Aines Castro-Prieto,[1] Bettina Wachter,[1] and Simone Sommer*,[1]

[1]Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany

*Corresponding author: E-mail: sommer@izw-berlin.de.

Associate editor: Scott Edwards

Introduction

Host genetic diversity plays an important role in buffering populations against widespread epidemics (Altizer et al. 2003). Nevertheless, there are an increasing number of species for which the extent of genetic variability and the ability to respond to diseases or environmental changes differs markedly from expectations (Amos and Harwood 1998). Immunocompetence is influenced by genetic factors such as the major histocompatibility complex (MHC) and environmental factors (Frankham et al. 2002). MHC genes are responsible for the adaptive immune response in vertebrates and are thereby involved in modulating host resistance to emerging pathogens (Hill 1998). They encode MHC class I (MHC I) and MHC class II (MHC II) cell-surface glycoproteins that bind and present intracellular (e.g., virus) and extracellular (e.g., bacteria) foreign peptides, respectively, to T-cell receptors to elicit an adequate immune response (Doherty and Zinkernagel 1975). Very high patterns of diversity at MHC loci among vertebrates (Garrigan and Hedrick 2003; Sommer 2005; Piertney and Oliver 2006) are interpreted as an adaptation to detect and present a wide array of peptides from rapidly evolving pathogens (Yuhki and O'Brien 1990b). Consequently, MHC diversity could be the ultimate response to selection in the face of unpredictable or temporally varying disease outbreaks (Altizer et al. 2003). Balancing selection is suggested to maintain variation at MHC loci (Hedrick 1994) driven mainly by pathogenic (Hedrick 2002a) and reproductive selective pressures (Sommer et al. 2002).

Most natural populations reveal high MHC diversity in terms of allele numbers and the extent of sequence variation among alleles as well as levels of heterozygosity (Klein 1986; Hedrick 2003a). By contrast, bottlenecked populations of, for example, Scandinavian beavers (Castor fiber; Ellegren et al. 1993), fallow deer (Cervus dama; Mikko et al. 1999), and Northern elephant seals (Mirounga angustirostris; Weber et al. 2004) exhibit low or no detectable polymorphisms in MHC genes and yet have survived and even increased in numbers with no apparent indications of increased susceptibility to infectious diseases (but see Radwan et al. 2010). By contrast, bottlenecked populations of desert bighorn sheep (Ovis aries) are highly susceptible to many infectious diseases despite showing high levels of MHC diversity (Gutierrez-Espeleta et al. 2001). Therefore, the influence of the extent of MHC diversity on the ability to respond to pathogenic challenges and, consequently, to the viability and survival of bottlenecked populations is not conclusive and still unclear (Edwards and Potts 1996; Hedrick 2003b; Radwan et al. 2010).

For decades, cheetahs (Acinonyx jubatus) have been considered a classic example in conservation genetics because of their relatively limited genomic diversity observed at neutral loci (e.g., minisatellites, microsatellites, and mitochondrial

DNA; Menotti-Raymond and O'Brien 1993, 1995; Freeman et al. 2001; but see Driscoll et al. 2002) and adaptive loci (e.g., allozymes and MHC I and II; O'Brien et al. 1983; Yuhki and O'Brien 1990b, 1994; Drake et al. 2004). The reduced levels of genetic variation in cheetahs have been attributed to several bottleneck events in the history of the species (Menotti-Raymond and O'Brien 1993; Driscoll et al. 2002). Whether the low genetic variation is of relevance in a conservation context has been controversial (Caughley 1994; Caro and Laurenson 1994; O'Brien 1994; Laurenson et al. 1995; May 1995).

Low genetic diversity particularly at MHC loci has been associated with high susceptibility to infectious diseases in cheetahs (O'Brien et al. 1985, 1986; O'Brien and Evermann 1988). However, increased susceptibility has only been observed in captive cheetahs (Evermann et al. 1988; Heeney et al. 1990), whereas free-ranging cheetahs from Eastern and Southern Africa show robust health (Caro 1994; Munson et al. 2004, 2005; Thalwitzer et al. 2010). In Namibia, where the largest free-ranging cheetah population in the world occurs (Marker-Kraus et al. 1996), recent studies on seroprevalence and necropsies have shown no clinical symptoms of acute viral diseases in cheetahs and a good general health status (Munson et al. 2004, 2005; Thalwitzer et al. 2010). This suggests that the immunocompetence of this cheetah population is not limited by their capacity to respond effectively to viral challenges (Thalwitzer et al. 2010) and that the observed difference in immunocompetence between captive and free-ranging cheetahs might be a consequence of unfavorable husbandry conditions resulting in stress and reduced immunocompetence, as previously suggested (Caro and Laurenson 1994; Merola 1994). The apparent robust immunocompetence of free-ranging Namibian cheetahs, however, still raises the question whether this population has a higher level of MHC diversity than previously observed (Drake et al. 2004) or than reported from other cheetah populations (Yuhki and O'Brien 1990b), or whether the cheetah in its natural environment represents an example of a species with low MHC variability and yet low disease susceptibility.

Early MHC-based studies on cheetahs have used 1) indirect methods (skin graft experiments; O'Brien et al. 1985), 2) low-resolution molecular methods to quantify MHC diversity (restriction fragment length polymorphism analysis [RFLP] of MHC I genes; Yuhki and O'Brien 1990b), or 3) high-resolution molecular methods but small sample sizes (sequence analysis of MHC I clones from two individuals, Yuhki and O'Brien 1994; reference strand–mediated conformational analysis [RSCA] of MHC II-DRB genes in 25 individuals, Drake et al. 2004).

Here, we use high-resolution molecular methods (single-strand conformation polymorphism [SSCP] analysis, cloning and sequencing) in 149 Namibian cheetahs to identify the genetic diversity in MHC I and MHC II-DRB loci. Additionally, we 1) validate the expression of the observed alleles, 2) investigate the phylogenetic relationship of MHC alleles to assign them into putative loci, and 3) test for signatures of positive selection. The results from this
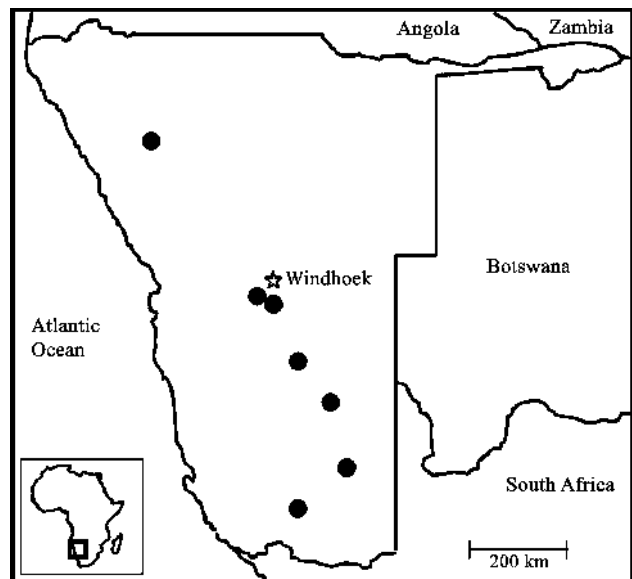

FIG. 1. Schematic map showing the origin (dots) of the cheetah samples in Namibia.

study will contribute to clarify the much-debated cheetah's classic example of reduced genetic diversity compromising the survival of the species, particularly in relation to infectious disease vulnerability.

Materials and Methods

Sampling of Namibian Cheetahs
Between 2002 and 2008, 149 wild-born cheetahs (including 121 free-ranging individuals inhabiting commercial livestock or game farmlands in Namibia and 28 wild-caught individuals kept in private farms) (fig. 1) were trapped, immobilized, and examined for their overall health status, and then sampled and released as described in Thalwitzer et al. (2010). Blood samples were collected and centrifuged, and the leucocyte pellets were stored in liquid nitrogen until later genomic DNA isolation. For expression analysis, PAXgene blood RNA tubes (Qiagen, Hilden, Germany) were filled with blood from 33 individuals, incubated at room temperature for 24 h and stored at −20°C until further processing.

Nucleic Acid Isolation and cDNA Synthesis
Genomic DNA was isolated using the DNeasy Extraction Kit (Qiagen) and RNA was isolated from 2.5 ml whole blood using the PAXgene blood RNA kit (Qiagen) following the manufacturer's instructions. To ensure the removal of genomic DNA from the isolated RNA, a second DNA digestion was performed using the DNase I RNase-free Set (Fermentas, St Leon-Rot, Germany). The cDNA synthesis was obtained using 200 U of RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) in a 20 μl reaction tube containing 2 μg total RNA as a template and 1 μl Oligo(dT)$_{12-18}$ primer (0.5 $\mu g/\mu l$; Invitrogen, Karlsruhe, Germany), 0.5 μl ribonuclease inhibitor (20 U/μl), 2 μl

Oligonucleotide primers | **Sequence (5'- 3')**

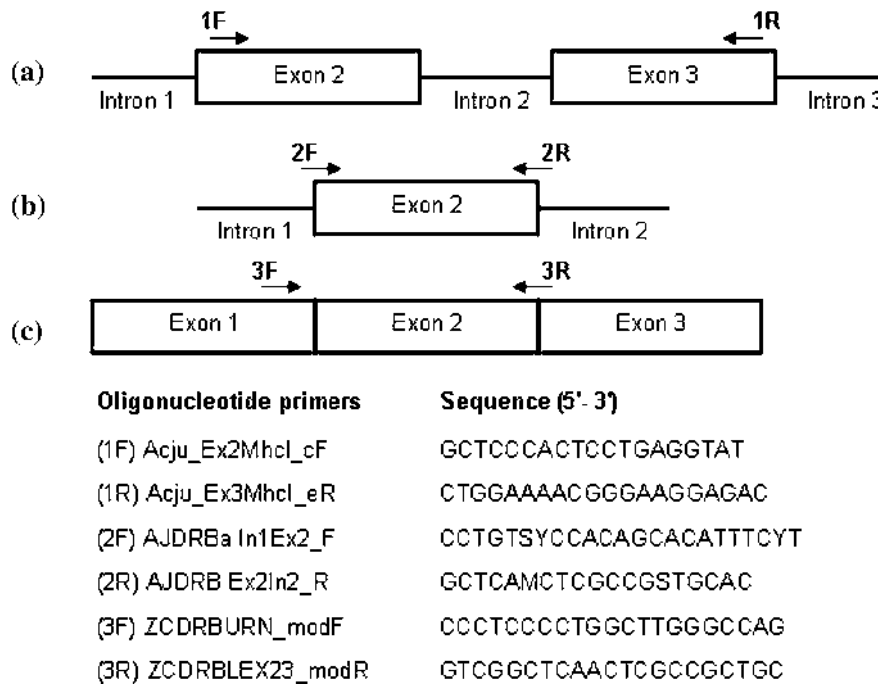| (1F) Acju_Ex2Mhcl_cF | GCTCCCACTCCTGAGGTAT |
(1R) Acju_Ex3Mhcl_eR	CTGGAAAACGGGAAGGAGAC
(2F) AJDRBa In1Ex2_F	CCTGTSYCCACAGCACATTTCYT
(2R) AJDRB Ex2In2_R	GCTCAMCTCGCCGSTGCAC
(3F) ZCDRBURN_modF	CCCTCCCCTGGCTTGGGCCAG
(3R) ZCDRBLEX23_modR	GTCGGCTCAACTCGCCGCTGC

FIG. 2. Position and sequences of PCR primers to amplify the indicated fragments of MHC I genes based on (a) genomic DNA as well as cDNA, and MHC II-DRB genes based on (b) genomic DNA, and (c) cDNA.

dNTP mix (10 mM), 4 μl buffer 5×, and RNase free water. The reaction was incubated at 42 °C for 60 min.

Primer Design and Amplification of MHC I and II Loci

We focused on the highly polymorphic second and third exons of MHC I genes encoding the alpha 1 (α_1) and alpha 2 (α_2) domains of class I proteins, respectively (Bjorkman et al. 1987), as well as the second exon of MHC II-DRB genes encoding the beta 1 (β_1) domain of class II proteins (Brown et al. 1993). These regions include the functionally important antigen-binding sites (ABS), that is, amino acid positions postulated to interact directly with the foreign antigens.

Our primer design aimed to isolate the maximum possible number of MHC I and II-DRB alleles in A. jubatus. Primer set 1 (F, R), binding the second and third exons of MHC I genes, was designed from homologous transcript sequences available from other felid species including the cheetah (GenBank accession numbers AJU07665 and AJU07666) (fig. 2a). The intronic fragment between the second and third exons is highly conserved as observed in human MHC I introns (Cereb et al. 1996, 1997) and thereby can be a better indicator of locus specificity than polymorphic coding regions. Primer set 2 (F, R), binding the second exon of MHC II-DRB genes, was designed from homologous sequences in other felids available in GenBank, including forward and reverse intron–exon boundary primers, and obtained almost the entire sequence of the exon 2 (fig. 2b). Polymerase chain reaction (PCR) amplifications were run in a final volume of 20 μl including 10–100 ng DNA, 0.375 μM of each primer, 1.75 μM dNTP mix, 2.5 μl buffer 10× and 0.5 U Taq polymerase (MP Biomedicals, Irvine, CA). The thermal profile consisted of an initial denaturation at 94 °C for 5 min, 35 cycles of 1 min at 94 °C, 1 min at 60/61 °C, 2 min at 72 °C with a final extension period at 72 °C for 10 min in a T Gradient and T Professional Thermocycler (Biometra, Göttingen, Germany).

MHC Genotyping

Of the 149 cheetahs, 108 and 139 individuals, respectively, were genotyped for their MHC I and II-DRB constitution. Individual amplicons were screened by SSCP analysis (Orita et al. 1989). This method can detect variants separated by only a single base difference (Sunnucks et al. 2000). For denaturation, 2–4 μl PCR products were mixed with 6 μl loading dye (10 μl formamide + 2.5 μl xylencyanol 1%), heated at 95 °C for 5 min and cooled on ice for 5 min. They were loaded on 15% nondenaturing polyacrylamide gels (ETC, Kirchentellinsfurt, Germany) and run on a horizontal cooling electrophoresis system (Amersham Pharmacia, Freiburg, Germany) setting the following conditions: 200 V, 10 mA, 10 W for 20 min followed by 450 V, 30 mA, 20 W for 3:45 hours and 4:30 hours at a constant temperature of 10 °C and 15 °C depending on the primer set 1 and 2, respectively. Gels were fixed and silver-stained using PlusOne DNA Silver Staining Kit (Amersham Pharmacia) following the manufacturer's recommendations. Distinctive single-strand bands were excised from the gel, eluted in 30 μl TBE buffer 1× and incubated for at least 3 h. A volume of 2–4 μl of the elution was added to 20 μl PCR mix and re-amplified as described above for 30 cycles. PCR products were purified with the BigDye XTerminator Purification Kit (Applied Biosystems, Foster City, CA) and directly sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems)

on an ABI PRISM 310 (Applied Biosystems). The PCR-SSCP analysis was conducted at least twice per individual sample on different gels to confirm its banding pattern reproducibility. All known SSCP patterns were run as references on each SSCP gel.

Because some individuals revealed a complex MHC I SSCP banding pattern, a second approach was conducted to identify the genotype. PCR products were generated with a proofreading polymerase (Hotstar Hifidelity polymerase; Qiagen), purified using a PeqGold Cycle Pure Kit (Peqlab Biotechnologie, Erlangen, Germany), and cloned into a pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen) following the manufacturer's protocol. Twenty-four recombinant clones per individual were selected and PCR amplified using the vector primers T7 and M13 rev. Cloned PCR products were purified and directly sequenced with the vector primers as described above. The criteria used to define a sequence as a true allele were based on its occurrence in at least two independent PCR reactions derived from the same or different individuals and/or confirmation by SSCP. Allele sequences were named according to the nomenclature rules set by Klein et al. (1990).

Expression Analyses

Qualitative expression analyses were conducted to validate the expression of the observed MHC I and II-DRB alleles. cDNA was obtained from nine selected individuals showing unique MHC I and II-DRB genomic genotypes and PCR amplified using the primer set 1 (F, R) for MHC I genes and modified primers 3F and 3R from Bowen et al. (2004) for MHC II-DRB genes (fig. 2c). Both primer sets were intron spanning to detect the amplification of genomic DNA contaminants. PCR reactions and temperature profiles were conducted as described above. All amplified cDNA products were analyzed through SSCP, cloning, and sequencing as described above.

Data Analysis

Nucleotide sequences were edited based on their forward and reverse consensus chromatograms using Chromas Pro Version 1.33, aligned and coding regions translated into deduced amino acid sequences using ClustalW as implemented in MEGA 3.1 (Kumar et al. 2004). The histocompatibility nature of the sequences was verified through a homology analysis using BlastN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from NCBI (http://www.ncbi.nlm.nih.gov).

We used MEGA 3.1 to detect the number of variable, conserved, and parsimony-informative sites (e.g., sites with at least two different nucleotides or amino acids) to compute the mean number of nucleotide and amino acid differences and derive the overall mean genetic distances of nucleotide sequences based on Kimura's two-parameter evolutionary distances as well as Poisson-corrected amino acid distances. MEGA 3.1 was also used to calculate the relative rates of nonsynonymous (d_N) and synonymous (d_S) nucleotide substitutions within and outside the ABS according to Nei and Gojobori (1986) with the Jukes and Cantor (1969) correction for multiple hits. The relative

rates of d_N and d_S were confirmed for the normal distribution and compared with a t-test (two-tailed based on a significance level of $\alpha = 0.05$) in SPSS version 16.0. Defined ABS codon positions were inferred from human MHC I (Bjorkman et al. 1987; Bjorkman and Parham 1990) and MHC II-DRB (Brown et al. 1993; Stern and Wiley 1994) molecules. The standard errors of the estimates were obtained through 1,000 bootstrap replicates.

The phylogenetic relationships among the MHC I and II-DRB alleles were reconstructed under two different model-based approaches (maximum likelihood [ML] and Bayesian, respectively) to assign them into putative loci. Domestic cat Felis catus MHC I and II-DRB sequences (GenBank accession number EU153401) were used as outgroups. First, ML trees were generated using PAUP 4.0 beta version (Swofford 2002). The implemented MODELTEST 3.7 (Posada and Crandall 1998) was applied to choose a model and the estimated parameters that explained best the nucleotide sequence evolution of the data. The likelihood settings for both MHC I and II-DRB alleles corresponded to the model GTR $+ \Gamma$ (general time-reversible $+$ gamma distribution of rates with four categories). The robustness of the ML tree was estimated through 1,000 bootstrap replicates. Second, we adopted a Bayesian inference approach using Mr. Bayes 3.1 (Ronquist and Huelsenbeck 2003). For MHC I, we generated partitioned Bayesian analyses (Brandley et al. 2005; Monaghan et al. 2007) by separating the combined matrix into seven partitions based on the individual mode of evolution of each codon position at the coding (exon 2 and exon 3) and noncoding (intron 2) regions. A nonpartition Bayesian analysis (all data in a single partition) was also conducted for comparison purposes. In both partition and nonpartition analyses, tree searches were conducted using a GTR $+$ invariable $+ \Gamma$ model and its estimated parameter values. Each Bayesian phylogenetic analysis consisted of 5×10^6 generations with a random starting tree, default priors and two runs of four heated and one cold Markov chains (heating $= 0.20$) sampled every 1,000 generations. A burn-in of 1.25×10^6 generations was selected based on the average standard deviation of split frequencies as well as by plotting $-\ln L$ against generation time. The MHC II-DRB alleles represented a single coding (exon 2) region; therefore, we conducted regular Bayesian analyses (no partition) using the same settings as described above.

Pairwise sequence comparison analysis of MHC I and II-DRB alleles, respectively, were conducted against a domestic cat MHC I and II-DRB annotated genomic sequence (2,973,765 bp) available in GenBank (accession number EU153401; Yuhki et al. 2008) using two different alignment tools: BlastN from NCBI and Exonerate (http://www.ebi.ac.uk/%7Eguy/exonerate/).

Results

MHC Class I and Class II-DRB Diversity and Expression Analysis

Ten unique MHC I exon 2 (249 bp), intron 2 (141/200/202 bp), and exon 3 (263/265 bp) nucleotide sequences were

FIG. 3. Amino acid sequence alignment of expressed MHC I alleles from Namibian cheetahs aligned to *AJUMHCAJUI3* (Yuhki and O'Brien 1994, GenBank accession number U07666). Numbers indicate the amino acid positions of α_1 and α_2 domains according to the human MHC I sequence. Asterisks indicate putative ABS (Bjorkman et al. 1987; Bjorkman and Parham 1990). Dots indicate identity to the top sequence.

identified in 108 Namibian cheetahs (supplementary fig. S1, Supplementary Material online). Eight of these alleles, *Acju-MHCI*02*, *Acju-MHCI*04–09* and *Acju-MHCI*12*, were novel (GenBank accession numbers GU971407–14), whereas *AJUMHCAJUI1* and *AJUMHCAJUI3* were previously described (Yuhki and O'Brien 1994). The homology analysis through a BlastN search revealed that all sequences shared high similarity with partial sequences of MHC I genes in other felids included in GenBank (e.g., domestic cat, ocelot *Leopardus pardalis*, Asiatic lion *Panthera leo persica*). The number of unique sequences observed per individual ranged from four to six, indicating that at least three MHC I loci were amplified. All genomic DNA sequences except for *Acju-MHCI*12* were also observed in cDNA, validating their expression and functionality. The sequence *Acju-MHCI*12* showed two insertions in exon 3 that changed the open reading frame of the transcript sequence. Therefore, this allele is likely to represent a pseudogene (i.e., a no longer expressed gene). The putative amino acid translation of the expressed MHC I sequences is shown in figure 3 (GenBank accession numbers PROTEIN).

The alignment of expressed MHC I exon 2 (249 bp) and exon 3 (263 bp) nucleotide sequences revealed 36 and 40 variable sites, respectively (Table 1, supplementary fig. S1, Supplementary Material online). The corresponding amino acid sequence consisted of 83 amino acids in the α_1 domain (positions 8–90; fig. 3) and 87 amino acids in the α_2 domain (positions 91–177; fig. 3). Of 22 and 17 variable sites along the α_1 and α_2 domains, respectively, 11 and 8 were located in important positions for antigen binding (fig. 3).

The overall mean number of nucleotide differences between MHC I exon 2 and exon 3 coding regions was similar (differing by an average of 0.3 nucleotides), whereas the mean number of amino acid differences was higher in the α_1 than in the α_2 domain (differing by an average of three amino acids). The overall mean genetic distance was 0.07 for both exon 2 and exon 3, whereas it increased to 0.14 and 0.09 when considering the amino acid sequences for the α_1 and α_2 domains, respectively. The mean nucleotide and amino acid distances were always much higher in the ABS than in the non-ABS, indicating that genetic diversity is mainly owing to changes occurring at positions important for antigen recognition (table 1).

The MHC I intron 2 sequences showed two deletions in one allele with the other alleles having 202 bp except for the presumed pseudogene *Acju-MHCI*12* (141 bp length) (supplementary fig. S1, Supplementary Material online). Few nucleotide differences were observed, conferring to 97% of similarity (genetic distance: 0.03 ± 0.01) among the intronic sequences of expressed MHC class I alleles (table 1).

Four MHC II-DRB exon 2 (243/246 bp) alleles were identified in 139 Namibian cheetahs (supplementary fig. S2, Supplementary Material online). Partial sequences (235/238 bp) of the four alleles *AcjuFLA-DRB1*ha14–17* (accession numbers AY312960–63) were previously described (Drake et al. 2004). The number of alleles observed per individual ranged from two to four, suggesting the presence of at least two DRB loci in *A. jubatus*. All alleles were detected at the genomic and cDNA level and, therefore, represented functional alleles.

The alignment of these MHC II-DRB exon 2 sequences revealed 41 variable sites (table 1, supplementary fig. S2, Supplementary Material online). This corresponded to 81/82 amino acids of the β_1 domain (positions 9–90; fig. 4). Of 25 variable sites 15 were located in important positions for antigen binding (fig. 4). The overall mean genetic

Table 1. Overall mean genetic distances (± standard errors) of nucleotide and amino acid sequences of expressed MHC I (nine) and MHC II-DRB (four) alleles in 108 and 139 Namibian cheetahs, respectively.

MHC	Region	Length	V	C	Si	%Si	No. differences	Genetic distances		
								All sites	ABS	Non-ABS
Class I	Exon 2	249	36	213	21	8.43	17.07 ± 2.71	0.07 ± 0.01	0.19 ± 0.05	0.04 ± 0.01
	Exon 3	263	40	223	13	4.94	16.73 ± 2.42	0.07 ± 0.01	0.18 ± 0.05	0.05 ± 0.01
	Intron 2	200/202	12	190	3	1.48	4.80 ± 1.41	0.03 ± 0.01	—	—
	Alpha 1	83	22	61	13	15.66	10.67 ± 1.95	0.14 ± 0.03	0.40 ± 0.11	0.08 ± 0.03
	Alpha 2	87	17	70	4	4.60	7.60 ± 1.79	0.09 ± 0.02	0.33 ± 0.12	0.05 ± 0.02
Class II-DRB	Exon 2	243/246	41	205	6	2.44	22.17 ± 3.20	0.10 ± 0.02	0.20 ± 0.04	0.06 ± 0.01
	Beta 1	81/82	25	57	5	6.10	14.33 ± 2.43	0.20 ± 0.04	0.50 ± 0.13	0.10 ± 0.03

NOTE.—V, variable; C, conserved; Si, parsimony-informative sites; %Si, Si percentage. The mean number (± standard error) of differing nucleotides and amino acids among the MHC I and MHC II-DRB alleles, respectively, are shown. Genetic distances are presented for all sites, ABS and non-ABS.

distance was 0.10 for exon 2 and 0.20 for the β_1 domain among all DRB alleles observed in the population (table 1). Nucleotide and amino acid mean distances were much higher at ABS compared with non-ABS (table 1).

Phylogenetic analyses and Putative MHC Class I and Class II-DRB Loci

The seven-partition and no-partition Bayesian models generated the same topologies, indicating that the mode of evolution of the codon positions in the coding (exons 2 and 3) and the noncoding (intron 2) regions of the cheetah's MHC I alleles are similar.

Both Bayesian and ML approaches distinguished four well-supported clusters corresponding to the putative MHC I loci in cheetahs (Fig. 5a). The number of alleles observed per single putative locus in all genotyped individuals supports this assumption because no individuals had more than two alleles from cluster B or cluster D. Clusters A and C are two putative loci represented by only one allele each. Because the allele in cluster C was nonfunctional (Acju-MHCI*12, see above), this result suggests that the alleles observed in A. jubatus belong to three functional and one nonfunctional MHC I loci.

Pairwise sequence comparisons assigned the ten cheetahs' MHC I alleles as the best hits to three classical (i.e., ubiquitously expressed, FLAI-E, FLAI-H and FLAI-K) and one nonclassical (i.e., nonubiquitously transcribed, FLAI-J) MHC I loci described for the domestic cat (Yuhki et al. 2008), thereby corresponding to the three functional and one nonfunctional putative MHC I loci of the cheetah.

The phylogenetic analysis of MHC II-DRB exon 2 alleles using Bayesian and ML approaches distinguished three well-supported clusters corresponding to putative functional MHC II-DRB loci (fig. 5b). Here, the number of alleles observed per single putative locus in the genotyped individuals also supports this classification because all individuals carried either one or two of the alleles in cluster C and all showed the allele in cluster B and cluster A, which represent monomorphic but functional MHC II-DRB loci.

Pairwise sequence comparisons assigned the cheetahs' MHC II-DRB alleles as the best hits to two (FLA-DRB3 and FLA-DRB4) of three functional MHC II-DRB loci described in the domestic cat MHC genome (Yuhki et al. 2008).

Testing for Positive Selection

To identify the signatures of long-term (historical) positive selection, we calculated the rates of nonsynonymous (d_N) and synonymous (d_S) substitutions for ABS and non-ABS positions (table 2). Pairwise comparisons among the MHC I alleles of exon 2 revealed a higher d_N than d_S in ABS ($t = 3.40$, degrees of freedom [df] = 28, $P < 0.01$) and no difference in non-ABS. The d_N:d_S ratio for ABS was much higher than one indicating positive selection. In exon 3, there was no difference between d_N and d_S in ABS, whereas d_N was lower than d_S in non-ABS ($t = -3.63$, df = 15.12, $P < 0.01$). The d_N was nine times higher in ABS (0.18) than in non-ABS (0.02) (Table 2; $t = 4.16$, df = 14.62, $P = 0.001$). Pairwise comparisons among the MHC II-DRB exon 2 alleles revealed no significant difference between the d_N and d_S in ABS and non-ABS (table 2) but as in MHC I the d_N rate was five times higher in ABS (0.21) than in non-ABS (0.04) (table 2; $t = 3.96$, df = 10, $P < 0.01$).

Discussion

Functional MHC Diversity in Namibian Cheetahs
We thoroughly examined levels of MHC I and II-DRB diversity in a large sample of Namibian cheetahs. Because the
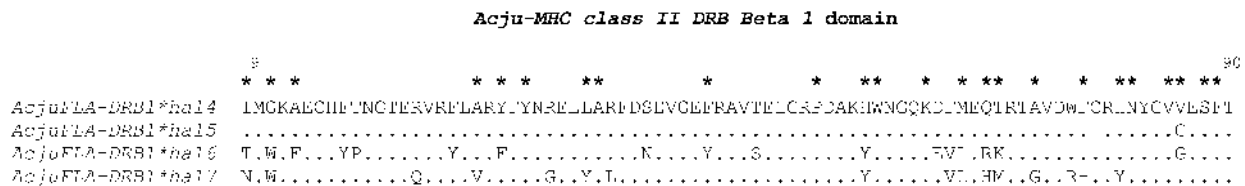
Acju-MHC class II DRB Beta 1 domain

```
                      9                                                                              90
                      * * *           * * *     **          *        *   **   *  *  **  *   *  **  ** **
AcjuFLA-DRB1*ha14   IMGKAECHFUNCTERVRFLARYTYNRELLARFDSLVGEFRAVTELCRFDAKIWNGQKDTMEQTRTAVDWTCRTNYCVVESFT
AcjuFLA-DRB1*ha15   ......................................................................................C....
AcjuFLA-DRB1*ha16   T.W.F...YP.......Y...F............N....Y....S.......Y.....FVT.RK...............G....
AcjuFLA-DRB1*ha17   N.W..........Q...V.....G..Y.L...............Y......VI.HV..G...R-..Y..........
```

FIG. 4. Amino acid sequence alignment of expressed MHC II-DRB alleles from Namibian cheetahs aligned to AcjuFLA-DRB1*ha14 (Drake et al. 2004, GenBank accession number AY312960). Numbers indicate the amino acid positions of the β_1 domain according to the human MHC II-DRB sequence. Asterisks indicate putative ABS (Brown et al. 1993). Dots indicate identity to the top sequence and minuses indicate an amino acid deletion.
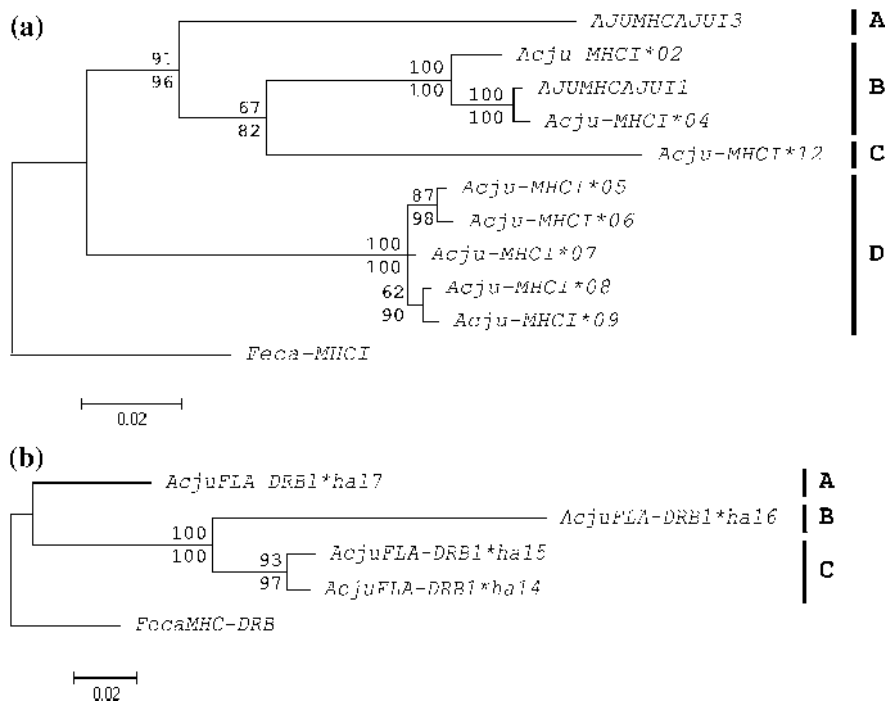
FIG. 5. Phylogenetic relationships among the MHC I (*a*) and MHC II-DRB (*b*) alleles detected in Namibian cheetahs. Numbers above and below the branches refer to bootstrap and posterior probabilities values, respectively. The trees were rooted using a MHC-annotated genomic sequence of the domestic cat (GenBank accession number EU153401).

individuals were sampled across the country, we assume that the number of alleles observed in this study represent largely the extent of MHC variation in Namibian cheetahs. The expression of all but one observed MHC alleles confirmed their adaptive functionality.

We detected ten MHC I alleles in 108 Namibian cheetahs. The observed MHC I diversity in terms of the number of alleles is higher compared with a previous study of the species (two alleles detected in two individuals; Yuhki and O'Brien 1994). This difference is clearly because of the larger number of individuals analyzed in this study. The number of MHC I alleles that occurred in the Namibian cheetah population was still relatively low compared with other natural populations of mammalian species (table 3). In felids, a total of 52 alleles were detected in 25 lions from an Asiatic and Afro-Asiatic hybrid population in India (Sachdev et al. 2005). However, the criteria of the authors to accept clone sequences as true alleles did not follow

a conservative approach and thereby we cannot exclude the possibility that the allele diversity in the 25 lions was overestimated. Recently, a total of 14 alleles were detected in only 14 Bengal tigers from India (Pokorny et al. 2010). Despite differences in the number of alleles among all populations of felid species, the overall nucleotide diversity, rates of nonsynonymous and synonymous substitutions along all sites of the second exon of MHC I alleles were similar (Table 3).

In contrast to MHC I, no additional MHC II-DRB alleles were detected in the 139 cheetahs of this study compared with a RSCA direct sequencing approach that found five alleles in 25 cheetahs of different origins, including Namibian (Drake et al. 2004). Drake et al. (2004) identified an extra allele (GenBank accession number AY312964) in a single individual, which was not present in any of the individuals we genotyped. Thus, we confirmed the previously observed low levels of MHC II-DRB diversity in cheetahs.

Table 2. Nonsynonymous (d_N) and synonymous (d_S) substitutions ($\pm$ standard error) as well as their ratio in ABS and non-ABS assuming concordance with the human MHC I and MHC II-DRB sequences (Bjorkman and Parham 1993; Brown et al. 1993).

MHC	Region	Sites	N	d_N	d_S	d_N/d_S	P
Class I	Exon 2	ABS	18	0.23 ± 0.08	0.08 ± 0.06	2.87	<0.01
		Non-ABS	65	0.04 ± 0.01	0.04 ± 0.02	1.00	0.71
		All	83	0.08 ± 0.02	0.05 ± 0.02	1.60	0.04
	Exon 3	ABS	16	0.18 ± 0.07	0.15 ± 0.13	1.20	0.51
		Non-ABS	71	0.02 ± 0.01	0.13 ± 0.04	0.15	<0.01
		All	87	0.05 ± 0.01	0.13 ± 0.03	0.38	0.02
Class II-DRB	Exon 2	ABS	24	0.21 ± 0.05	0.15 ± 0.08	1.40	0.35
		Non-ABS	58	0.04 ± 0.01	0.13 ± 0.05	0.31	0.06
		All	82	0.09 ± 0.02	0.13 ± 0.04	0.69	0.25

NOTE.—N is the number of codons in each category. P is the probability that d_N and d_S are different using a t-test.

Table 3. Examples of allelic diversity (± standard errors) at MHC I and MHC II-DRB loci in natural populations of mammalian species.

Species	N	Origin	MHC locus	MHC typing method	A	L	Allelic diversity		
							pi	d_N	d_S
Carnivores									
Cheetah[a] (*Acinonyx jubatus*)	108	Namibia	Class I	SSCP, cloning and sequencing	10	3	0.07 ± 0.01	0.08 ± 0.02	0.05 ± 0.02
	139	Namibia	Class II-DRB	SSCP, cloning and sequencing	4	2	0.10 ± 0.02	0.09 ± 0.02	0.13 ± 0.04
Asiatic lion[b] (*Panthera leo persica*)	25	Gir Forest, India	Class I	Cloning and sequencing	52	5	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
Bengal tiger[c] (*Panthera tigris tigris*)	14	Northern, western and central India	Class I	Cloning and sequencing	14	4	0.07 ± 0.01	0.07 ± 0.02	0.06 ± 0.02
	16		Class II-DRB	Cloning and sequencing	4	2	0.10 ± 0.02	0.10 ± 0.02	0.12 ± 0.04
Eurasian lynx[d] (*Lynx lynx*)	16	China	Class II-DRB	Cloning and sequencing	13	3	0.08 ± 0.01	0.07 ±0.01	0.12 ± 0.03
Domestic cat[e] (*Felis catus*)	36	Worldwide	Class II-DRB	Cloning and sequencing	61	3	0.14 ± 0.01	0.13 ± 0.02	0.17 ± 0.03
Grey wolf[f] (*Canis lupus*)	175	North America	Class II-DRB	PCR-direct sequencing	17	1	0.08 ± 0.01	0.09 ± 0.02	0.04 ± 0.02
African wild dog[g] (*Lycaon pictus*)	368	East and Southern Africa	Class II-DRB	RSCA, cloning and sequencing	17	1	0.06 ± 0.01	0.07 ± 0.02	0.04 ± 0.01
Coyote[h,] (*Canis latrans*)	49	California, Texas and North Carolina	Class II-DRB	SSCP, cloning and sequencing	17	1	0.09 ± 0.01	0.08 ± 0.02	0.05 ± 0.02
Noncarnivores									
Striped mouse[i] (*Rhabdomys pumilio*)	58	Southern Kalahari	Class II-DRB	SSCP and sequencing	20	1	0.11 ± 0.05	0.10 ± 0.02	0.06 ± 0.02
Yellow-necked mouse[j] (*Apodemus flavicollis*)	146	Northern Germany	Class II-DRB	SSCP and sequencing	27	1	0.21 ± 0.02	0.12 ± 0.03	0.05 ± 0.02
Mouse lemur[k] (*Microcebus murinus*)	228	Southeastern Madagascar	Class II-DRB	SSCP and sequencing	14	1	0.13 ± 0.02	0.15 ± 0.03	0.14 ± 0.03
Humans[l] (*Homo sapiens sapiens*)	264	Southern China	Class I (HLA-B)	SSOP	50	1	NA	NA	NA
			Class II-DRB1	SSOP	28	1	NA	NA	NA

NOTE.—N, number of individuals; A, number of alleles; L, minimum number of loci amplified; pi = nucleotide diversity; d_N, nonsynonymous substitutions rate; d_S, synonymous substitutions rate; NA, no available information; SSOP, sequence-specific oligonucleotide probes analysis. Overall pi, d_N, and d_S were estimated for all sites of the second exon of both MHC I and MHC II-DRB alleles and only putatively functional alleles were included in the calculations. Data from>
[a] present study,
[b] Sachdev et al. (2005),
[c] Pokorny et al. (2010),
[d] Wang et al. (2009);
[e] Yuhki and O'Brien (1997);
[f] Kennedy et al. (2007);
[g] Marsden et al. (2009);
[h] Hedrick, Lee, et al. (2000), Hedrick, Lee, et al. (2002);
[i] Froeschke and Sommer (2005);
[j] Meyer-Lucht and Sommer (2005);
[k] Schad and Sommer (2005);
[l] Trachtenberg et al. (2007).

Compared with other natural populations of mammalian species, the number of MHC II-DRB alleles detected in cheetahs was also low (Table 3). However, the nucleotide diversity with only four MHC II-DRB alleles in cheetahs was similar to other felids and relatively high compared with other canids that showed even more alleles. Contrary to canids and other noncarnivore species (including rodents and primates), felids showed higher rates of synonymous over nonsynonymous substitutions in all sites of the second exon of MHC II-DRB alleles (table 3). This is remarkable as most natural populations of vertebrates show an opposite pattern (Bernatchez and Landry 2003).

The comparison of MHC variation between populations of different species must be interpreted carefully because the extent of MHC polymorphism varies strongly among and between different taxonomic orders of mammalian populations (Kelley et al. 2005), probably related to their demographic history, degree of admixture, and selective factors. In addition, the number of detected alleles is influenced by the sample size, the number of populations analyzed, and the number of MHC loci investigated. Therefore, such aspects should be considered in comparative analyses of MHC diversity among populations from different species.

The extent of functional diversity in terms of amino acid sequence variation at the α_1 and α_2 domains of the MHC I transcripts of cheetahs (14% and 9%, respectively) is comparable with the homologous sequences of the domestic cat (12% and 12%, respectively; Yuhki and O'Brien 1990a), ocelot (17% and 14%, respectively; Yuhki and

O'Brien 1994), Asiatic lion (17% and 15%, respectively; Sachdev et al. 2005), and Bengal tiger *Panthera tigris tigris* (13% and 8%, respectively; Pokorny et al. 2010). The distribution of genetic variation along the MHC I transcripts in Namibian cheetahs revealed a higher amino acid divergence at the α_1 domain (14%) compared with the α_2 domain (9%; table 1). Most of the functional variation was concentrated in the second half of the α_1 domain, whereas the substitutions in the α_2 domain were dispersed. This pattern is consistent with MHC I transcripts from other felids (e.g., domestic cat, ocelot, Asiatic lion; Yuhki and O'Brien 1990a, 1994; Sachdev et al. 2005).

Noncoding regions evolving under neutral conditions can be expected to exhibit high genetic polymorphism. The noncoding intronic sequences between the second and third exons of MHC I loci, however, revealed a low diversity (3%; table 1). This is because noncoding regions in close proximity to regions evolving under strong selection are homogenized over evolutionary time by the results of recombination and subsequent genetic drift (Hughes 2000). This pattern is consistent with intronic sequences flanking coding regions at human MHC I loci (Cereb et al. 1996, 1997).

The cheetah MHC II-DRB amino acid sequence variation (20%) is similar to the homologous sequences of the domestic cat (19%; Yuhki and O'Brien 1997), ocelot (18%, GenBank accession numbers AAF70955–64), margay *Leopardus wiedii* (19%, GenBank accession numbers AAF71016–25) and Bengal tiger (18%; Pokorny et al. 2010), but relatively higher than the Eurasian lynx *Lynx lynx* (14%; Wang et al. 2009).

Number of Putative MHC Loci in Cheetahs

Phylogenetic analysis of MHC I alleles indicated the presence of four putative loci in *A. jubatus*. In domestic cats, 19 MHC I loci were characterized, from which three were tentatively assigned as classical MHC I genes: *FLAI-E*, *FLAI-H*, and *FLAI-K* (Yuhki et al. 2008). Alleles from the three functional putative MHC I loci (cluster A, cluster B, and cluster D) in cheetahs can be considered orthologous to domestic cat classical MHC I loci, suggesting that the cheetah's putative loci likely represent classical MHC I loci. The distinctive amino acid positions of classical MHC I molecules in humans were also present in the cheetah transcript sequences (Bjorkman and Parham 1990). The single not expressed locus in the cheetah (cluster C) was highly homologous to a nonclassical MHC I locus in the domestic cat (*FLAI-J*).

Phylogenetic analysis of MHC II-DRB alleles indicated the presence of three functional putative loci in the cheetah. Thus, the DRB locus in cheetahs has gone through duplication events as previously observed by Drake et al. (2004). Gene duplication is regarded as an important mechanism for generating MHC diversity and has been observed in many taxa (Klein, Sato, ÓhUigin 1998), including felids (O'Brien and Yuhki 1999). The number of putative MHC II-DRB loci suggested for cheetahs is consistent with the three (*FLA-DRB1*, *FLA-DRB3*, and *FLA-DRB4*) functional MHC II-DRB loci observed in domestic cats (Yuhki et al.

2007, 2008). However, it was not possible to orientate all MHC II-DRB alleles according to well-characterized homologous sequences identified in domestic cats as we did for the class I loci. The inconsistencies observed between the cheetah and domestic cat MHC class II-DRB loci might be because of a high rate of recombination events among the DRB alleles in cheetahs, as suggested previously (Drake et al. 2004).

Patterns of Historical Positive Selection

The excess of nonsynonymous over synonymous substitutions in the ABS of MHC class I exon 2 alleles from Namibian cheetahs provides evidence that positive selection operated to retain variation in these important parts (Hughes and Nei 1988, 1989). This result is consistent with the positive selection patterns observed at MHC I molecules during Felidae evolution (Yuhki and O'Brien 1990a). Positive selection is stronger at exon 2 than at exon 3, which is attributed to the structural principles that govern the peptide-binding motifs of MHC I molecules (Zhang et al. 1998). Still, the d_N rate of exon 3 was higher in the ABS than in the non-ABS, implying that selection processes also determined the variation of ABS here.

Similar to MHC I exon 3, no significant difference between nonsynonymous and synonymous substitutions in the ABS of MHC II-DRB exon 2 was observed. Nonsynonymous substitutions were more frequently observed in the ABS than in the non-ABS, indicating that selection was likely acting upon ABS at least in historical times. Whereas similar results were obtained for the Eurasian lynx (Wang et al. 2009) and Bengal tiger (Pokorny et al. 2010), this variation pattern is rare in MHC II-DRB loci. Most mammalian populations living under natural conditions show significant higher rates of d_N compared with d_S in ABS (Bernatchez and Landry 2003; Sommer 2005) including the domestic cat (Yuhki and O'Brien 1997). It is unlikely that pseudogenes led to higher d_S and biased the d_N:d_S ratio (Satta 1993) because we confirmed the expression of all DRB alleles. Another explanation might be that the ABS in *A. jubatus* MHC II-DRB molecules are different from those in humans. However, analyses of the sequence variation of MHC I and II-DRB transcripts conducted in other felid species showed similar ABS locations as in human molecules (Yuhki et al. 1989; Yuhki and O'Brien 1994).

Current hypotheses interpret low MHC polymorphism as a consequence of reduced selection pressure, constraints caused by mating systems, or bottleneck effects (Sommer et al. 2002). Reduced selection imposed by pathogens due to host ecological and behavioral factors is highly unlikely for cheetahs because the observed d_N:d_S ratios at the ABS indicate the presence of pathogen-driven selection occurring over thousands of generations (Piertney and Oliver 2006).

Our results indicate that historical positive selection was strong enough to maintain moderate MHC I diversity in the species. Positive selection having a stronger effect on variation than genetic drift has also been observed in other bottlenecked species (e.g., Nicolas Island fox *Urocyon littoralis dickeyi*, Aguilar et al. 2004; Hawaiian honeycreepers

[Drepanidinae], Jarvi et al. 2004). At the same time, the effect of genetic drift might have been strong enough to counteract balancing selection at the MHC II-DRB loci in the species, suggesting different intensities of selection operating at different MHC loci. If so, selection could maintain functional variation more effectively at the MHC I than at the MHC II loci. Different selection intensities at MHC loci have also been shown in humans where MHC I loci revealed higher selection coefficients than MHC II loci (Satta et al. 1994).

The Effect of Demographic Events on MHC Diversity in Cheetahs

The cheetah's poor overall genetic makeup has been mainly attributed to a severe ancient bottleneck (at the end of the last ice age, 10–12,000 years ago) and a more recent (20th century) anthropogenic-related bottleneck event with subsequent inbreeding (O'Brien et al. 1987; Menotti-Raymond and O'Brien 1993). The Namibian cheetah population has been subject to high levels of removals through trophy hunting, export, or conflict with local farmers in the past century (Marker-Kraus et al. 1996). However, as management practices have gradually changed, the level of removals has dropped significantly (Marker et al. 2003). Currently, there is no accurate estimate of the population size but the consensus is between 3,100 and 5,800 individuals (Hanssen and Stander 2004).

A study using 83 neutral microsatellite loci have revealed that the genetic diversity in current African cheetah populations is as high as in other outbred populations or species (Driscoll et al. 2002). A more detailed study on the patterns of neutral diversity using 38 microsatellite loci in 89 cheetahs throughout Namibia revealed 3 to 10 alleles per locus, with no deviation from Hardy–Weinberg expectations. The limited differentiation among the geographical regions suggests that the Namibian cheetahs form a large panmictic population (Marker et al. 2008). Our study on the adaptive MHC loci conducted in the same cheetah population revealed ten MHC I alleles with one to five alleles per locus and four MHC II-DRB alleles with one or two alleles per locus. The preservation of few but highly divergent and functional MHC I and II-DRB alleles that survived the bottleneck could be interpreted as balancing selection shaping MHC diversity in the current population (Hedrick 2003b, but see Ejsmond and Radwan 2009). This pattern is consistent with the mechanism of divergent allele advantage (Wakeland et al. 1990) because high divergence among alleles can result in a wider array of pathogen-derived antigens being recognized by the host population. Thus, the low levels of MHC I and II-DRB diversity observed in the Namibian cheetah population can be attributed to demographic processes rather than an absence of or reduced selection on MHC. Other examples of bottlenecked mammalian populations that showed the same pattern include carnivores (e.g., red wolf *Canis rufus*, Hedrick et al. 2002b), ungulates (e.g.. European and North American moose, *Alces alces*, Mikko and Andersson 1995; Arabian oryx, *Oryx leucoryx*, Hedrick, Parker, et al. 2000; European

bison, *Bison bison*, Radwan et al. 2007), and rodents (Malagasy giant rat, *Hypogeomys antimena*, Sommer 2003; Eurasian beaver, *C. fiber*, Babik et al. 2005).

A scenario of historic rather than recent reduction in the population size of cheetahs appears better suited to explain fixation of four MHC I and II-DRB alleles observed in the species. Assuming that 3,100 Namibian cheetahs represent the lowest population estimate (Hanssen and Stander 2004), that approximately half of the individuals are breeding adults, and that there is an equal sex ratio, an empirical estimate of the current effective population size $N_e = 1,550$ cheetahs (Storz et al. 2002). However, because N_e can reach 1/10 of the census size in wildlife populations (Frankham 1995a), we must consider a lower limit of $N_e = 310$ cheetahs. The fixation of a neutral nuclear gene is expected after $4N_e$ generations (Nichols 2001), which represents some 1,240–6,200 generations or 2,976–14,880 years according to a generation time of 2.4 years in the cheetah (Kelly et al. 1998). These time scales likely are underestimates because balancing selection acting on MHC genes could slow fixation by one or two orders of magnitude (Klein, Sato, Nagl, et al. 1998b).

Correlation between Immune Gene Diversity and Immunocompetence of Free-Ranging Namibian Cheetahs

The extent of genetic diversity required to ensure the long-term viability of the natural population remains a fundamental question in conservation genetics (Miller and Lambert 2004). A number of studies have indicated that bottlenecked populations exhibit a highly reduced MHC I and II variation and predicted that these populations have a low immune adaptability and a high risk for disease outbreaks and extinction (Frankham 1995b; O'Brien and Evermann 1988). However, the effect of reduced MHC variation on the long-term viability of bottlenecked populations has remained unclear (reviewed in Radwan et al. 2010). MHC-based studies have been useful in explaining some of the variation in disease resistance in free-ranging animal populations (Sommer 2005). Nevertheless, few studies on bottlenecked populations have associated levels of MHC diversity with the occurrence of diseases. For example, O'Brien et al. (1985; 1986) associated an outbreak of FIP (feline infectious peritonitis) in a captive population of cheetahs with the lack of variation at MHC loci measured indirectly by calculating the time of allograft rejection between unrelated cheetahs. Siddle et al. (2007) assumed an increased susceptibility to DFTD (devil facial tumor disease) in a free-ranging bottlenecked population of Tasmanian devils *Sarcophilus harrisii* because of the loss of MHC I diversity. By contrast, Giese and Hedrick (2003) found no evidence of MHC heterozygosity associated with mortality caused by a novel pathogen in the endangered Gila topminnow *Poeciliopsis occidentalis*. Mainguy et al. (2007) did not observe an increased susceptibility to disease in a bottlenecked population of Canadian mountain goats *Oreamnos americanus* in which only two MHC II-DRB alleles were retained.

The results from this study also suggest that low levels of MHC variation observed in the Namibian cheetah population do not limit or compromise their immunocompetence against (infectious) diseases. This population tested seropositive for feline calcivirus (FCV), feline parvovirus (FPV), feline herpesvirus, canine distemper virus (CDV), feline corona virus (FCoV), and rabies with high seropositivity in the northern part of the population; 65% and 48% of the investigated cheetahs were seropositive against FCV and FPV, respectively (Munson et al. 2004; Thalwitzer et al. 2010). Despite seropositivity for several viruses, no clinical or pathological evidence for infectious diseases were detected in living or dead cheetahs (Munson et al. 2004, 2005; Thalwitzer et al. 2010). The social structure of cheetahs with small group sizes or solitary individuals (Caro 1994) prevents a high rate of pathogen transmission and reinfection; however, transmission of, for example, FPV, FCoV, and CDV is facilitated through indirect contact at marking trees where cheetahs deposit feces (Marker-Kraus et al. 1996; Thalwitzer et al. 2010). Also, interspecific virus transmission might occur via free-ranging or domestic non-vaccinated carnivore species acting as pathogen reservoirs that potentially come into contact with free-ranging cheetahs in the Namibian farmlands (Thalwitzer et al. 2010).

Our results are not consistent with the cheetah's classic example of low MHC genetic diversity associated with high susceptibility to diseases (O'Brien et al. 1985, 1986; O'Brien and Evermann 1988; Yuhki and O'Brien 1990b) and supports the idea that the cheetahs' paradigm of disease vulnerability is pertinent only to captive populations and likely to be enhanced by extrinsic (e.g., stress) rather than genetic factors (Caro and Laurenson 1994; Merola 1994; Terio et al. 2004). The long-term survival of free-ranging cheetahs in Namibia seems more likely to depend on human-induced rather than genetic factors. However, it cannot be ruled out that levels of MHC variation observed in the Namibian cheetahs might limit a prosperous immunocompetence in the case of an emerging disease scenario because none of the remaining alleles might be able to present antigens of a novel parasitic type (Altizer et al. 2001; Radwan et al. 2010).

Because MHC diversity does not account for all the genetic susceptibility effects to an infectious disease in a population (Jepson et al. 1997), investigating non-MHC immune-relevant genes (e.g., cytokines and Toll-like receptors) might add to our understanding of how host genetic variation correlates with resistance to pathogens in wildlife populations (Acevedo-Whitehouse and Cunningham 2006). We also recommend extending the structural variance approach with quantitative measurements of transcript levels of immune-relevant genes (Bowen et al. 2006; Axtner and Sommer 2009; Weyrich et al. 2010) to provide a timely and relevant measure of altered host immune potential and environmental stress.

Supplementary Material

Supplementary figures S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acevedo-Whitehouse K, Cunningham AA. 2006. Is MHC enough for understanding wildlife immunogenetics? *Trends Ecol. Evol.* 21: 433–438.

Aguilar A, Roemer G, Debenham S, Binns M, Garcelon D, Wayne R. 2004. High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. *Proc Natl Acad Sci U S A.* 101:3490–3494.

Altizer S, Foufopoulos J, Gager A. 2001. Conservation and diseases. In: Levin SA editor. Encyclopedia of Biodiversity. San Diego (CA): Academic Press. p. 109–126.

Altizer S, Harvell D, Friedle E. 2003. Rapid evolutionary dynamics and disease threats to biodiversity. *Trends Ecol Evol.* 18:589–596.

Amos W, Harwood J. 1998. Factors affecting levels of genetic diversity in natural populations. *Philos Trans R Soc Lond Ser B.* 353:177–186.

Axtner J, Sommer S. 2009. Validation of internal reference genes for quantitative real-time PCR in a non-model organism, the yellow-necked mouse, Apodemus flavicollis. *BMC Res. Notes* 2:264.

Babik W, Durka W, Radwan J. 2005. Sequence diversity of the MHC DRB gene in the Eurasian beaver (*Castor fiber*). *Mol Ecol.* 14: 4249–4257.

Bernatchez L, Landry C. 2003. MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *J Evol Biol.* 16:363–377.

Bjorkman PJ, Parham P. 1990. Structure, function, and diversity of class-I major histocompatibility complex-molecules. *Annu Rev Biochem.* 59:253–288.

Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. 1987. The foreign antigen-binding site and T-Cell recognition regions of class-I histocompatibility antigens. *Nature* 329:512–518.

Bowen L, Aldridge B, Beckmen K, Gelatt T, Rea L, Burek K, Pitcher K, Stott JL. 2006. Differential expression of immune response genes in Steller sea lions (*Eumetopias jubatus*): an indicator of ecosystem health? *Ecohealth* 3:109–113.

Bowen L, Aldridge BM, Gulland F, Van Bonn W, DeLong R, Melin S, Lowenstine LJ, Stott JL, Johnson ML. 2004. Class II multiformity generated by variable MHC-DRB region configurations in the

California sea lion (*Zalophus californianus*). *Immunogenetics* 56: 12–27.

Brandley MC, Schmitz A, Reeder TW. 2005. Partitioned Bayesian analyses, partition choice, and the phylogenetic relationships of scincid lizards. *Syst Biol.* 54:373–390.

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC. 1993. Three-dimensional structure of the human class-II histocompatibility antigen HLA-DR1. *Nature* 364:33–39.

Caro TM. 1994. Cheetahs of the Serengeti plains: group living in an asocial species. Chicago: The University of Chicago Press.

Caro TM, Laurenson MK. 1994. Ecological and genetic-factors in conservation—a cautionary tale. *Science* 263:485–486.

Caughley G. 1994. Directions in conservation biology. *J Anim Ecol.* 63:215–244.

Cereb N, Hughes AL, Yang SY. 1997. Locus-specific conservation of the HLA class I introns by intra-locus homogenization. *Immunogenetics* 47:30–36.

Cereb N, Kong Y, Lee S, Maye P, Yang SY. 1996. Nucleotide sequences of MHC class I introns 1, 2, and 3 in humans and intron 2 in nonhuman primates. *Tissue Antigens.* 47:498–511.

Doherty PC, Zinkernagel RM. 1975. Biological role for major histocompatibility antigens. *Lancet* 1:1406–1409.

Drake GJC, Kennedy LJ, Auty HK, Ryvar R, Ollier WER, Kitchener AC, Freeman AR, Radford AD. 2004. The use of reference strand-mediated conformational analysis for the study of cheetah (*Acinonyx jubatus*) feline leucocyte antigen class II DRB polymorphisms. *Mol Ecol.* 13:221–229.

Driscoll CA, Menotti-Raymond M, Nelson G, Goldstein D, O'Brien SJ. 2002. Genomic microsatellites as evolutionary chronometers: a test in wild cats. *Genome Res.* 12:414–423.

Edwards S, Potts W. 1996. Polymorphism of genes in the major histocompatibility complex: implications for conservation genetics of vertebrates. In: Smith T, Wayne R, editors. Molecular genetic approaches in conservation. New York: Oxford University Press. p. 214–237.

Ejsmond MJ, Radwan J. 2009. Mhc diversity in bottlenecked populations: a simulation model. *Conserv Genet.* doi:10.1007/s10592-009-9998-6.

Ellegren H, Hartman G, Johansson M, Andersson L. 1993. Major histocompatibility complex monomorphism and low-levels of DNA-fingerprinting variability in a reintroduced and rapidly expanding population of beavers. *Proc Natl Acad Sci U S A.* 90: 8150–8153.

Evermann JF, Heeney JL, Roelke ME, Mckeirnan AJ, O'Brien SJ. 1988. Biological and pathological consequences of feline infectious peritonitis virus-infection in the cheetah. *Arch Virol.* 102:155–171.

Frankham R. 1995a. Effective population size/adult sizes ratios in wildlife: a review. *Genet Res.* 66:95–107.

Frankham R. 1995b. Inbreeding and extinction—a threshold effect. *Conserv Biol.* 9:792–799.

Frankham R, Ballou JD, Briscoe DA. 2002. Introduction to Conservation Genetics. Cambridge. Cambridge University Press.

Freeman AR, Machugh DE, McKeown S, Walzer C, McConnell DJ, Bradley DG. 2001. Sequence variation in the mitochondrial DNA control region of wild African cheetahs (*Acinonyx jubatus*). *Heredity* 86:355–362.

Froeschke G, Sommer S. 2005. MHC class II DRB variability and parasite load in the striped mouse (*Rhabdomys pumilio*) in the Southern Kalahari. *Mol Biol Evol.* 22:1254–1259.

Garrigan D, Hedrick PW. 2003. Perspective: detecting adaptive molecular polymorphism: lessons from the MHC. *Evolution* 57:1707–1722.

Giese AR, Hedrick PW. 2003. Genetic variation and resistance to a bacterial infection in the endangered Gila topminnow. *Anim Conserv.* 6:369–377.

Gutierrez-Espeleta GA, Hedrick PW, Kalinowski ST, Garrigan D, Boyce WM. 2001. Is the decline of desert bighorn sheep from infectious disease the result of low MHC variation? *Heredity* 86:439–450.

Hanssen L, Stander P. 2004. Namibia Large Carnivore Atlas Report vol. 1. Windhoek (Namibia): Predator Conservation Trust, Ministry of Environment and Tourism, Division of Speciality Support Services.

Hedrick PW. 1994. Evolutionary genetics of the major histocompatibility complex. *Am Nat.* 143:945–964.

Hedrick PW. 2002. Pathogen resistance and genetic variation at MHC loci. *Evolution.* 56:1902–1908.

Hedrick PW. 2003a. A heterozygote advantage. *Science* 302:57.

Hedrick PW. 2003b. The major histocompatibility complex (MHC) in declining populations: an example of adaptive variation. In: Holt W, Pickard A, Rodger J, Wildt D, editors. Reproduction science and integrated conservation.. Cambridge: Cambridge University Press. p. 97–113.

Hedrick PW, Lee RN, Garrigan D. 2002. Major histocompatibility complex variation in red wolves: evidence for common ancestry with coyotes and balancing selection. *Mol Ecol.* 11:1905–1913.

Hedrick PW, Lee RN, Parker KM. 2000. Major histocompatibility complex (MHC) variation in the endangered Mexican wolf and related canids. *Heredity* 85:617–624.

Hedrick PW, Parker KM, Gutierrez-Espeleta GA, Rattink A, Lievers K. 2000. Major histocompatibility complex variation in the Arabian oryx. *Evolution* 54:2145–2151.

Heeney JL, Evermann JF, McKeirnan AJ, Marker-Kraus L, Roelke ME, Bush M, Wildt DE, Meltzer DG, Colly L, Lukas J. 1990. Prevalence and implications of feline coronavirus infections of captive and free-ranging cheetahs (*Acinonyx jubatus*). *J Virol.* 64:1964–1972.

Hill AVS. 1998. The immunogenetics of human infectious diseases. *Annu Rev Immunol.* 16:593–617.

Hughes AL. 2000. Evolution of introns and exons of class II major histocompatibility complex genes of vertebrates. *Immunogenetics* 51:473–486.

Hughes AL, Nei M. 1988. Pattern of nucleotide substitution at major histocompatibility complex class-I loci reveals overdominant selection. *Nature* 335:167–170.

Hughes AL, Nei M. 1989. Nucleotide substitution at major histocompatibility complex class-II loci—evidence for over-dominant selection. *Proc Natl Acad Sci U S A.* 86:958–962.

Jarvi SI, Tarr CL, McIntosh CE, Atkinson CT, Fleischer RC. 2004. Natural selection of the major histocompatibility complex (MHC) in Hawaiian honeycreepers (Drepanidinae). *Mol Ecol.* 13: 2157–2168.

Jepson A, Banya W, SisayJoof F, HassanKing M, Nunes C, Bennett S, Whittle H. 1997. Quantification of the relative contribution of major histocompatibility complex (MHC) and non-MHC genes to human immune responses to foreign antigens. *Infect Immun.* 65:872–876.

Jukes TH, Cantor CR. 1969. Evolution of protein molecules. In: Munro HN, editor. Mammalian protein metabolism. New York: Academic Press. p. 21–132.

Kelly MJ, Laurenson MK, FitzGibbon CD, Collins DA, Durant SM, Frame GW, Bertram BCR, Caro TM. 1998. Demography of the Serengeti cheetah (*Acinonyx jubatus*) population: the first 25 years. *J Zool.* 244:473–488.

Kelley J, Walter R, Trowsdale J. 2005. Comparative genomics of major histocompatibility complexes. *Immunogenetics* 56:683–695.

Kennedy LJ, Angles JM, Barnes A, Carmichael LF, Radford AD, Ollier WER, Happ GM. 2007. DLA-DRBI, DQAI, and DQBI alleles and haplotypes in North American gray wolves. *J Hered.* 98: 491–499.

Klein J. 1986. Natural History of the Major Histocompatibility Complex. New York: Wiley.

Klein J, Bontrop RE, Dawkins RL, Erlich HA, Gyllensten UB, Heise ER, Jones PP, Parham P, Wakeland EK, Watkins DI. 1990. Nomenclature for the major histocompatibility complexes of different species—a proposal. Immunogenetics 31:217–219.

Klein J, Sato A, Nagl S, O'hUigín C. 1998. Molecular trans-species polymorphism. Annu. Rev. Ecol. Syst. 29:1–21.

Klein J, Sato A, ÓhUigin C. 1998. Evolution by gene duplication in the major histocompatibility complex. Cytogenet. Cell Genet. 80: 123–127.

Kumar S, Tamura K, Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinformatics. 5:150–163.

Laurenson MK, Caro TM, Gros P, Wielebnowski N. 1995. Controversial cheetahs? Nature 377:392.

Mainguy J, Worley K, Cote SD, Coltman DW. 2007. Low MHC DRB class II diversity in the mountain goat: past bottlenecks and possible role of pathogens and parasites. Conserv Genet. 8: 885–891.

Marker-Kraus LL, Kraus D, Barnett D, Hurlbut S. 1996. Cheetah survival on Namibian farmlands. Windhoek (Namibia): Cheetah Conservation Fund.

Marker LL, Mills MGL, Macdonald DW. 2003. Factors influencing perceptions of conflict and tolerance towards cheetahs on Namibian farmlands. Conserv Biol. 17:1290–1298.

Marker LL, Wilkerson AJP, Sarno RJ, Martenson J, Breitenmoser-Würsten C, O'Brien SJ, Warren EJ. 2008. Molecular genetic insights on cheetah (Acinonyx jubatus) ecology and conservation in Namibia. J Hered. 99:2–13.

Marsden CD, Mable BK, Woodroffe R, Rasmussen GSA, Cleaveland S, McNutt JW, Emmanuel M, Thomas R, Kennedy LJ. 2009. Highly endangered African wild dogs (Lycaon pictus) lack variation at the major histocompatibility complex. J Hered. 100:S54–S65.

May RM. 1995. The cheetah controversy. Nature 374:309–310.

Menotti-Raymond M, O'Brien SJ. 1993. Dating the genetic bottleneck of the African cheetah. Proc Natl Acad Sci U S A. 90:3172–3176.

Menotti-Raymond MA, O'Brien SJ. 1995. Evolutionary conservation of ten microsatellite loci in four species of Felidae. J Hered. 86: 319–322.

Merola M. 1994. A reassessment of homozygosity and the case for inbreeding depression in the cheetah, Acinonyx jubatus: implications for conservation. Conserv Biol. 8:961–971.

Meyer-Lucht Y, Sommer S. 2005. MHC diversity and the association to nematode parasitism in the yellow-necked mouse (Apodemus flavicollis). Mol Ecol. 14:2233–2243.

Mikko S, Andersson L. 1995. Low major histocompatibility complex class-II diversity in European and North-American moose. Proc Natl Acad Sci U S A. 92:4259–4263.

Mikko S, Roed K, Schmutz S, Andersson L. 1999. Monomorphism and polymorphism at MHC DRB loci in domestic and wild ruminants. Immunol Rev. 167:169–178.

Miller HC, Lambert DM. 2004. Genetic drift outweighs balancing selection in shaping post-bottleneck major histocompatibility complex variation in New Zealand robins (Petroicidae). Mol Ecol. 13:3709–3721.

Monaghan MT, Inward DJG, Hunt T, Vogler AP. 2007. A molecular phylogenetic analysis of the Scarabaeinae (dung beetles). Mol Phylogenet Evol. 45:674–692.

Munson L, Marker L, Dubovi E, Spencer JA, Evermann JF, O'Brien SJ. 2004. Serosurvey of viral infections in free-ranging Namibian cheetahs (Acinonyx jubatus). J Wildl Dis. 40:23–31.

Munson L, Terio KA, Worley M, Jago M, Bagot-Smith A, Marker L. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (Acinonyx jubatus) populations. J Wildl Dis. 41:542–548.

Nei M, Gojobori T. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol. 3:418–426.

Nichols R. 2001. Gene trees and species trees are not the same. Trends Ecol Evol. 16:358–364.

O'Brien SJ. 1994. The cheetah's conservation controversy. Conserv Biol. 8:1153–1155.

O'Brien SJ, Evermann JF. 1988. Interactive influence of infectious disease and genetic diversity in natural populations. Trends Ecol Evol. 3:254–259.

O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M, Wildt DE. 1985. Genetic basis for species vulnerability in the cheetah. Science 227:1428–1434.

O'Brien SJ, Wildt DE, Bush M. 1986. The cheetah in genetic peril. Sci Am. 254:84–92.

O'Brien SJ, Wildt DE, Bush M, Caro TM, Fitzgibbon C, Aggundey I, Leakey RE. 1987. East-African cheetahs—evidence for two population bottlenecks. Proc Natl Acad Sci U S A. 84:508–511.

O'Brien SJ, Wildt DE, Goldman D, Merril CR, Bush M. 1983. The cheetah is depauperate in genetic variation. Science 221: 459–462.

O'Brien SJ, Yuhki N. 1999. Comparative genome organization of the major histocompatibility complex: lessons from the Felidae. Immunol Rev. 167:133–144.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci U S A. 86:2766–2770.

Piertney SB, Oliver MK. 2006. The evolutionary ecology of the major histocompatibility complex. Heredity 96:7–21.

Pokorny I, Sharma R, Goyal SP, Mishra S, Tiedemann R. 2010. MHC class I and MHC class II DRB gene variability in wild and captive Bengal tigers (Panthera tigris tigris). Immunogenetics 62:667–679.

Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817–818.

Radwan J, Biedrzycka A, Babik W. 2010. Does reduced MHC diversity decrease viability of vertebrate populations? Biol Conserv. 143:537–544.

Radwan J, Kawalko A, Wójcik JM, Babik W. 2007. MHC-DRB3 variation in a free-living population of the European bison, Bison bonasus. Mol Ecol. 16:531–540.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Sachdev M, Sankaranarayanan R, Reddanna P, Thangaraj K, Singh L. 2005. Major histocompatibility complex class I polymorphism in Asiatic lions. Tissue Antigens. 66:9–18.

Satta Y. 1993. How the ratio of nonsynonymous to synonymous pseudogene substitutions can be less than one. Immunogenetics 38:450–454.

Satta Y, Ohuigin C, Takahata N, Klein J. 1994. Intensity of natural selection at the major histocompatibility complex loci. Proc Natl Acad Sci U S A. 91:7184–7188.

Schad J, Sommer S. 2005. Parasite burden and constitution of major histocompatibility complex in the Malagasy mouse lemur. Microcebus murinus. Evolution 59:439–450.

Siddle HV, Kreiss A, Eldridge MDB, Noonan E, Clarke CJ, Pyecroft S, Woods GM, Belov K. 2007. Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. Proc Natl Acad Sci U S A. 104:16221–16226.

Sommer S. 2003. Effects of habitat fragmentation and changes of dispersal behaviour after a recent decline on the genetic variability of noncoding and coding DNA of a monogamous Malagasy rodent. Mol Ecol. 12:2845–2851.

Sommer S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Front Zool.* 2:16.

Sommer S, Schwab D, Ganzhorn JU. 2002. MHC diversity of endemic Malagasy rodents in relation to geographic range and social system. *Behav Ecol Sociobiol.* 51:214–221.

Stern LJ, Wiley DC. 1994. Antigenic peptide binding by class-I and class-II histocompatibility proteins. *Structure* 2:245–251.

Storz JF, Ramakrishnan U, Alberts SC. 2002. Genetic effective size of a wild primate population: influence of current and historical demography. *Evolution* 54:817–829.

Trachtenberg E, Vinson M, Hayes E, Hsu YM, Houtchens K, Erlich H, Klitz W, Hsia Y, Hollenbach J. 2007. HLA class I (A, B, C) and class II (DRB1, DQA1, DQB1, DPB1) alleles and haplotypes in the Han from southern China. *Tissue Antigens.* 70:455–463.

Sunnucks P, Wilson ACC, Beheregaray LB, Zenger K, French J, Taylor AC. 2000. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol Ecol.* 9:1699–1710.

Swofford DL. 2002. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sunderland (MA): Sinauer Associates.

Terio KA, Marker L, Munson L. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. *J Wildl Dis.* 40:259–266.

Thalwitzer S, Wachter B, Robert N, Wibbelt G, Muller T, Lonzer J, Meli ML, Bay G, Hofer H. 2010. Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland. *Clin Vaccine Immunol.* 17:232–238.

Wakeland EK, Boehme S, She JX, Lu CC, McIndoe RA, Cheng I, Ye Y, Potts WK. 1990. Ancestral polymoprhisms of MHC class II genes: divergent allele advantage. *Immunol Res.* 9:115–122.

Wang X, Wei K, Zhang Z, Xu X, Zhang W, Shen F, Zhang L, Yue B. 2009. Major histocompatibility complex class II DRB exon-2 diversity of the Eurasian lynx (*Lynx lynx*) in China. *J Nat Hist.* 43:245–257.

Weber DS, Stewart BS, Schienman J, Lehman N. 2004. Major histocompatibility complex variation at three class II loci in the northern elephant seal. *Mol Ecol.* 13:711–718.

Weyrich A, Axtner J, Sommer S. 2010. Selection and validation of reference genes for real-time RT-PCR studies in the non-model species *Delomys sublineatus*, an endemic Brazilian roedent. *Biochem Biophys Res Commun.* 392:145–149.

Yuhki N, Beck T, Stephens R, Neelam B, O'Brien SJ. 2007. Comparative genomic structure of human, dog, and cat MHC: HLA, DLA, and FLA. *J Hered.* 98:390–399.

Yuhki N, Heidecker GF, O'Brien SJ. 1989. Characterization of MHC cDNA clones in the domestic cat - diversity and evolution of class-I genes. *J Immunol.* 142:3676–3682.

Yuhki N, Mullikin JC, Beck T, Stephens R, O'Brien SJ. 2008. Sequences, annotation and single nucleotide polymorphism of the major histocompatibility complex in the domestic cat. *PLoS One.* 3:e2674.

Yuhki N, O'Brien SJ. 1990a. DNA recombination and natural selection pressure sustain genetic sequence diversity of the Feline MHC class-I genes. *J Exp Med.* 172:621–630.

Yuhki N, O'Brien SJ. 1990b. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proc Natl Acad Sci U S A.* 87:836–840.

Yuhki N, O'Brien SJ. 1994. Exchanges of short polymorphic DNA segments predating speciation in feline major histocompatibility complex class-I genes. *J Mol Evol.* 39:22–33.

Yuhki N, O'Brien SJ. 1997. Nature and origin of polymorphism in feline MHC class II DRA and DRB genes. *J Immunol.* 158:2822–2833.

Zhang C, Anderson A, DeLisi C. 1998. Structural principles that govern the peptide-binding motifs of class I MHC molecules. *J Mol Biol.* 281:929–947.