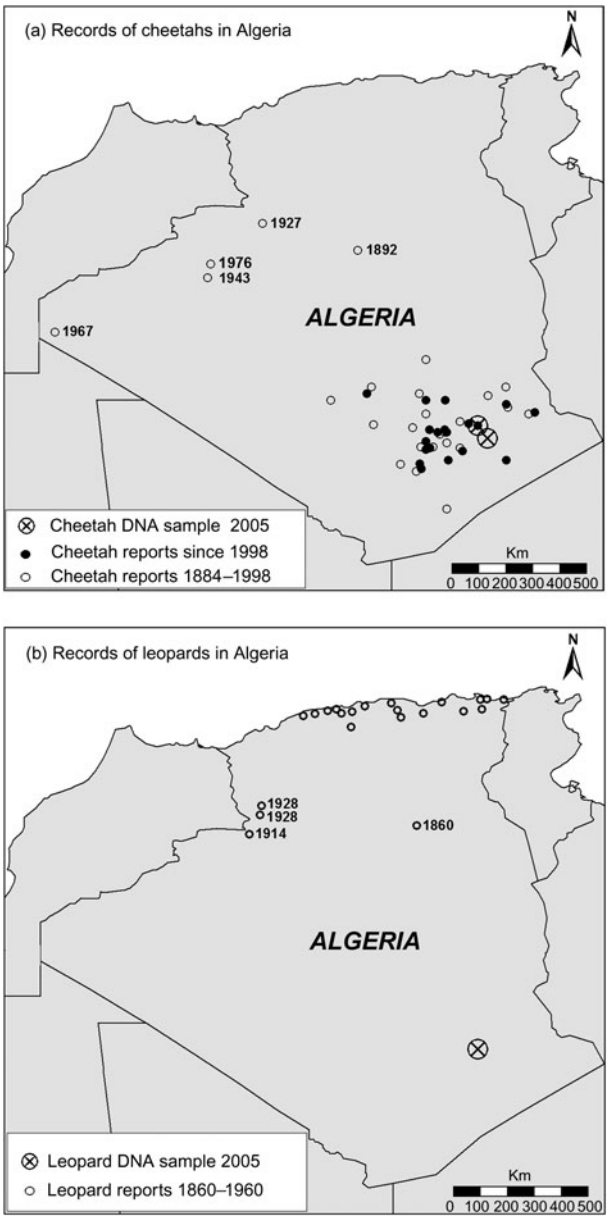

FIG. 1 The Ahaggar samples compared to the historical distributions of (a) cheetah and (b) leopard in Algeria. Records are from Kowalski & Rzebik-Kowalska (1991), Hamdine et al. (2003) and this 2005 study. The scats of the eight cheetahs found in 2005 are shown by two symbols, the northern representing five cheetahs and that to the south-east, three.

the presence of large carnivores in this part of Algeria has significant implications. The northern African subspecies of cheetah *A. jubatus heckii* is categorized as Critically Endangered on the IUCN Red List (IUCN, 2008), and documented information on the presence of cheetah and other carnivores in this region would provide impetus for conservation efforts.

A joint Sahelo-Saharan Interest Group/Office du Parc National d'Ahaggar team surveyed the central zone of the Ahaggar National Park in 2005 (Wacher et al., 2005). We collected 42 samples of unknown yet georeferenced carnivore scats.

To establish reference samples from known species, we extracted DNA from the tissue of five animals from the Zoological Society of London (ZSL) tissue bank: a cheetah, a leopard, a dwarf mongoose *Helogele parvula*, a banded mongoose *Mungos mungo* and a fennec fox *Vulpes zerda*. Sequences were also collected from the literature via GenBank (Benson et al., 2008). DNA was extracted using the Qiagen QIAmp Stool and Qiagen Tissue extraction kits according to the manufacturer's guidelines. To avoid contamination with tissue samples, scat samples were extracted under a clean fume hood in a different area of the laboratory. The polymerase chain reaction (PCR) was used to amplify the mitochondrial target region of the 12S and cytochrome b genes, using the primer combinations 12S1/12S4 and cytb1/cytb2 (Janczewski et al., 1995). PCR amplification was carried out in a total volume of 10 μL containing 1μL of genomic DNA (approximate concentration 2–10 ng μL^{-1}) in 50 mM KCl, 2.0 mM $MgCl_2$, 10 mm Tris HCl (pH = 8.8), 1 mM dNTP mix, 2.5–5 units of *Taq* DNA polymerase, and 2.5 pmoles of each primer. Following this the products were purified using the Qiagen PCR purification kit according to the manufacturer's guidelines and subsequently sequenced using the ABI BigDye chemistry and 3100 Automatic Sequencer (Applied Biosystems, Foster City, USA). The identity of each sample was assigned by BLASTING (BLASTn) the sequences on GenBank to look for homologous sequences within the database.

DNA was successfully extracted from c. 75% (30/42) of the collected samples and 12S and/or cytochrome b sequences were produced for all these individuals. Even after three attempts to extract usable DNA, that of the remaining samples was too degraded for reliable construction of either sequence. For each sample we sequenced 208–583 base pairs (bp) of mitochondrial 12S and cytochrome b protein coding genes in both directions. Table 1 shows the BLAST results for the cheetah and leopard samples with the species match and accession numbers of the top match on GenBank. The e-values are all essentially zero, showing that these matches were highly significant (see table legend for explanation of e-value). Eight of the samples matched cheetah sequences and one matched a leopard. Of the remaining 21 samples 20 matched sequences from a variety of non-felid carnivores. These included 14 canids, *Canis* spp. (possibly domestic dogs), five common genets *Genetta genetta*, a mongoose (species unresolved), and potentially a wild cat *Felis silvestris*.

The eight cheetah samples were then screened for the presence of 8 dinucleotide microsatellite loci, following the tube approach of Taberlet et al. (1996). Eight loci, characterized in the domestic cat *Felis catus* (Menotti-Raymond et al., 1999), were chosen on the basis of their high polymorphism and easy scorability. Genotyping was

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TABLE 1 The results of the BLASTn search on GenBank (Benson et al., 2008) for cheetah *Acinonyx jubatus* and leopard *Panthera pardus* samples. Identity is the extent to which two nucleotide sequences are invariant. The e-value describes the number of hits one can expect to see by chance when searching a database with a sequence of a given length. An e-value of 1 can be interpreted as meaning that in a database of the current size one may expect to see one match with a similar score purely by chance. Therefore the closer to zero the e-value, the more significant the match is.

			12S match		Cyt. b match	
Putative sample ID	BLASTn species match	Accession number	Identity	E-value	Identity	E-value
P. pardus	*P. pardus*	EF551002.1			417/424	0.00E+00
A. jubatus	*A. jubatus*	AY463959.1	214/217	1.00E-10	464/469	0.00E+00
A. jubatus	*A. jubatus*	AY463959.1	216/217	0.00E+00	410/415	0.00E+00
A. jubatus	*A. jubatus*	AY463959.1	203/208	0.00E+00	464/469	0.00E+00
A. jubatus	*A. jubatus*	AY463959.1	214/217	1.00E-110	464/469	0.00E+00
A. jubatus	*A. jubatus*	AY463959.1	214/217	1.00E-110	356/365	0.00E+00
A. jubatus	*A. jubatus*	AY463959.1	214/217	3.00E-108		
A. jubatus	*A. jubatus*	AY463959.1	216/217	2.00E-115		
A. jubatus	*A. jubatus*	AY463959.1	214/217	1.00E-110	467/473	0.00E+00

carried out using fluoro-labelled primers and performing 40 cycles of PCR amplification in a 6 μL reaction volume. This contained 1 μL ($\leq$10 ng) DNA, 0.5 μL (0.2 μM) of each primer and 4 μL of PCR QIAGEN Master Mix which provides a final concentration of 3mM $MgCl_2$. The primers, labeled with specific dyes with different allele sizes, were amplified and run separately with a size standard (Liz 500) on a ABI 3100 Automatic Sequencer (Applied Biosystems, Foster City, USA) running GENEMAPPER (Applied Biosystems, Foster City, USA). The reaction was repeated at least three times for each sample to establish a consensus genotype. Reference samples were included on each PCR allowing the standardization of allele size measurements across runs. Where a consensus genotype could not be estimated, or if no reliable alleles could be scored (for example due to poor quality of the DNA), the samples were recorded as having 'any allele' for that locus (nine out of the 64 loci). Alleles were compared across the eight loci using *GIMLET* (Valiere, 2002), which finds the proportion of identical alleles for each pair of cheetahs. Two individuals are considered to have identical alleles at a locus if at least one allele at that locus is the same in both individuals. This method therefore assumes that similar alleles are identical by descent. Despite this conservative approach, no two cheetahs shared more than two thirds of their alleles across the eight loci. Therefore all cheetah samples are from separate individuals.

Two potential sources of error are contamination and the identification of a species that was not present. The leopard reference sequence and Algerian leopard sample differed by three out of 424 bases, whilst 28 bases differed from an African lion sequence, the only other *Panthera* species in Africa (sequence from GenBank). No cheetah sequence differed from another by more than three (out of 217) bases of 12S, or five (out of 429) bases of cytochrome b, compared to seven base differences between *F. catus* and the cheetahs for the 12S and 36 base differences with the cytochrome b between the same species. It is therefore highly unlikely that we assigned the incorrect felid to the samples. The presence of within species sequence differences between the reference and Algerian samples in both the cheetahs and the leopard, together with the microsatellite diversity in the cheetahs and the precautions taken to ensure scat DNA was extracted in a separate and sterile area, suggest that the Algerian sequences are unlikely to be the result of contamination.

Based on the most complete compilation of literature on Algerian mammals (Kowalski & Rzebik-Kowalska, 1991) our findings are the first direct evidence of wild leopards in Algeria for almost 50 years, and should be an incentive for further studies in the surrounding areas of Aïr and Termit in Niger and the eastern Chadian desert. Our results also demonstrate that it is feasible to collect unknown carnivore scat samples, extract their DNA and sequence specific mitochondrial genes to identify species. Assuming the presence of generic primer combinations, species can thus be identified solely from scat samples collected in the field. Earlier methods of identifying species from unknown scats involved deducing the species from outward morphological appearance, a method subjective and prone to error. Davison et al. (2002) found that even expert naturalists failed to distinguish correctly the scat of pine marten *Martes martes* and fox *Vulpes vulpes*.

Genetic studies are increasingly made on scat from unknown species but these involve assaying for only a few candidate species (e.g. stone and pine martens, Pilot et al., 2007; bobcats, bears and martens, Long et al., 2007). Although there are examples of methods using restriction fragment length polymorphism PCR (RFLP-PCR; Livia et al., 2007) or species-specific primers (Dalen et al., 2004) to distinguish several species, the power of our method comes from its ability to identify species in all taxa across both major branches of the Carnivora. Although not as

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cheap as RFLP-PCR, the method also benefits from being accurate and reproducible. It is therefore a powerful and reliable tool for assessing carnivore biodiversity, especially when the number of scats collected is low. More generally, this method will be especially effective in remote areas where animal density is low and where individuals are rarely seen, two characteristics inherent to threatened species.

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Biographical sketches

The research for this project comprised two teams, both of which are committed to the interdisciplinary conservation of wildlife in Africa. The first consisted of a field team, mainly based in Algeria but containing members from both the Office du Parc National de l'Ahaggar and the Zoological Society of London (ZSL) in the UK. Farid Belbachir, Tim Wacher, Laurie Marker, Koen De Smet, Amel Belbachir-Bazi, Amina Fellous and Mohamed Belghoul are all involved in the conservation of large mammals and carnivores in Algeria, and elsewhere in Africa, and collected the ecological observations and scat used in this study. George Busby and Dada Gottelli work in the conservation genetics laboratories in the Institute of Zoology at ZSL and performed the genetic identification of the scat. Farid Belbachir and Sarah Durant continue to work together to combine the expertise of these two teams.