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Gene 338 (2004) 111–119

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

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Analysis of the mitochondrial genome of cheetahs (*Acinonyx jubatus*) with neurodegenerative disease

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Received 14 January 2004; received in revised form 1 April 2004; accepted 28 May 2004

Received by G. Pesole

Abstract

The complete mitochondrial genome of *Acinonyx jubatus* was sequenced and mitochondrial DNA (mtDNA) regions were screened for polymorphisms as candidates for the cause of a neurodegenerative demyelinating disease affecting captive cheetahs. The mtDNA reference sequences were established on the basis of the complete sequences of two diseased and two nondiseased animals as well as partial sequences of 26 further individuals. The *A. jubatus* mitochondrial genome is 17,047-bp long and shows a high sequence similarity (91%) to the domestic cat. Based on single nucleotide polymorphisms (SNPs) in the control region (CR) and pedigree information, the 18 myelopathic and 12 non-myelopathic cheetahs included in this study were classified into haplotypes I, II and III. In view of the phenotypic comparability of the neurodegenerative disease observed in cheetahs and human mtDNA-associated diseases, specific coding regions including the tRNAs leucine UUR, lysine, serine UCN, and partial complex I and V sequences were screened. We identified a heteroplasmic and a homoplasmic SNP at codon 507 in the subunit 5 (*MTND5*) of complex I. The heteroplasmic haplotype I-specific valine to methionine substitution represents a nonconservative amino acid change and was found in 11 myelopathic and eight non-myelopathic cheetahs with levels ranging from 29% to 79%. The homoplasmic conservative amino acid substitution valine to alanine was identified in two myelopathic animals of haplotype II. In addition, a synonymous SNP in the codon 76 of the *MTND4L* gene was found in the single haplotype III animal. The amino acid exchanges in the *MTND5* gene were not associated with the occurrence of neurodegenerative disease in captive cheetahs.

Keywords: Mitochondrial DNA; Heteroplasmy; Mitochondrial disease; Myelopathy; Real-time PCR; Single nucleotide polymorphism

1. Introduction

Numerous cases of neurodegenerative and multisystemic disease of unknown origin have been described in juvenile and adult cheetahs in the European Endangered Species Program (EEP; Walzer et al., 1998, 2003). This neurodegenerative disease characterised by upper motor neurone lesions, progressive ataxia, hind limb paresis and paralysis, and bilateral symmetric myelin degeneration associated with axonal degeneration of the spinal cord is described as cheetah myelopathy (Walzer et al., 2003). Despite extensive postmortem examinations, the aetiology of this neurodegeneration and of the possibly high disease prevalence in cheetahs remains unclear (Kotsch et al., 2002; Walzer et al., 2003). Neurodegenerative diseases of unknown cause have also been observed in the South African and North American captive cheetah populations (Munson et al., 1999), in snow leopard cubs (Robert et al., 2003) and in

Abbreviations: mtDNA, mitochondrial DNA; SNP, single nucleotide polymorphism; *MTND1–6* and *4L*, mitochondrial NADH dehydrogenase subunits 1–6 and 4L; *MTCO3*, cytochrome *c* oxidase subunit 3; *MTATP8* and *6*, ATPase subunits 8 and 6; CR, control region; OLR, origin of light strand replication; tRNA, transfer RNA; RS, repeat sequence; OXPHOS, oxidative respiratory system; ARMS, amplification refractory mutation system; NTAC, nontarget amplification control.

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doi:10.1016/j.gene.2004.05.020

domestic animal species (Brenner et al., 1997; Palmer and Cavanagh, 1995; Toenniessen and Morin, 1995).

Besides idiopathic origin, the major causes for neurodegeneration include environmental, pathogenic and genetic factors (Jellinger, 2003). The genetic factors include nuclear and mitochondrial DNA (mtDNA) defects or derangements in the interaction of the two genomes. Nuclear DNA defects as such cause a large group of neurodegenerative diseases (Jellinger, 2003). Beyond that, mutated nuclear gene products targeted to mitochondria can affect the organellar function and replication and thus can impair almost any aspect of cellular function and oxidative energy metabolism (Schon and Manfredi, 2003). The mitochondria harbour the oxidative respiratory system (OXPHOS), and therefore disorders arising from defects in the nucleus and mitochondria encoded subunits of OXPHOS are also referred to as 'mitochondrial' diseases. Since the discovery of these mitochondrial diseases, more than 100 point mutations and innumerable mtDNA rearrangements have been identified as cause for various types of progressive neurodegenerative and multisystemic diseases (DiMauro and Schon, 2001).

The neurodegenerative disease of cheetahs shows symptoms comparable to human mitochondrial diseases, e.g., complex I (NADH dehydrogenase) deficiencies, myoclonic epilepsy with ragged red fibres (MERRF), mitochondrial encephalomyopathy, lactic acidosis and strokelike episodes (MELAS) and neuropathy, ataxia, retinitis pigmentosa (NARP). In addition, ataxia in humans has been associated with large-scale deletions in the mtDNA, e.g., the 'common deletion' of 4977 bp representing over 50% of deletions in the human mtDNA (Mita et al., 1990).

In this study we addressed the issue of a possible extrachromosomal genetic cause for the phenotype of neurodegenerative disease in the EEP cheetah population. Due to the progressive course of this disease resulting in the exclusion of myelopathic animals from breeding programs, we were not able to demonstrate the presence or absence of Mendelian inheritance for this trait. In view of the phenotypic comparability of the progressive neurodegeneration in the cheetah and human mitochondrial diseases, we investigated whether mutations in the cheetah mtDNA were associated with the myelopathic phenotype. For this purpose we determined the complete mitochondrial genome of two myelopathic and two non-myelopathic cheetahs and selected coding regions as candidate genes for analysis including homo- and heteroplasmic base substitutions and deletions.

2. Materials and methods

2.1. Biological material

The international studbook numbers (Marker-Kraus, 2001) of the cheetahs included in this study, dates of birth and death, information on the animal origin, type of sample specimen and the occurrence of the myelopathic phenotype are included in Table 1.

2.2. DNA isolation

For the determination of the *A. jubatus* mtDNA reference sequence, DNA was isolated from mitochondria-enriched tissue fractions to exclude amplification from nuclear mitochondrial pseudogenes (Tourmen et al., 2002). Native tissue samples (10 g) from liver, right muscle quadriceps, central nervous system and cardiac muscle were taken immediately after euthanization and transported to the laboratory in ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris–HCl (pH 7.4). The mitochondrial fraction was obtained by differential centrifugation (Fleischer and Kervina, 1974) and used for mtDNA isolation.

Total cellular DNA was extracted from blood and native or paraffin embedded tissue samples. Paraffin samples were cut from 2 × 2-cm blocks into 5–10-μm sections and extracted with 1-ml xylene. DNA isolations were carried out using the NucleoSpin® Blood or Tissue kits (Machery & Nagel, Dueren, Germany) resulting in 60 μl containing ≤20-μg DNA.

2.3. PCR and sequencing

Amplification primers were designed with Primer Express (Applied Biosystems, Vienna, Austria) using cheetah sequences for *MTATP8*, *MTND5* and *MT16SrRNA* (GenBank accession nos. AF241817, AF241816 and AF141815) and conserved regions determined by aligning mitochondrial genes of related felid species (*Felis catus*, *Panthera leo*, *P. tigris*, *P. pardus*, *P. onca*; GenBank accession nos. U20753, AF006458, AF006460, AF006444 and AF006442).

Control region (CR) sequences, excluding the two repeat elements RS2 (Freeman et al., 2001) and RS3 (Lopez et al., 1996), were applied for haplotype classification. Five partially overlapping amplicons generated with the primer pairs CR1f (16,252–16,273)/CR1r (16,503–16,473), CR2f (16,787–16,810)/CR2r (17,029–17,008), CR3f (16,961–16,985)/CR3r (187–166), CR4f (35–52)/CR4r (236–218) and CR5f (644–662)/CR5r (895–877) and fitting the reduced maximum amplification length due to formalin fixation before paraffin embedding, were sequenced.

The analysis of the mitochondrial leucine UUR, serine UCN and lysine tRNAs and the partial amplification of the complexes I and V were performed with the primer pairs LEUf (3366–3387)/LEUr (3673–3654), SERf (7512–7533)/SERr (7860–7843), LYSf (8401–8425)/LYSr (8720–8696), N4Lf (10,933–10,954)/N4Lr (11,334–11,316), $N5_1$f (14,032–14,052)/$N5_1$r (14,295–14,275), $N5_2$f (14,077–14,100)/$N5_2$r (14,357–14,335), A8f (8639–8662)/A8r (8937–8915), $A6_1$f (9204–9225)/$A6_1$r (9552–9531) and $A6_2$f (9466–9490)/$A6_2$r (9875–9856). The num-

Table 1
Details on the *A. jubatus jubatus* individuals

Haplotype	Cheetah no.	Sample specimen	Myelopathy	Studbook number	Origin	Dates of birth and death	Cause of death
I	1	muscle, liver CNS, blood	+	4050	F1	22.03.98/31.10.00	E
I	2	muscle, liver CNS, blood	+	4237	F1	03.06.00/31.10.00	E
I	3a	blood[a]	–	3671	A1	27.10.96/05.03.02	E
I	3b	muscle	+	3671	A1	27.10.96/05.03.02	E
I	4	blood	–	3672	A1	27.10.96/alive	NA
I	5	liver	+	4353	F2	22.03.01/29.04.01	E
I	6	liver	+	4508	F2	17.10.01/01.03.02	E
I	7	liver	–	4509	F2	17.10.01/18.12.01	aortic rupture
I	8	liver	–	4510	F2	17.10.01/17.12.01	aortic aneurysm
I	9	muscle	+	4513	F2	17.10.01/01.03.02	E
I	10	lung	–	1463	F1	1983/07.11.00	senile
I	11	muscle	–	1755	F1	1986/06.06.02	senile
I	12	blood	–	3285	F1	20.09.94/alive	NA
I	13	blood	–	3722	F1	18.12.96/alive	NA
I	14	muscle	+	4058	F1	14.04.99/07.06.02	E
I	15	liver	+	4059	F1	14.04.99/07.06.02	E
I	16	blood	–	4060	F1	14.04.99/alive	NA
I	17	blood	–	4487	F1	18.08.01/alive	NA
I	18	blood	–	not reg; ID 3383	F1	2002/alive	NA
I	19	blood	+	1775	A1	14.10.88/29.05.02	E
I	20	blood	+	1921	A1	18.05.89/29.05.02	E
I	21	CNS	+	3619	A1	25.05.96/11.11.96	E
I	22	CNS	+	3799	A1	19.09.97/24.08.98	E
I	23	muscle	–	3147	A3	05.11.93/23.09.02	renal failure
I	24	CNS (PET)	+	2819	A1	1987/05.06.99	E
I	25	CNS (PET)	+	3800	A1	19.09.97/24.11.98	E
I	26	CNS (PET)	+	4363	UAE	1987/01.06.96	E
I	27	CNS (PET)	+	not reg; ID Fota 1	IRE	01.06.97/21.08.97	E
II	28	muscle	+	not reg; ID Douma	F3	01.06.02/11.09.02	E
II	29	CNS	+	1976	D	17.12.86/21.04.98	E
III	30	CNS	–	not reg; ID S/1571	A2	1997/1997	mega-colon

PET: paraffin embedded tissue; not reg.: not registered in the International Studbook 2001; ID: local identification; F1, F2, F3: La Palmyre, Peaugres, Montpellier (France); A1, A2, A3: Salzburg, Herberstein, Vienna (Austria); D: Saarbrücken (Germany); UAE: Dubai (United Arabian Emirates); IRE: Fota (Ireland); NA: not applicable; E: euthanized due to severe symptoms of myelopathy.

[a] Blood sample taken 1.5 years before disease onset.

bering (5′–3′) is according to the GenBank accession no. AF344830.

PCR was performed in a 25-μl volume containing 60 mM Tris–HCl (pH 8.5), 15 mM $(NH4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 600 nM of each primer, 1.25 U *Taq* polymerase (Invitrogen, Lofer, Austria), and 1.5-μl (10–30 μg) DNA template. Conditions for the amplification of the approximately 700-bp PCR fragments on the RoboCycler® (Stratagene, Amsterdam, Holland) were: 5 min/95 °C, 1 min/50 to 59 °C, 3 min/72 °C and 38 cycles of 30 s/95 °C, 45 s/50 to 59 °C, 80 s/72 °C. PCR products were purified with a binding buffer containing 7 M Guanidinium–HCl and 200 mM MES (pH 5.6) and MultiScreen®-FB Opaque Plates (Millipore, Vienna, Austria), and sequenced with the ABI PRISM® BigDye Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems). The amplification primers were applied for PCR product sequencing of both strands. Unincorporated dyes were removed with the Sephadex™ G-50 Superfine System (Sigma-Aldrich, Vienna, Austria) and MultiScreen®-HV Clear Plates (Millipore). Capillary electrophoresis of the sequencing reactions was performed on the MegaBACE 1000 automated DNA sequencer (Amersham, Buckinghamshire, Great Britain). Electropherograms were reevaluated by visual inspection using the computer programs Sequence Navigator Version 1.0.1 and Autoassembler Version 2.1 (Applied Biosystems). Numbering and determination of the gene boundaries within the cheetah mtDNA were

Table 2
Haplotype classification of the 30 cheetahs

Haplotype	Cheetah no.	mtDNA region	Substitution (amino acid change)
I	#1–27	CR	wild type
	#1, 2, 5–12, 14–18, 24–27	MTND5	G14175G/A (V507M)[a]
II	#28, 29	CR	G16,828A
		MTND5	T14,176C (V507A)[b]
III	#30	CR	G16,828A
		CR	T159C
		CR	A203G
		MTND4L	G11021A (no)

[a] Nonconservative.
[b] Conservative.

performed on the basis of the *F. catus* mtDNA (GenBank accession no. NC_001700). The classification of amino-acid substitutions was performed as described (Dayhoff et al., 1978).

2.4. Amplification refractory mutation system (ARMS) allele-specific real-time PCR

ARMS allele-specific quantitative real-time PCR (Steinborn et al., 2000) was used to quantify the relative amount of the heteroplasmic single nucleotide polymorphism (SNP). PCR assays of 25 µl contained 60 mM Tris–HCl (pH 8.5), 15 mM $(NH4)_2SO_4$, 0.2 mM dATP, dCTP, dGTP and 0.4 mM dUTP, 300 nM of each primer, 150 nM of the fluorogenic probe, 1.25 units of *Taq* polymerase, 3.5 mM $MgCl_2$ and 1.5-µl template DNA. Real-time PCR was performed with the ABI PRISM™ Sequencing Detection System 7900 (Applied Biosystems). The threshold cycle (C_T) values, i.e., the C_T was defined as the cycle at which an amplification plot reached a significant threshold, were determined using the Sequence Detector 2.1 software (Applied Biosystems). The allele-specific ARMS (Newton et al., 1989) reverse primer was designed to discriminate between the alleles G and A at nt 14,175 (GenBank accession no. AF344830). To further inhibit amplification of the nontarget allele, a mismatch at position 3 from the 3′ end of the primer was arbitrarily introduced. This substitution generating a mismatch with both alleles at this bi-allelic site is printed in capital letter: ARMS (gag ttt taa gtt ttt ggc Aac, 14,175–14,196). The oligonucleotides TM1 (14,103–14,122) and TM2 (14,169–14,149) were used to quantify the total amount of mtDNA. The hydrolysis probe TMP (14,243–14,267) was labelled at the 5′ end with 6-carboxyfluorescein (6FAM™) and at the 3′ end with 6-carboxytetramethylrhodamine (TAMRA™). ARMS allele-specific real-time PCR was performed with 5 min at 95 °C, 40 cycles for 15 s at 95 °C, 30 s at 52 °C and 30 s at 72 °C. To test the reliability of the PCR, each sample was amplified in duplicates with a standard deviation of threshold cycle (C_T) values not exceeding 0.5. Samples with C_T values >32 were amplified in quadruplicates and the highest and lowest values were excluded. Each experiment included a standard curve (regression coefficient R^2>0.991), a non-template control and a nontarget amplification control (NTAC). The NTAC consisted of the synthetic oligonucleotide (14,103–14,195; MWG Biotech, Ebersberg, Germany) including the nontarget allele A at nt 14,175 and carrier salmon sperm DNA (Invitrogen). The percentage of the G allele at 14,175 was determined by the "Comparative C_T method" (ABI PRISM 7700 sequence detection system, Applied Biosystems, 1997) using the equation: $\text{Heteroplasmy} = 100\% / (1+E)^{\Delta\Delta C_T}$, where the efficiency $(E) = 10^{-1/s} - 1$. The slope (s) being a measure for the efficiency of the PCR reaction is determined by the standard curve $y = sx + b$. ARMS allele-specific real-time PCR targeted the G-allele at 14,175. Assuming a bi-allelic locus, we calculated for the percentage of the mutant allele A: 100% – allele G. Statistical analyses were performed with SPSS Version 9.0.1 (SPSS, Chicago, USA).

2.5. Screening for mtDNA deletions

As deletions often occur within direct repeats of mtDNA sequences (Mita et al., 1990), the complete mitochondrial genomes of cheetahs #1–4 were analysed

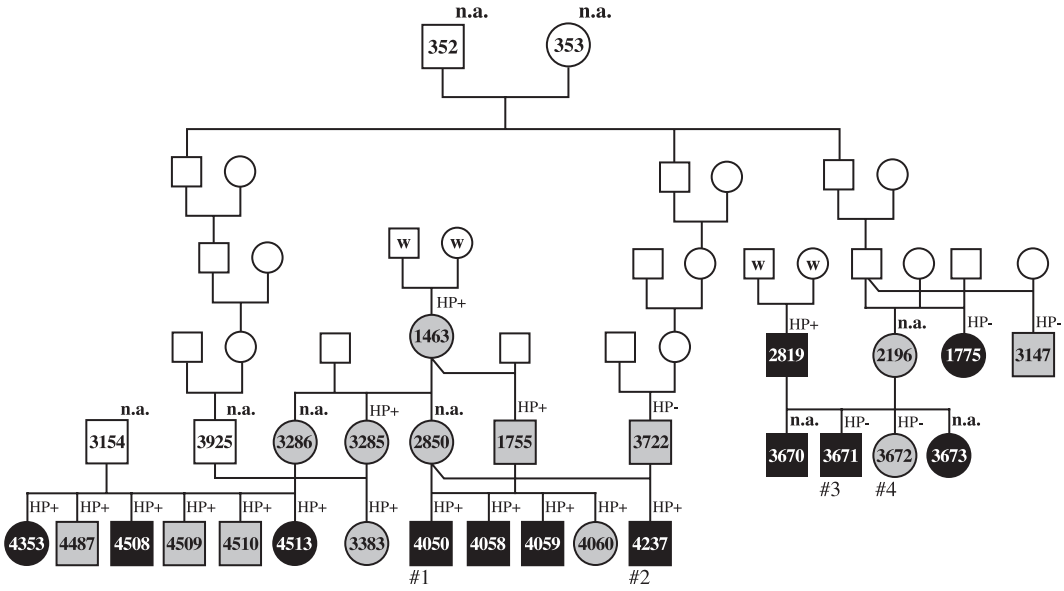

Fig. 1. Pedigree illustrating the relationship of cheetahs #1 to 4 used for the determination of the mitochondrial genome, the transmission of the myelopathic phenotype and the heteroplasmy *G14,175A*. Cheetah #3 is presented with the myelopathic phenotype after developing neurodegenerative symptoms in the course of the study. Square: male; circle: female; white: health status unknown; black: myelopathic; grey: non-myelopathic; w: wild-born (Namibia); HP – : homoplasmic at 14,175 (wild-type, *14,175G*); HP+: heteroplasmic at 14,175 (*G14,175A*); n.a.: not analysed (no material available).

with the Repeat Masker Server Version 07.07.02 (http://ftp.genome.washington.edu/).

Long-range PCR to screen for large-scale mtDNA deletions was performed with the primer pair LR1 (16,183–16,206)/LR2 (15,728–15,708) and the TripleMaster® PCR System (Eppendorf, Wesseling-Berzdorf, Germany). Following DNA denaturation for 3 min at 93 °C, amplification was performed for 27 cycles (15 s/93 °C, 20 s/56 °C, 17 min/68 °C). During the last 17 cycles the extension time was increased by 20 s per cycle. PCR products were run on a 0.7% agarose gel at 3 V/cm for 18 h.

2.6. GenBank accession numbers

Complete mtDNA sequences (AF344830, cheetah #1; AY463959, cheetah #2), partial sequences of the mitochondrial control region (AY463960, cheetah #18; AY463961, cheetah #29; AY463962, cheetah #30) and partial sequences of polymorphic mitochondrial genes (AY486335, cheetah #29; AY486336, cheetah #30) were submitted to the GenBank.

3. Results

3.1. Analysis of the complete mtDNA sequence and mtDNA haplotyping of A. jubatus

Relevant biological data of the cheetahs ($n = 30$) analysed are presented in Table 1. The mitochondria-enriched liver fractions from myelopathic cheetahs #1 and 2 and blood samples from non-myelopathic animals #3a and 4 were used

Table 3
The composition of the complete mitochondrial genome of the cheetah

Gene	Nucleotide position		Number	Size	Codon		5′ intervening spacer
	From	To	(bp)[a]	Start	Stop[b]		
$tRNA^{Phe}$	893	962	70				
rRNA-12S	963	1922	960				
$tRNA^{Val}$	1923	1990	68				
rRNA-16S	1991	3562	1572				
$tRNA^{Leu\ (UUR)}$	3563	3637	75				AC
ND1	3640	4596	957	ATG	TA–[b]		
$tRNA^{Ile}$	4596	4664	69				
$tRNA^{Gln}$	4662	4735	74 (L)				A
$tRNA^{Met}$	4737	4805	69				
ND2	4806	5847	1042	ATT	T–[b]		
$tRNA^{Trp}$	5848	5915	68				CATACCAAACTGCCT
$tRNA^{Ala}$	5931	5999	69 (L)				
$tRNA^{Asn}$	6001	6073	73 (L)				
RepOrigin OLR	6074	6105	32				
$tRNA^{Cys}$	6106	6171	66 (L)				
$tRNA^{Tyr}$	6172	6238	67 (L)				T
CO1	6240	7784	1545	ATG	TAA		
$tRNA^{Ser\ (UCN)}$	7783	7852	70 (L)				TTAA
$tRNA^{Asp}$	7857	7925	69				
CO2	7925	8608	684	ATG	TAA		ATT
$tRNA^{Lys}$	8612	8679	68				T
ATP8	8681	8884	204	ATG	TAA		
ATP6	8842	9522	681	ATG	TAA		
CO3	9522	10,305	784	ATG	T–[b]		
$tRNA^{Gly}$	10,306	10,374	69				
ND3	10,375	10,721	347	ATA	TA–[b]		
$tRNA^{Arg}$	10,722	10,790	69				
ND4L	10,791	11,087	297	ATG	TAA		
ND4	11,081	12,458	1378	ATG	T–[b]		
$tRNA^{His}$	12,459	12,527	69				
$tRNA^{Ser\ (AGY)}$	12,528	12,586	59				
$tRNA^{Leu\ (CUN)}$	12,587	12,656	70				
ND5	12,657	14,477	1821	ATA	TAA		
ND6	14,461	14,988	528 (L)	ATG	TAA		
$tRNA^{Glu}$	14,989	15,057	69 (L)				
CytB	15,061	16,200	1140	ATG	AGA		TTA
$tRNA^{Thr}$	16,201	16,270	69				
$tRNA^{Pro}$	16,271	16,337	67 (L)				
CR	16,338	892	1602				

[a] (L) describes the genes encoded on the L-strand.
[b] Signifies an incomplete termination codon.

to determine a reference mitochondrial genome. In the course of the study, cheetah #3a developed symptoms of neurodegenerative disease and was therefore euthanized (Table 1). The cross-check of muscle tissue from the euthanized animal (termed #3b) showed no differences to the sequence determined from the blood sample.

The classification of the cheetahs into haplotypes I to III ($n=27$, $n=2$ and $n=1$, respectively) was based on SNPs of mitochondrial control region sequences (Table 2) and pedigree analysis (Fig. 1). Haplotype I included 25 related and two unrelated cheetahs, whereas haplotypes II and III included only individuals with unknown parental origin. Fig. 1 illustrates the pedigree of the related haplotype I animals and the transmission of the myelopathic phenotype; the presence and absence of myelopathy were histopathologically confirmed. Mendelian inheritance of the myelopathy could not be demonstrated because of the progressive aetiopathology and insufficient sample size.

The *A. jubatus* mtDNA is 17,047-bp long and is composed of 33.1% A, 25.8% C, 13.6% G and 27.5% T, respectively. The numbering and determination of the gene boundaries within the mitochondrial genome of cheetah #1 (Table 3) were performed on the basis of the *F. catus* mtDNA (GenBank accession no. NC_001700). Similar to other mammals, the *A. jubatus* mtDNA is composed of 13 protein-coding genes resembled by structural open reading frames, 22 tRNAs, the 12S- and 16S-rRNA and the regulatory control region. Sequence comparisons to the cat and human (GenBank accession nos. NC_001700 and J01415) showed an overall homology of 91% and 80%, respectively. Protein translation is generally initiated by the start codon ATG (Met) except for *MTND2* which is initiated at ATT (Ile). The cheetah mtDNA lacks complete termination codons in the genes *MTND1*, *MTND2*, *MTCO3*, *MTND3* and *MTND4*. As in transcripts of human peptide-coding mitochondrial genes, *MTND1*, *MTND2*, *MTCO3* and *MTND3* contain a stop codon created by posttranscriptional polyadenylation. Therefore, most stop codons in the cheetah mtDNA are TAA. Furthermore, coding sequences overlap between the *MTATP8* and *MTATP6*, the *MTND4* and *MTND4L*, and the *MTND5* and *MTND6* genes, respectively. The CR contains the RS2 and RS3 repeat units as described in other cheetah subspecies (Freeman et al., 2001 and references therein). The light strand encodes the *MTND6* gene and the tRNAs glutamine, alanine, aspargine, cysteine, tyrosine, serine UCN, glutamic acid and proline. The origin of light strand replication (OLR; 6074 to 6105) contains a poly-A stretch; A is repeated nine times in cheetah #4 and 10 times in animals #1–3.

3.2. Analysis of genetic variation in the cheetah mtDNA

Cheetah mtDNA regions were selected for further analysis according to human mtDNA regions associated with mitochondrial diseases. The seven mitochondrially encoded complex I subunits of three myelopathic cheetahs (#1, 2 and 3b) were compared with those of three nondiseased control animals (#3a, 4 and 23). Cheetahs #3, 4 and 23 were genetically invariant across these seven genes. In contrast, cheetahs #1 and 2 were polymorphic in the *MTND5* gene. The SNP was heteroplasmic and consisted of a G to A transition at position 14,175 (*G14,175A*) leading to a nonconservative amino acid change valine to methionine (*V507M*). This

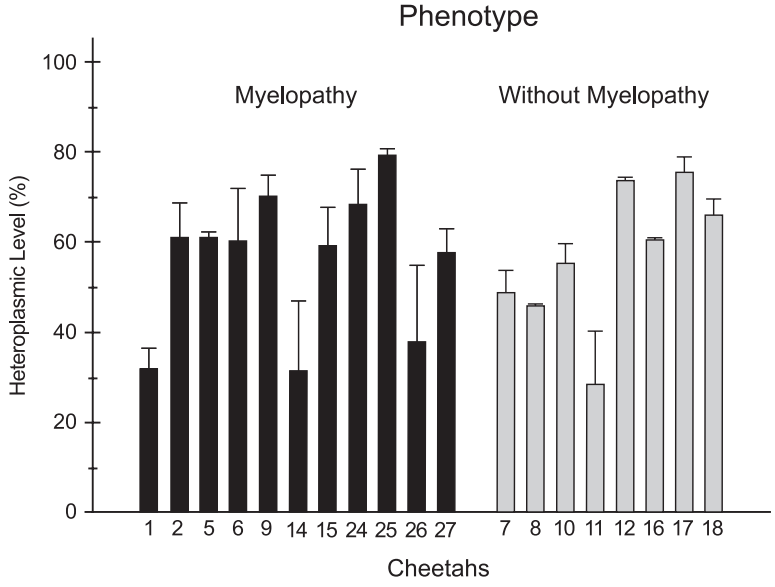

Fig. 2. Quantification of the heteroplasmic level *G14,175A*. The heteroplasmic levels range from 32% to 81% in myelopathic animals (given in black) and from 29% to 76% in cheetahs without confirmed myelopathy (given in grey). Due to the limited availability of postmitotic tissue samples, the issue of an intraindividual difference in the heteroplasmic level of postmitotic versus mitotic tissues could not be addressed (e.g., cheetah #1: muscle (32.1% ± 4.5) and CNS (64.4% ± 0.9) versus liver (46.5% ± 9.1) and blood (40.7% ± 9.4); cheetah #2: muscle (61.2% ± 7.7) and CNS (31.9% ± 2.2) versus liver (19.6% ± 6.6) and blood (23.9% ± 2.1)).

heteroplasmy was detected in 17 other animals of haplotype I. The individual levels of the heteroplasmy were quantified by ARMS allele-specific real-time PCR and are given in Fig. 2. The heteroplasmic animals represented both clinical phenotypes ($n=11$ myelopathic, $n=8$ non-myelopathic), and neither the occurrence (chi-square test: $p=0.551$) nor the percentage ($p=0.440$) of heteroplasmy was significantly associated with the neurodegenerative disease in the examined cheetahs. Furthermore, no significant correlation between the heteroplasmy and the different tissue samples was found (Kruskal–Wallis test: $p=0.823$). The comparison of the heteroplasmic level with the age of the individual animals resulted in a nonsignificant negative correlation (Spearman Rho-test: -0.419, $p=0.301$).

Complex I partial sequence analysis of all cheetahs revealed another homoplasmic polymorphic variant in the *MTND5* gene (*T14,176C*) of cheetahs #28, 29 leading to the conservative amino acid change valine to alanine in the codon 507. The conservative and nonconservative amino-acid exchanges at the codon 507 were not significantly correlated with the occurrence of the myelopathy (chi-square test: $p=0.571$). An additional SNP in the *MTND4L* gene of cheetah #30 without an amino acid substitution was found (Table 2).

As the clinical symptoms of myelopathic cheetahs could be compared with the human mitochondrial diseases MERRF, MELAS and NARP (DiMauro and Schon, 2001), the mtDNA regions associated with these diseases were screened in all animals. No genetic variation for the $tRNA^{Leu(UUR)}$, $tRNA^{Ser(UCN)}$, $tRNA^{Lys}$ and in the partial sequences (8680–8850 and 9280–9800) of the complex V genes was found (data not shown).

Large-scale deletions in the human mtDNA have been identified as a cause for several mitochondrial diseases and most deletion breakpoints occur within direct repeat sequences (Mita et al., 1990). The mtDNA of the cheetah includes three direct repeats of 4, 5 and 6 bp in length (3562–3565/3641–3644; 4660–4664/4731–4735; 7782–7787/7854–7859). As demonstrated in Fig. 3, the amplification products of the approximately 16.5-kb mtDNA fragments from three myelopathic (#1, 5, and 28) and three non-myelopathic cheetahs (#7, 8, and 18) did not show any additional shorter bands indicating the absence of large-scale deletions.

4. Discussion

This study established the complete sequence of the mitochondrial genome of *A. jubatus* and performed analysis of polymorphic sites in coding regions of animals with and without myelopathy. Myelopathy in the cheetah is a progressive neurodegenerative disease of the spinal cord, which affects cheetahs in captivity in Europe and the Middle East and has an unknown aetiology (Kotsch et al., 2002; Walzer et al., 2003). In view of the phenotypic similarities of the diseases in the EEP cheetah population with human mtDNA-associated diseases, the cheetah mitochondrial genome was analysed to reveal a possible extrachromosomal genetic basis for the myelopathy.

Generally, the cheetah is unusual among Felidae in exhibiting high genetic uniformity at a variety of genetic loci including the mtDNA. This is caused by a population bottleneck about 12,000 years ago (Driscoll et al., 2002 and references therein). The cheetah mtDNA shows a high sequence similarity (91%) to the domestic cat (Lopez et al., 1996) and possesses several features also observed in other mammalian species (Bibb et al., 1981; Xu and Arnason, 1994). Due to an accumulation of repeat sequences and AT-rich intergenic spacers, the length of non-coding and intergenic regions can vary considerably within a genus (Burger et al., 2003), a fact also observed in the CR and OLR of the cheetah.

Based on pedigree information and polymorphisms in the CR, all animals included in this study were classified into three haplotypes and the majority of the cheetahs were considered to be related. Due to the progressive course of the neurodegenerative disease, a heritability for this trait could not be shown. The maternal transmission of the heteroplasmy observed at the site 14,175 could be demonstrated for the related haplotype I animals, apart from one cheetah of wild-born parental origin. In general, mtDNA is maternally inherited, although paternal mtDNA inheritance was found in a single case with mitochondrial myopathy (Schwartz and Vissing, 2002; Taylor et al., 2003; Filosto et al., 2003).

The sequence analysis of four complete and 26 partial cheetah mitochondrial genomes identified two SNPs at codon 507 in the *MTND5* and one SNP at position 76 in the *MTND4L* gene. These genes belong to the mitochon-

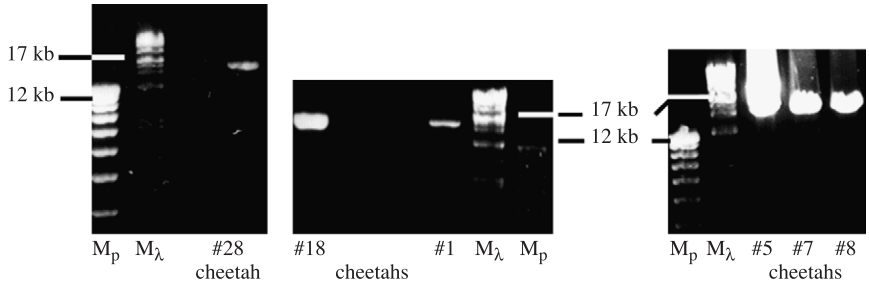

Fig. 3. Deletion screening via long range PCR. M_p = 1-kb Plus Ladder (Invitrogen); M_λ = Lambda Mix Marker, 19 (MBI Fermentas, St. Leon-Rot, Germany).

drial complex I for which mutations have been reported as one of the most common causes of mitochondrial encephalomyopathies (DiMauro and Schon, 2001). One SNP in the *MTND5* consisted of a nonconservative valine to methionine substitution and was found heteroplasmic in 19 cheetahs belonging to haplotype I. The other polymorphic *MTND5* variant leading to the conservative amino acid exchange valine to alanine was found in a homoplasmic state in two cheetahs of haplotype II. In addition, a synonymous homoplasmic polymorphism in the *MTND4L* was demonstrated in the single haplotype III animal. Comparative alignment between different species indicated that the amino acid at codon 507 in the *MTND*5 gene is moderately conserved throughout mammalian evolution, i.e., methionine is encoded by the bovine and alanine by the feline mtDNA (GenBank accession nos. NC_001567 and NC_001700). Nevertheless the nonconservative amino acid exchange valine to methionine probably affects the three-dimensional structure of the resulting ND5 protein, as valine contains only a simple aliphatic side chain, whereas methionine includes a thioether (-S-) group in its side chain. Despite the heteroplasmy was found at high levels (32–79%), only 11 of 19 cheetahs with the mutant genotype had developed myelopathy and statistical analysis did not reveal a significant correlation between the heteroplasmic amino acid exchange and the myelopathy. Pathogenic mutations are usually, but not invariably, heteroplasmic and a minimum critical number of mutant mtDNA molecules must be present before tissue specific dysfunction and clinical signs appear—a concept termed threshold effect (DiMauro and Schon, 2001). Homoplasmic exchanges of mtDNA may additionally produce different phenotypes. Interactions between nuclear and mtDNA modify functions of the nervous system, and thus it is suggested that mitochondrial polymorphism in general is probably not as neutral as was previously believed (Roubertoux et al., 2003). The major processes inducing neurodegeneration are considered to be multifactorial (Jellinger, 2003) and thus a contribution of mtDNA mutations in the aetiology of the cheetah myelopathy cannot be excluded. A possible mechanism of the aetiopathogenesis could be explained by the oligo-genetic model of nuclear–mitochondrial interaction which involves a primary mitochondrial mutation, not sufficient to induce pathology, and a nuclear modifier contributing to the pathogenic effect of the mtDNA mutation (Carelli et al., 2003).

Future research should consider a chromosomal genetic cause and the complex nuclear–mitochondrial interactions and investigate whether nuclear modifier genes are involved. The depletion of mtDNA in postmitotic tissue samples can be determined by a PCR-based assay measuring the mtDNA to nuclear DNA ratio (Miller et al., 2003). The spectrophotometrical analysis of mitochondrial enzyme activity (Barrientos, 2002) can indicate whether a neurodegenerative disease is related to mitochondria, and thus examinations of mitochondrial enzyme functions should be performed. Extensive viral, bacterial and parasitic examinations without a distinctive indication of association with the cheetah myelopathy were recently published (Walzer et al., 2003). Still, future research should consider further pathogenic origins, e.g., the neurotropic strain of coronavirus (JHM-MHV) leading to demyelisation in the CNS of mice (Ramakrishna et al., 2002) or autoimmune associated causes of demyelisation.

In conclusion, this study reports the complete mtDNA of *A. jubatus* and describes one heteroplasmic and two homoplasmic SNPs in the mitochondrial complex I of cheetahs with and without neurodegenerative disease. Our results showed no significant correlation between the SNPs and the cheetah myelopathy and thus these mutations as singular cause for the neurodegeneration appear unlikely. It remains to be clarified whether the neurodegenerative disease in cheetahs is multifactorial involving a contribution of these mtDNA polymorphisms.

Acknowledgements

We are grateful to Verena Kotsch, Anna Kübber-Heiss, Angelika Url and Nadia Robert for providing cheetah samples, essential information and great support, to Katrin Staniek for instruction in the preparation of mtDNA from the mitochondria-enriched fractions, to Irene Sommerfeld-Stur for statistical analysis, to Claus Vogl for pedigree analysis, to Christian Schlötterer for support, to Walter Rossmanith for valuable discussion, and to Steven Weiss for comments on the manuscript. P.A.B. was funded by a grant (Doktorandenstipendium #20888) of the Austrian Academy of Science.

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