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Diversity and Evolutionary Patterns of Immune Genes in Free-Ranging Namibian Leopards (*Panthera pardus pardus*)

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Abstract

The genes of the major histocompatibility complex (MHC) are a key component of the mammalian immune system and have become important molecular markers for fitness-related genetic variation in wildlife populations. Currently, no information about the MHC sequence variation and constitution in African leopards exists. In this study, we isolated and characterized genetic variation at the adaptively most important region of MHC class I and MHC class II-DRB genes in 25 free-ranging African leopards from Namibia and investigated the mechanisms that generate and maintain MHC polymorphism in the species. Using single-stranded conformation polymorphism analysis and direct sequencing, we detected 6 MHC class I and 6 MHC class II-DRB sequences, which likely correspond to at least 3 MHC class I and 3 MHC class II-DRB loci. Amino acid sequence variation in both MHC classes was higher or similar in comparison to other reported felids. We found signatures of positive selection shaping the diversity of MHC class I and MHC class II-DRB loci during the evolutionary history of the species. A comparison of MHC class I and MHC class II-DRB sequences of the leopard to those of other felids revealed a trans-species mode of evolution. In addition, the evolutionary relationships of MHC class II-DRB sequences between African and Asian leopard subspecies are discussed.

Key words: African leopard, MHC class I, MHC class II, Panthera pardus, positive selection

Carnivores, especially members of the Felidae and Canidae families, belong to the most threatened taxa by pathogens within all mammalian species (Pedersen et al. 2007). This is partly because many carnivore populations are seriously endangered by anthropogenic factors such as human population expansion, alteration, and loss of habitat. These factors influence disease ecology by disrupting historically stable host–pathogen interactions and/or introducing highly virulent pathogens leading to potential epizootic events (Murray et al. 1999; Smith et al. 2009; Munson et al. 2010). Host populations have evolved numerous ways of immune responses to overcome infectious challenges imposed by pathogens (Acevedo-Whitehouse and Cunningham 2006). A key component of the mammalian immune system is the major histocompatibility complex (MHC), a genetic region responsible for the adaptive immune response and integral to host resistance to emerging pathogens (Hill 1998; Kumanovics et al. 2003). The MHC is a cluster of genes that code for cell surface pathogen and T-cell receptor recognition proteins. The MHC class I (MHC I) and class II (MHC II) proteins bind and present short peptides derived from intracellular (e.g., virus) and extracellular (e.g., bacteria) pathogens, respectively, to cytotoxic and T helper cells, thereby triggering a cascade of immune responses (Klein 1986). 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 (Doherty and Zinkernagel 1975). Positive selection is suggested to maintain MHC variation over generations (Hedrick 1994) driven mainly by pathogenic pressures (Hedrick 2002) and sexual selection (Sommer et al. 2002). The retention of MHC allelic lineages for longer evolutionary periods than expected under neutrality (i.e., trans-species polymorphism) occurs only in systems evolving under positive selection and is a typical

mode of evolution of MHC genes (Klein et al. 1998). Apart from selection, other mechanisms such as mutation, recombination, gene conversion, and drift may affect the evolution of MHC genes, although their relative contributions are still uncertain (Richman et al. 2003). MHC-based studies have been useful in explaining some of the variation in disease resistance of free-ranging animal populations (reviewed in Sommer 2005). Given that MHC variation reflects evolutionary relevant and adaptive processes in natural populations, it has become of great importance in evolutionary ecology and conservation (Sommer 2005; Piertney and Oliver 2006).

Leopards (*Panthera pardus*) have the largest geographic distribution among free-ranging cats, suggesting they are highly adaptable to different habitats ranging from desert to rainforest (Henschel et al. 2008; Macdonald et al. 2010). Leopards are still distributed across most of their historic range that covers Africa, central and southeast Asia, and Eurasia (Nowell and Jackson 1995). However, the number of leopards has declined considerably in the last century due to anthropogenic pressures that have resulted in heavily fragmented and isolated leopard populations (Uphyrkina et al. 2001). Currently, most leopard subspecies are categorized as "near threatened" or "critically endangered" according to the International Union for Conservation of Nature Red List of Threatened Species (Henschel et al. 2008). The African leopard (*P. p. pardus*) is the most common of 9 revised leopard subspecies (Miththapala et al. 1996; Uphyrkina et al. 2001) and probably the most abundant large felid in Africa. In Namibia, free-ranging leopards (together with cheetahs *Acinonyx jubatus*) are the most abundant large carnivores, with an estimated population size ranging approximately between 5000 and 10 500 individuals (Hanssen and Stander 2004). They are widely distributed but mainly concentrated in the northern and central parts of the country (Mendelsohn et al. 2009; Hanssen and Stander 2004), where they inhabit unprotected areas on privately owned commercial livestock or game farmlands (Marker-Kraus et al. 1996). In contrast to some free-ranging large carnivore populations in Africa that have been afflicted by epizootics, such as lions (*P. leo*; Roelke-Parker et al. 1996) and wild dogs (*Lycaon pictus*; Kat et al. 1995), African leopards seem to have escaped from large-scale declines due to epizootics in the past (Spong et al. 2000). The apparent low rate of horizontal pathogen transmission in leopards has been mainly attributed to their solitary lifestyle (Bailey 1993; Stander et al. 1997). However, a solitary lifestyle does not protect an individual from generalist or vector-borne pathogens because the individual may contact the agent in prey, from the environment, or through encounters with other species (Munson et al. 2010). For example, canine distemper viruses originating from nonvaccinated domestic dogs (*Canis familiaris*) can emerge in highly virulent forms resulting in major epizootics (Roelke-Parker et al. 1996; Carpenter et al. 1998). On Namibian farmland, domestic and wildlife species use overlapping areas, and it has been suggested that pathogens might be transmitted between different species (Thalwitzer et al. 2010). Thus, knowledge of adaptive genetic variation related to disease resistance such as the MHC may be pertinent to the African leopard conservation (Hedrick 2001). Previous MHC-related studies on leopards have been performed only on small scale for both MHC class I (southern blot analysis of one individual, Yuhki et al. 1989) and MHC class II (sequence analysis of clones of 1 individual, Wang et al. 2008; sequence analysis of clones of 7 individuals, Wei et al 2010). Currently, no information about the MHC sequence variation and constitution of free-ranging African leopards exists.

Here we describe genetic variation at the most relevant adaptive region of MHC I and MHC II-DRB genes in free-ranging African leopards from Namibia. We also investigate the evolution of these immune genes by testing for signatures of historical positive selection, recombination, and trans-species mode of evolution. Finally, the evolutionary affinities of MHC II-DRB sequences between African and Asian leopards are examined. Our study provides basic information for designing future studies on MHC variation in free-ranging leopard populations with different demographic histories and parasite exposures. This will further contribute to a better understanding of the evolutionary significance and conservation implications of MHC in free-ranging felids.

Materials and Methods

Sampling and DNA Isolation

Between 2002 and 2010, tissue samples (including full blood, cardia gastri, duodenum, kidney, liver, muscle, or skin/hair) from 25 individual leopards (15 males and 10 females) were collected in commercial livestock or game farmland in east-central Namibia. Leopards originated from the Windhoek, Okahandja, Gobabis, and Omaruru districts (Figure 1). All leopard samples were collected from wild-born free-ranging individuals. The relatedness of the individuals was not known except for 1 female with her 2 cubs. The samples were collected from individuals immobilized for a health check ($N = 12$) and legally killed by trophy hunters or farmers ($N = 13$). Four leopards were immobilized with a mixture of ketamine (4.0 mg/kg; Kyron Laboratories, Benrose, RSA) and xylazine (5.0 mg/kg; Bayer, Isando, RSA) and 8 with a mixture of ketamine (3.0 mg/kg; Kyron Laboratories) and medetomidine (0.05 mg/kg; Novartis, Spartan, RSA). All leopards were reversed with atipamezole (0.25 mg/kg; Novartis). Genomic DNA was isolated from the tissue samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturers' instructions.

PCR and Single-Stranded Conformation Polymorphism Analysis

We focused our study on the MHC I and II-DRB genes, particularly on those regions comprising the functionally important antigen-binding sites (ABS), that is, amino acid positions postulated to interact directly with foreign peptides. The second and third exons of MHC I genes

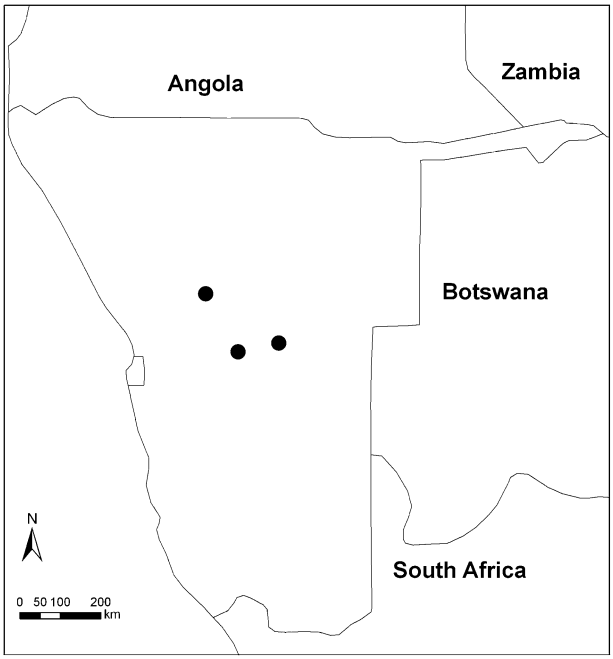

Figure 1. Schematic map showing the origin (dots) of the African leopard samples in Namibia.

encode the alpha 1 (α_1) and alpha 2 (α_2) extracellular domains, respectively, comprising the ABS of MHC I proteins (Bjorkman et al. 1987; Bjorkman and Parham 1990). The polymorphism observed in exon 2 is higher than in exon 3 in most mammal species including felids (e.g., domestic cat *Felis catus*, cheetah *A. jubatus*, ocelot *Leopardus pardalis*, Asiatic lion *P. l. persica*, and Bengal tiger *P. tigris tigris*; Yuhki and O'Brien 1990, 1994; Sachdev et al. 2005; Pokorny et al. 2010; Castro-Prieto et al. 2011). Therefore, we amplified the second exon (229 bp) of MHC I genes using the primers Acju_Ex2MhcI_cF (5′-GCTCCCACTC-CCTGAGGTAT-3′; Castro-Prieto et al. 2011) and Papa_Ex2MhcI_kR (5′-GGAKTCGCTCTGGTTG-TAGT-3′) designed from MHC I transcript sequences available from other felid species in GenBank. We also amplified the second exon (246 bp) of MHC II-DRB genes that encodes the beta 1 (β_1) extracellular domain of MHC II proteins (Brown et al. 1993) using the primers AJDRBaI-n1Ex2_F (5′-CCTGTSYCCACAGCACATTTCYT-3′) and AJDRBEx2In2_R (5′-TCAMCTCGCCGSTGCAC-3′; Castro-Prieto et al. 2011). 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 deoxynucleotide triphosphate mix, 10× 2.5 µl buffer, 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, and 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 I and II-DRB variation was screened through single-stranded conformation polymorphism (SSCP) analysis (Orita et al. 1989). This method can detect variants separated by only a single base difference (Sunnucks et al. 2000). SSCP analysis as described elsewhere (Castro-Prieto et al. 2011) was followed by sequence analysis of the distinctive single-strand bands. The PCR-SSCP analysis was conducted at least twice per individual sample on different gels to confirm its banding pattern reproducibility. The criteria used to define a sequence as a true allele were based on its occurrence in at least 2 independent PCR reactions derived from the same or different individuals. Allele sequences were named according to the nomenclature rules set by Klein et al. (1990).

Data Analysis

To examine patterns of sequence variation, nucleotide sequences were edited based on their forward and reverse consensus chromatograms using Chromas Pro Version 1.33 (Technelysium Pty Ltd), aligned, and coding regions translated into deduced amino acid sequences using CLUSTAL W as implemented in MEGA 3.1 (Kumar et al. 2004). The MHC-like nature of the sequences was verified through a homology analysis using blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Combinations of MHC sequences that are inherited together were referred to as haplotypes in this study. We used MEGA 3.1 to compute the mean number of nucleotide and amino acid differences, overall mean genetic distances of nucleotide sequences based on Kimura's 2-parameter (K2P) evolutionary distances, and of amino acids based on Poisson corrected distances.

To examine signatures of positive selection acting on MHC I and II-DRB sequences, we used 2 different approaches. First, we calculated by pairwise comparison the relative rates of nonsynonymous (d_N) and synonymous (d_S) nucleotide substitutions within and outside the ABS inferred from human MHC I (Bjorkman et al. 1987; Bjorkman and Parham 1990) and MHC II-DRB (Brown et al. 1993) molecules according to Nei and Gojobori (1986) with the Jukes and Cantor (1969) correction for multiple hits as implemented in MEGA 3.1. Standard errors of the estimates were obtained through 1000 bootstrap replicates. The relative rates of d_N and d_S did not deviate from normality (Kolmogorov–Smirnow Z test) and were compared with a 2-tailed t-test based on a significance level of $\alpha = 0.05$ in SPSS Version 16.0. Second, we used a maximum likelihood (ML) approach to detect species-specific positively selected codon sites ($d_N/d_S > 1$) using CODEML as implemented in PAML 4 (Yang 2007). The program estimates heterogeneous ω ($=d_N/d_S$) ratios among sites of aligned sequences applying different models of codon evolution as described in Yang et al. (2005). Neutral models M1a ($\omega_0 < 1$, $\omega_1 = 1$) and M7 ($0 < \omega < 1$) were compared with positive selection models M2a ($\omega_2 > 1$) and M8 ($0 < \omega < 1, \omega > 1$). M7 and M8 models are robust against the impact of recombination that can potentially generate false positives in the detection of positive selection (Anisimova et al. 2003). The models were evaluated using a likelihood ratio test (LRT; Nielsen and Yang 1998). To test the significance ($\alpha = 95\%$), the LRT statistic (twice the difference between the 2 negative

log likelihoods: $D = 2[Lb - La]$) was compared with the χ^2 distribution with the degrees of freedom (df) equal to the difference in the number of parameters between the models compared. Potential positively selected sites were identified at the 95% confidence level by both the Naive empirical Bayes (NEB) and the Bayes empirical Bayes (BEB) procedures (Zhang et al. 2005).

To detect the presence of recombination or gene conversion in the MHC I and II-DRB sequences of African leopards, we used the program GENECONV (Sawyer 1989). This recombination program is able to handle alignments of homologous sequences from the same locus or multiple loci (Posada 2002). GENECONV is based on the nucleotide substitution distribution to detect sequence fragments that were likely to have undergone recombination. Global and pairwise permutation tests (100 000 replicates) were used to assess significance. No mismatches were accepted, and *P* values were corrected for multiple comparisons.

We conducted model-based likelihood tree searches including the African leopard and other homologous sequences from different felid lineages available in GenBank to elucidate their relationships and to test for evidence of trans-species polymorphism. Sequences from Canidae species were used as out-groups to root the trees. The likelihood method included a Bayesian inference (BI) approach using MR BAYES 3.1 (Ronquist and Huelsenbeck 2003). The best fitting models of DNA evolution were selected based on the Akaike information criterion using MODELTEST (Posada and Crandall 1998) in combination with PAUP*4.0 (Win 32/DOS Beta Version 4; Swofford 1998). Those models corresponded to the transversional substitution model (TVM) with gamma shape distribution (TVM + Γ, $\alpha = 0.46$) for MHC I sequences and the general time reversible (GTR) with gamma shape distribution and a proportion of invariable sites (GTR + I + Γ, $\alpha = 0.63$ and I = 0.19) for MHC II-DRB sequences. The TVM model is a special case of the GTR model and is not yet implemented in MR BAYES; thus, we used the second best fitting GTR model (GTR + Γ) instead. BI trees were constructed based on the selected models and their estimated parameter values. Bayesian analysis run for 5×10^6 generations with a random starting tree and 2 runs of 4 heated and 1 cold Markov chains (heating = 0.20) sampled every 1000 generations. Burn-in corresponded to the first 20% of sampled trees based on the average standard deviation of split frequencies as well as by plotting the likelihood scores against generation time. The presence of similar sequences in different species does not always indicate trans-species polymorphism but rather convergent evolution (O'hUigiín 1995). To minimize the influence of convergence, we conducted tree searches based only on third codon positions (as most third position transitions are synonymous) of MHC I and II-DRB exon 2 sequences as described above (trees not shown). Trans-species polymorphism is likely when the tree topologies including all sites and those including only the third codon positions are similar. Finally, the evolutionary affinities between African and Asian leopard MHC II-DRB sequences were examined in a similar way through tree searches including the sequences from this study and all available DRB sequences from Asian leopards in GenBank. This was not possible for MHC I due to missing information in Asian leopards.

Results

MHC I

A total of 6 unique MHC I exon 2 nucleotide sequences (GenBank accession numbers HQ318105–10) were detected in 25 free-ranging African leopards from Namibia. They shared highest similarity (>95%) to homologous sequences from other felid species (e.g., domestic cat, ocelot, Asiatic lion, and Bengal tiger) available in GenBank and thus confirmed the MHC-like nature of the isolated MHC I sequences in *P. p. pardus*. The observed sequences grouped into 6 haplotypes (Table 1a). Between 2 and 6 sequences were detected per individual (Table 1a), indicating that our primers amplified at least 3 MHC I loci in the species. All individuals shared the sequences *Papa-MHCI*04* and *Papa-MHCI*06*, whereas *Papa-MHCI*02* and *Papa-MHCI*03* were detected in 23 (92%), *Papa-MHCI*05* in 17 (68%), and Papa-*MHCI*01* in 1 (0.4%) of the 25 sampled individuals.

The nucleotide alignment of MHC I exon 2 (229 bp) sequences revealed a total of 46 (20.09%) variable sites. No indels causing shifts of the reading frame and/or stop codons were detected. The putative amino acid translation of this fragment corresponded to 76 amino acids of the α_1 domain (positions 8–83; Figure 2a) according to human MHC I molecules (Bjorkman et al. 1987; Bjorkman and Parham 1990). Out of these 76 amino acid sites, 26 (34.21%) were variable, and of those, 13 were located in putative important antigen-binding positions (Figure 2a). The mean number of pairwise nucleotide differences between pairs of sequences was 19.73 ± 2.56 ranging from 7 (*Papa-MHCI*01* vs. *Papa-MHCI*03*) to 33 (*Papa-MHCI*04* vs. *Papa-MHCI*06*), and the mean number of amino acid differences was 12.67 ± 2.26 ranging from 5 (*Papa-MHCI*01* vs. *Papa-MHCI*03*) to 23 (*Papa-MHCI*04* vs. *Papa-MHCI*06*). The overall mean genetic distance among all sites of the MHC I nucleotide and the amino acid sequences was 9% and 18%, respectively (Table 2). The mean genetic distances for putative ABS were much higher than for non-ABS (Table 2).

Pairwise comparisons among the MHC I exon 2 sequences revealed a higher d_N than d_S in putative ABS ($t = 1.97$, df = 28, $P = 0.05$), whereas in non-ABS, d_S exceeded d_N ($t = -3.55$, df = 28, $P < 0.01$) (Table 3). The higher d_N than d_S in ABS results in an average ratio of $d_N/d_S > 1$, indicating historical positive selection acting on ABS (Hughes and Nei 1988, 1989). Also, the d_N was 7 times higher in the ABS (0.29) compared with non-ABS (0.04) ($t = 6.31$, df = 14.63, $P < 0.001$), supporting that selection was acting on these sites in the past (Table 3). The ML approach indicated potential positive selection on specific codon sites of MHC I sequences. The alternative model M8 (positive selection) fitted the data significantly better than

Table 1 MHC I and MHC II-DRB haplotypes observed in 25 free-ranging African leopards from east-central Namibia

	MHC haplotypes	No. of sequences	No. of individuals						
(a)				MHC I sequences					
				*Papa-MHCI*01*	*Papa-MHCI*02*	*Papa-MHCI*03*	*Papa-MHCI*04*	*Papa-MHCI*05*	*Papa-MHCI*06*
	I	6	1	1	1	1	1	1	1
	II	5	15	0	1	1	1	1	1
	III	4	6	0	1	1	1	0	1
	IV	4	1	0	1	0	1	1	1
	V	3	1	0	0	1	1	0	1
	VI	2	1	0	0	0	1	0	1
(b)				MHC II-DRB sequences					
				*Papa-DRB*01*	*Papa-DRB*02*	*Papa-DRB*03*	*Papa-DRB*04*	*Papa-DRB*05*	*Papa-DRB*06*
	I	5	2	1	1	1	1	1	0
	II	4	6	1	1	1	1	0	0
	III	4	3	1	1	0	1	1	0
	IV	4	1	0	1	1	0	1	1
	V	3	3	1	1	0	1	0	0
	VI	3	2	0	1	1	0	0	1
	VII	3	2	0	1	1	0	1	0
	VIII	3	1	1	0	0	1	1	0
	IX	2	4	0	1	1	0	0	0
	X	2	1	0	0	1	0	1	0

the null model M7 (neutral selection) ($P = 0.02$; Supplementary Material, Supplementary Table 1). Eight potential sites were identified under significant positive selection (9, 52, 63, 66, 67, 70, 74, and 77) by NEB method, but only 2 of those (67 and 70) were identified by BEB method. All sites but site 52 were consistent with ABS from those of the human MHC I molecule (Bjorkman et al. 1987; Bjorkman and Parham 1990) (Figure 2a).

GENECONV detected 2 fragments significantly involved in recombination events in a global comparison (i.e., after multiple comparisons correction for all possible sequence pairs) of the MHC I sequences of African leopards. One significant fragment was in the sequences of *Papa-MHCI*03* and *Papa-MHCI*05* at nucleotide positions 6–140 (135 pb length) in the alignment, and the other fragment was in the sequences of *Papa-MHCI*01* and *Papa-MHCI*05* at nucleotide positions 1–125 (125 pb length) in the alignment.

Phylogenetic reconstruction of the African leopard MHC I sequences in relation to other felids was poorly resolved. MHC I felid sequences were, however, clustered in a well-supported monophyletic group and diverged from the

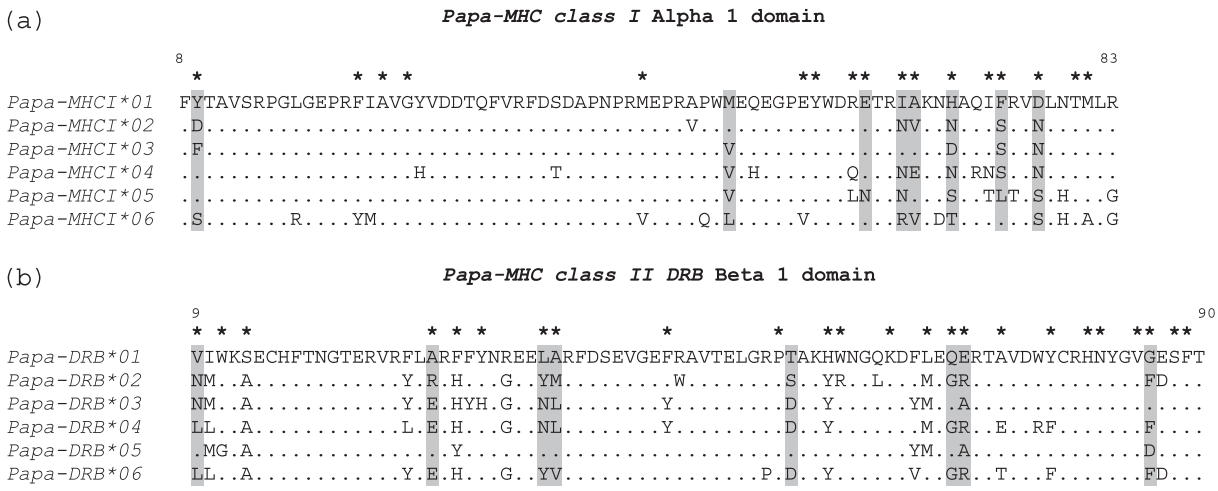

Figure 2. Deduced amino acid alignment of (**a**) MHC I and (**b**) MHC II-DRB sequences detected in 25 free-ranging African leopards. Dots indicate identity to the top sequence. Putative ABS (indicated with asterisks) in the alpha 1 (α1) and beta 1 (β1) domains were inferred from human MHC I (Bjorkman et al. 1987; Bjorkman and Parham 1990) and MHC II-DRB (Brown et al. 1993) molecules, respectively. Potential positively selected sites are shaded.

Table 2 Overall mean genetic distances (±standard errors) of 6 MHC I and 6 MHC II-DRB nucleotide and amino acid sequences detected in 25 African leopards

MHC gene	Region	Genetic distances		
		All	ABS	Non-ABS
MHC I	Exon 2	0.09 ± 0.01	0.27 ± 0.06	0.05 ± 0.01
	Alpha 1	0.18 ± 0.04	0.62 ± 0.16	0.09 ± 0.02
MHC II-DRB	Exon 2	0.12 ± 0.02	0.33 ± 0.06	0.05 ± 0.01
	Beta 1	0.21 ± 0.04	0.55 ± 0.12	0.10 ± 0.03

Distances are presented for all sites as well as only for the putative ABS and non-ABS.

canid out-group (gray wolf *C. lupus*) sequences (Figure 3). Leopard MHC I sequences showed a scattered distribution along the phylogram. They segregated independently from each other, and some clustered with sequences from other felid species with high statistical support (e.g., leopard *Papa-MHCI*04* with lion Pale AY909826, Pale AY909887, Pale AY909893, and Pale AY909873; leopard *Papa-MHCI*05* with cheetah Acju AJU07666 and tiger Pati HQ157994; leopard *Papa-MHCI*06* with lion Pale AY909889, Pale AY909880, and Pale AY909819; Figure 3). This pattern was consistent when only third codon positions (synonymous sites) were considered indicating trans-species polymorphism. Trans-species polymorphism was further supported with a sequence alignment including only polymorphic sites of exon 2 (Supplementary Material, Supplementary Figure 1). This comparison revealed short polymorphic sequence motifs throughout MHC I sequences in the African leopard that were also found in the sequences of other felid species. This sequence variation pattern is consistent with the mosaic structure previously observed between MHC I sequences in divergent felid species, which has been suggested as evidence of trans-species mode of retention of ancient variation through speciation processes in Felidae (Yuhki and O'Brien 1994).

MHC II-DRB

African leopards also showed a total of 6 distinct MHC II-DRB exon 2 nucleotide sequences (GenBank accession numbers HQ318099–104). Sequence *Papa-DRB*02* was previously observed in 2 Asian leopards from China (GenBank accession number FJ210710; Wei et al. 2010). Novel sequences shared highest similarity (>95%) to homologous sequences of other felid species (e.g., ocelot, lion, and tiger) available in GenBank. The observed sequences grouped into 10 haplotypes (Table 1b). Between 2 and 5 sequences were observed in a single individual (Table 1b), indicating that our primers amplified at least 3 MHC II-DRB loci in the species. Sequence *Papa-DRB*02* was detected in 23 (92%), *Papa-DRB*03* in 18 (72%), *Papa-DRB*01* and *Papa-DRB*04* in 15 (60%), *Papa-DRB*05* in 10 (40%), and *Papa-DRB*06* in 3 (12%) of the 25 sampled individuals.

The nucleotide alignment of MHC II-DRB exon 2 (246 bp) sequences revealed a total of 57 (23.17%) variable sites. No indels causing shifts of the reading frame and/or stop codons were detected. The putative amino acid translation of this fragment corresponds to 82 amino acids of the β_1 domain (positions 9–90; Figure 2b) according to human MHC II-DRB molecules (Brown *et al.* 1993). Out of 28 (34.15%) variable amino acid sites, 17 were located in important positions for antigen binding (Figure 2b). The mean number of pairwise nucleotide differences between pairs of sequences was 27.60 ± 3.31 ranging from 14 (*Papa-DRB*04* vs. *Papa-DRB*06*) to 37 (*Papa-DRB*01* vs. *Papa-DRB*03* and *Papa-DRB*04*), and the mean number of amino acid differences was 15.60 ± 2.58 ranging from 8 (*Papa-DRB*01* vs. *Papa-DRB*05*) to 20 (*Papa-DRB*05* vs. *Papa-DRB*06*). The overall mean genetic distance among all sites of the MHC II-DRB nucleotide and the amino acid sequences was 12% and 21%, respectively (Table 2). As with MHC I regions, genetic distances for putative ABS were higher than for non-ABS (Table 2).

Pairwise comparisons among the MHC II-DRB exon 2 sequences revealed similar d_N and d_S in putative ABS and non-ABS (Table 3). This results in an average ratio of d_N/d_S close to 1, indicating neutral selection acting on ABS inferred from the human sequence. However, as observed in MHC I, the d_N was 6 times higher in the ABS (0.32) compared with non-ABS (0.05) (Table 3; $t = 7.7$, df = 14.29, $P < 0.001$), indicating that positive selection was likely acting on these sites at least in the past (Table 3). Positive selection on specific codon sites of MHC II-DRB sequences was detected by the ML method. The alternative models M2a and M8 (positive selection) fitted the DRB data

Table 3 The average rates of nonsynonymous substitutions (d_N) and synonymous substitutions (d_S) with standard errors and their ratio in ABS and non-ABS assuming concordance with the human MHC I (Bjorkman and Parham 1990) and MHC II-DRB molecules (Brown *et al.* 1993)

MHC locus	Region	Site	N^a	d_N	d_S	d_N/d_S	P
MHC I	Exon 2	ABS	17	0.29 ± 0.06	0.17 ± 0.09	1.70	0.05
		non-ABS	59	0.04 ± 0.01	0.08 ± 0.03	0.50	0.01
		All	76	0.09 ± 0.02	0.10 ± 0.03	0.90	0.70
MHC II-DRB	Exon 2	ABS	24	0.32 ± 0.13	0.38 ± 0.29	0.84	0.48
		non-ABS	58	0.05 ± 0.01	0.06 ± 0.04	0.83	0.31
		All	82	0.12 ± 0.04	0.13 ± 0.07	0.92	0.73

P denotes the probability that d_N and d_S are different using a t-test.

[a] Number of codons in each category.

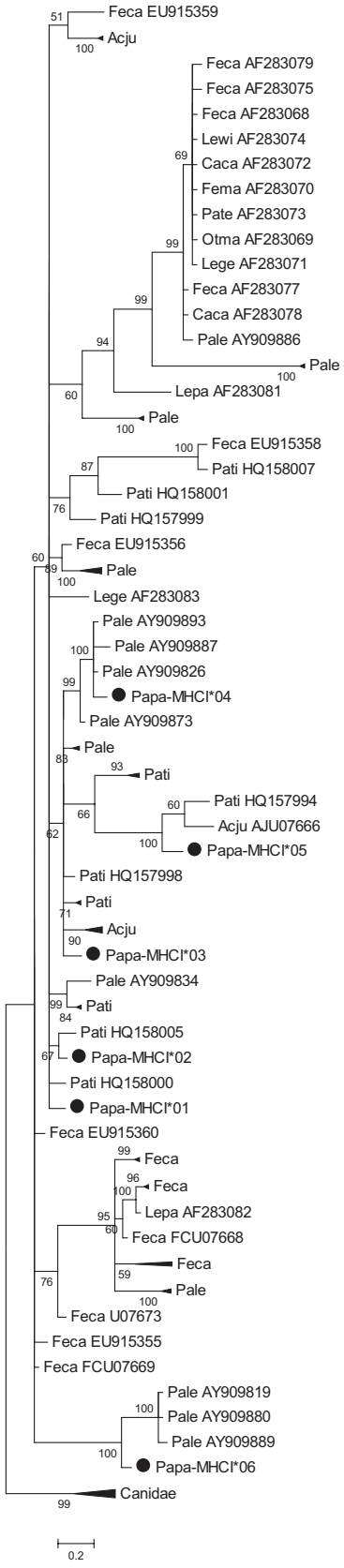

Figure 3. Phylogenetic relationships of the African leopard (*Papa*: *Panthera pardus pardus*) MHC I exon 2 sequences (indicated by circles) with a representative set of other felid sequences (*Acju*: *Acinonyx jubatus*, *Caca*: *Caracal caracal*, *Feca*: *Felis catus*, *Fema*: *F. margarita*, *Lege*: *Leopardus geoffroyi*, *Lepa*: *L. pardalis*, *Lewi*: *L. wiedii*, *Otma*: *Otocolobus manul*, *Pale*: *P. leo*, *Pati*: *P. tigris*, *Pate*: *Pardofelis temminckii*) followed by their corresponding GenBank accession numbers. We used canid sequences (*Calu*: *Canis lupus*) as out-group to root the tree. The 50% majority-rule tree from the Bayesian analysis is shown. Numbers refer to Bayesian posterior probability values.

significantly better than the null models M1a and M7 (neutral selection) ($P < 0.001$; Supplementary Material, Supplementary Table 1). Six potential sites were identified under significant positive selection (9, 28, 37, 38, 57, and 86) by both NEB and BEB methods. Two sites (70 and 71) were additionally identified by the BEB method. All sites but site 57 were consistent with ABS from those of the human DRB1 molecule (Brown et al. 1993) (Figure 2b).

GENECONV detected a single fragment significantly involved in recombination events in a global comparison of the MHC II-DRB sequences of African leopards. This fragment was in the sequences of *Papa-DRB*03* and *Papa-DRB*05* at nucleotide positions 155–232 (78 pb length) in the alignment.

Phylogenetic reconstruction of the African leopard MHC II-DRB sequences in relation to other felids is shown in Figure 4. MHC II-DRB felid sequences were monophyletic and clearly diverged from the canid out-group (domestic dog, African wild dog, gray wolf, and coyote *C. latrans*). The ancient origin of MHC II-DRB allelic lineage within felids was previously suggested by Yuhki and O'Brien (1997) and recently supported by Wei et al. (2010). African leopard MHC II-DRB sequences revealed a scattered distribution throughout the phylogram and segregated independently from each other. Some of these sequences clustered with those from other felid species. For example, sequences from the African leopard *Papa-DRB*01* and tiger Pati FJ210690–93 clustered with a high statistical support. This pattern was consistent when only third codon positions (synonymous sites) were considered. Furthermore, sequences from Asian leopard Papa FJ210700 and tiger Pati FJ210699 were identical, indicating an extreme case of trans-species polymorphism (Wei et al. 2010).

African leopard sequences were also strongly related to those from Asian leopard. For example, the African leopard *Papa-DRB*02* was identical to the Asian leopard Papa FJ210710 and strongly clustered with Asian leopard Papa FJ210711 (Figure 4), indicating that those sequences likely belong to a single locus that is present in both subspecies. Similarly, the African leopard *Papa-DRB*05* clustered with Asian leopard Papa FJ210700 (Figure 4). The sequences *Papa-DRB*03*, *Papa-DRB*04*, and *Papa-DRB*06* observed in African leopards did not show any close relationship to other Asian leopard sequences. However, when considering only synonymous sites, sequences *Papa-DRB*03* and *Papa-DRB*04*

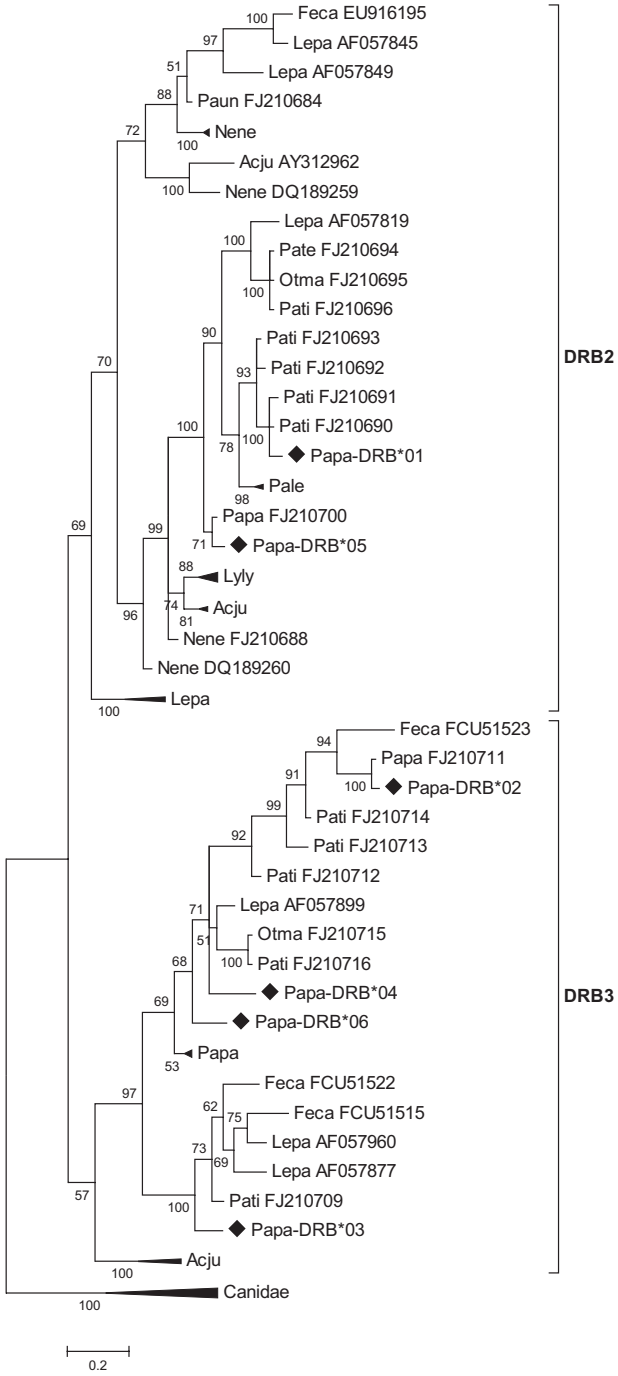

Figure 4. Phylogenetic relationships of the African leopard (*Papa*: *Panthera pardus pardus*) MHC II-DRB exon 2 sequences (indicated by diamonds) with a representative set of other felid sequences (*Acju*: *Acinonyx jubatus*, *Feca*: *Felis catus*, *Lepa*: *Leopardus pardalis*, *Lyly*: *Lynx lynx*, *Nene*: *Neofilis nebulosa*, *Otma*: *Otocolobus manul*, *Pale*: *P. leo*, *Pati*: *P. tigris*, *Paun*: *P. uncia*, *Pate*: *Pardofelis temminckii*) followed by their corresponding GenBank accession numbers. We used canid sequences (*Cafa*: *Canis familiaris*, *Cala*: *C. latrans*, *Calu*: *C. lupus*, *Lypi*: *Lycaon pictus*) as out-group to root the tree. DRB2 and DRB3 label 2 out of 5 well-defined DRB allelic lineages suggested for modern felid species (Yuhki and O'Brien 1997; Wei et al. 2010). Note that sequences from African leopard *Papa-DRB*02* and Asian leopard Papa FJ210710 are identical, and Asian leopard Papa FJ210700 is identical to tiger Pati FJ210699. The 50% majority-rule tree from the Bayesian analysis is shown. Numbers refer to Bayesian posterior probability values.

clustered together, suggesting that they might belong to the same locus, and sequence *Papa-DRB*06* grouped with Asian leopard Papa DQ189262–64, suggesting that they might belong to the same locus occurring in both subspecies (Figure 5). Nonetheless, as the 3 sequences Papa DQ189262–64 were isolated from a single individual, they belong to 2 different loci rather than 1 locus (Wang et al. 2008).

Discussion

Sequence Variation and Gene Duplication

In the present study, we isolated and described the genetic variation at MHC I and II-DRB genes observed in free-ranging African leopards from Namibia. A total of 6 sequences from at least 3 putative loci in both MHC I and II-DRB were detected in 25 individuals. There are only few MHC studies conducted in free-ranging felid populations and in most cases with unknown locus information, which make interspecific comparisons difficult. For example, Bengal tigers showed 14 sequences from at least 4 MHC I loci and 4 sequences from at least 2 MHC II-DRB loci in 16 individuals from different geographic regions in India (Pokorny et al. 2010). Asiatic lions showed 52 sequences from at least 5 MHC I loci in 25 individuals from the Gir Forest, India (Sachdev et al. 2005). However, in the latter study, 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 MHC I allelic diversity in the lions was overestimated. The levels of MHC diversity (in terms of number of sequences) in Namibian leopards detected in this study are relatively low at MHC I but similar at MHC II-DRB loci compared with Pantherinae species from the previously mentioned studies. This is unexpected because the amount of neutral genetic diversity revealed in leopards is higher or comparable to other big cats (e.g., lions, jaguars, and pumas), although it varies significantly across their geographic range (Uphyrkina et al. 2001). Moreover, the African leopard is the most genetically diverse leopard subspecies as revealed by mitochondrial DNA and neutral microsatellite markers (Spong et al. 2000; Uphyrkina et al. 2001). Therefore, we would expect also higher levels of MHC diversity in this subspecies. The low number of MHC sequences observed in leopards from this study, however, may be partly explained by artifacts due to sampling bias. Our sampling coverage was limited to individuals from east-central Namibia, and therefore, it may not reflect the extent of MHC variation of the whole population. Thus, the incorporation of additional samples from throughout Namibia is required to make an

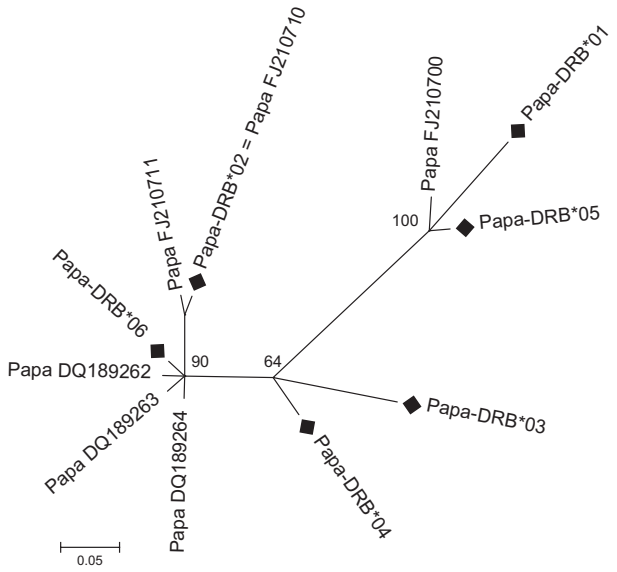

Figure 5. Unrooted phylogenetic tree including only third codon positions of the 6 MHC II-DRB exon 2 sequences detected in African leopards (indicated by diamonds, this study) and 6 Asian leopard sequences (Wang et al. 2008; Wei et al. 2010). Sequence *Papa-DRB*02* was observed in both subspecies. The 50% majority-rule tree from the Bayesian analysis is shown. Numbers refer to Bayesian posterior probability values. The branch lengths are proportional to distances, and the scale bar indicates percentage of divergence.

accurate estimate of the current MHC variation in this leopard population. Also, the use of multiple primer sets might expand the number of MHC genes or alleles recovered in the population. Additionally, the potential relatedness of the leopards sampled may have influenced our results. However, different collection sites and dates as well as the origin of the samples suggest that all but 3 samples belong to unrelated individuals. Also, the number and distribution of the MHC sequences observed in leopards from different collection sites did not show any pattern indicative of relatedness among them.

The extent of diversity in terms of amino acid sequence variation among MHC I sequences from African leopards (18%, Table 2) is higher than the one from domestic cats (12%; Yuhki and O'Brien 1990), African cheetahs (14%; Castro-Prieto et al. 2011), and Bengal tigers (13%; Pokorny et al. 2010), although similar to the one from ocelots (17%; Yuhki and O'Brien 1994) and Asiatic lions (17%; Sachdev et al. 2005). For MHC II-DRB sequences, the extent of diversity from African leopards (21%, Table 2) is higher than the one from Asian leopards (13%; Wang et al. 2008; Wei et al. 2010) and Eurasian lynx (14%; Wang et al. 2009), although similar to the one from domestic cats (19%; Yuhki and O'Brien 1997), African cheetahs (20%; Castro-Prieto et al. 2011), ocelots (18%; GenBank accession numbers AAF70955–64), margays (19%; GenBank accession numbers AAF71016–25), and Bengal tigers (18%; Pokorny et al. 2010).

Evidence of multiple loci at MHC I and II-DRB was revealed by the presence of up to 6 and 5 sequences, respectively, in a single leopard individual (Table 1a and 1b), indicating the occurrence of at least 3 gene copies in the species. This is consistent with 3 functional gene copies of MHC II-DRB observed in other felids such as domestic cats (Yuhki et al. 2008) and cheetahs (Castro-Prieto et al. 2011). Gene duplication is considered as an important mechanism to generate MHC diversity (Klein et al. 1998) and has been documented also in other mammal species (Yeager and Hughes 1999). Because of interlocus allelic exchange known to occur at MHC genes (Yeager and Hughes 1999), the accurate assignment of the observed sequences in *P. p. pardus* to specific loci is not possible without more detailed genomic information (e.g., a considerable longer fragment including introns). This may require a more challenging genotyping approach such as extensive cloning or next-generation sequencing analyses (Babik et al. 2009). Most MHC-related studies in nonmodel species face the problem of lacking detailed genomic information, which is a major constraint for detailed and accurate estimations of MHC polymorphism and molecular evolution analyses (Edwards et al. 1995). Nevertheless, an increasing number of MHC studies in wildlife species has been conducted addressing similar aspects as in the present study (e.g., Miller and Lambert 2004; Babik et al. 2005; Miller et al. 2007; Meyer-Lucht et al. 2008; Promerová et al. 2009; Bollmer et al. 2010; Pokorny et al. 2010). As in the previous studies, we treated our data for all statistical analyses as if all sequences observed in *P. p. pardus* corresponded to the same MHC I or MHC II-DRB locus and discussed the limitations of the analyses to make detail-oriented inferences on the MHC evolution in African leopards.

Patterns of Historical Positive Selection and Recombination

The observed excess of nonsynonymous over synonymous substitutions in putative ABS indicates historic positive selection most likely driven by pathogens acting on the second exon of MHC I sequences of African leopards (Hughes and Nei 1988). This is consistent with higher nucleotide and amino acid mean distances revealed at defined ABS compared with non-ABS of MHC I sequences (Table 2). In contrast to MHC I, no difference between nonsynonymous and synonymous substitutions in ABS was detected in the second exon of MHC II-DRB sequences of African leopards indicating neutral evolution (Nei 2005). Still, nucleotide and amino acid mean distances were higher at defined ABS compared with non-ABS (Table 2), suggesting that selection has favored amino acid changes in positions that are postulated to interact with peptides, at least in historical times. This variation pattern is rare in MHC II-DRB genes of most mammalian populations living under natural conditions, which frequently show significantly higher rates of d_N compared with d_S in ABS (Bernatchez and Landry 2003; Sommer 2005). However, our results are consistent with low d_N/d_S ratios in ABS observed across major allelic lineages of MHC II-DRB loci

in 8 putative extant Felidae lineages (Wei et al. 2010). Positive selection was also detected on specific codon sites of both MHC I and MHC II-DRB sequences of African leopards, as revealed by the ML method.

The critical role of positive selection shaping the diversity in MHC loci has been well documented in other mammals (reviewed by Sommer 2005; Piertney and Oliver 2006), including species from different felid lineages (e.g., domestic cat, ocelot, cheetah, and tiger; Yuhki and O'Brien 1990; 1997; O'Brien and Yuhki 1999; Pokorny et al. 2010; Castro-Prieto et al. 2011). Although signatures of positive selection on MHC loci were also observed in African leopards, it is more difficult to estimate precisely the magnitude of this selection considering the limitations of our data (e.g., unknown locus information and lack of expression patterns). Our study included sequences that belong to closely related but different loci, which may potentially result in underestimates of d_N and consequently bias the d_N/d_S ratios (Piertney and Oliver 2006). Such underestimations occur when the sequences compared differ by large numbers of synonymous substitutions, which leads to saturation of the corresponding estimates of d_N (Edwards et al. 1995). This is commonly observed when comparing highly divergent sequences and may potentially explain the unexpected low d_N/d_S ratios observed in MHC II-DRB of African leopards. The 6 sequences isolated are highly divergent (12%; Table 2) and correspond to at least 3 different loci in the species. Moreover, comparing sequences that do not correspond to classical MHC genes (i.e., highly polymorphic and ubiquitously expressed and thus functional) but rather nonclassical MHC genes (i.e., limited polymorphism and nonubiquitously expressed) or even pseudogenes (i.e., monomorphic and nonexpressed thus nonfunctional) results in a lower d_N/d_S ratio than expected (Hughes and Nei 1989). In our study, all sequences contain the conserved residues expected in functional MHC I and II-DRB alleles from humans (Kaufman et al. 1994), a reading frame with no terminal codons or frameshift mutations, suggesting that all sequences derived from this study likely correspond to functional MHC alleles. This assumption, however, cannot be confirmed until further expression analyses are conducted. Estimates of d_N/d_S ratios may also be compromised by the fact that ABS sites for a given allele may vary with the peptide it binds, as well as between alleles or species (Edwards et al. 1995). Our analysis was based on ABS defined for human's MHC molecules (Bjorkman et al. 1987; Brown *et al.* 1993) as no such information is available for felids. However, comparative sequence analysis has revealed an extraordinary similarity in the quantity and quality of MHC I and II-DRB polymorphism (Yuhki et al. 1989; Yuhki and O'Brien 1997).

Recombination or gene conversion has been previously suggested as an important mechanism in the origin and maintenance of MHC diversity in domestic cats (Yuhki and O'Brien 1990) and free-ranging felid species (Yuhki and O'Brien 1994; O'Brien and Yuhki 1999). In this study, the presence of recombination was detected in the history of both MHC I and II-DRB sequences from African leopards. The program GENECONV has been evaluated as having a high probability of inferring correctly recombination events (Posada 2002). The presence of PCR-induced recombinant sequences in African leopards was ruled out by comparing the products from 2 independent amplifications per individual sample. The occurrence of common sequence motifs between MHC I sequences of African leopards and other divergent felid species (Supplementary Material, Supplementary Figure 1) further supports recombinational mechanisms to generate mosaic structures previously observed among felid MHC I sequences (Yuhki and O'Brien 1994). The mosaic pattern structure, however, was rarely seen in feline MHC II-DRB sequences, suggesting different modes of evolution operate diversification of feline MHC I and MHC II-DRB genes (Yuhki and O'Brien 1997).

Phylogenetic Analysis and Trans-species Mode of Evolution

The MHC II-DRB sequences from felids included in this analysis were segregated into 2 major clusters that were not species or lineage specific (Figure 4). These clusters were consistent with 2 (DRB2 and DRB3) of 5 well-defined DRB allelic lineages suggested for modern felid species (Yuhki and O'Brien 1997; Wei et al. 2010). All sequences from African and Asian subspecies were segregated between these 2 allelic lineages. Wei et al. (2010) suggested DRB2 to be the oldest among all allelic lineages as it included all Pantherinae species as well as representative species from the extant felid lineages (except caracal *Caracal caracal*), indicating that DRB2 predates the felid ancestor diversification into modern felid species at around 10.8 Ma (Johnson et al. 2006). Leopard sequences *Papa-DRB*01* and *Papa-DRB*05* belong to the DRB2 allelic lineage, whereas the sequences *Papa-DRB*02*, *Papa-DRB*03*, *Papa-DRB*04*, and *Papa-DRB*06* belong to the DRB3 allelic lineage. We found 11 individuals with at least 3 of the 4 sequences in the DRB3 allelic lineage (Table 1b), which confirms that this allelic lineage is not restricted to a single locus. Yuhki and O'Brien (1997) previously suggested that recent duplication events occurred after the generation of this allelic lineage based on domestic cat sequences.

Phylogenetic analyses indicated that MHC I and MHC II-DRB alleles of African leopard were closer to those of other Pantherinae species such as lion and tiger than to each other (Figures 3 and 4). This pattern is commonly interpreted as trans-species polymorphism (Klein et al. 1998). Trans-species polymorphism in MHC I was further supported by common sequence motifs between African leopard and other divergent felid species (Supplementary Material, Supplementary Figure 1). The influence of convergent evolution on the phylogenetic analyses was minimized by comparing third codon positions of the second exon of MHC sequences. However, for quantifying the extent of trans-species polymorphism, it is required to expand the phylogenetic analyses to regions under less or no selection at these loci. Our results are consistent with a trans-species mode of evolution of MHC I loci (Yuhki and O'Brien 1994; Smith and Hoffman 2001) and MHC II-DRB

loci (Yuhki and O'Brien 1997; O'Brien and Yuhki 1999; Wang et al. 2008; Wei et al. 2010) previously suggested for the Felidae family. This result gives further evidence for the selective maintenance of MHC polymorphism.

Evolutionary Affinities of MHC II-DRB Sequences between African and Asian Leopards

The evolutionary affinities of MHC II-DRB sequences between African (Namibia) and Asian (China) leopard subspecies suggest the presence of at least 4 putative DRB loci in African and Asian leopards, but only 3 of those loci are likely to be shared between both subspecies (Figure 5). It is likely that the sequence *Papa-DRB*02* (=Papa FJ210710) detected in both African and Asian leopard subspecies belongs to a DRB gene copy that was already present in the last common ancestor of the modern leopard lineages. This is assumed to be dated before the Pliocene/Pleistocene (3.0 Ma) migrations from Asian-derived Panthera species toward Africa occurred (Johnson et al. 2006). The maintenance of this particular sequence during the evolutionary history of the species and its occurrence in 23 out of 25 individuals analyzed in this study suggests that it has played an important adaptive role likely related to pathogen recognition. A similar scenario is suggested for African leopard sequences *Papa-DRB*01* and *Papa-DRB*05* and Asian leopard sequence FJ21700 on the basis of their close relationship. Sequences *Papa-DRB*01* and *Papa-DRB*05* differ in 4 and 1 amino acid, respectively, from FJ21700, and all 3 sequences belong to the oldest DRB allelic lineage (DRB2) proposed among the felids. The high similarity between leopard sequences from Africa *Papa-DRB*06* and Asia Papa DQ189262–64 also suggests that this gene copy was present before the divergence of both subspecies. However, these sequences are not as old as the ones in the allelic lineage DRB2 because they belong to a more recent allelic lineage DRB3. The opposite scenario is likely for sequences *Papa-DRB*03* and *Papa-DRB*04*. They apparently belong to one, presumably more recent gene copy that evolved only within African leopards, because no identical or significantly closely related DRB sequences to Asian leopards were detected.

Implications for Conservation

The extent and patterns of adaptive genetic variation is crucial for the long-term survival of wildlife species and therefore of primary interest in conservation genetics (Hedrick 2001). The loss of adaptive MHC variation has the potential to affect the ability to mount a protective immune response (O'Brien and Evermann 1988; Hughes 1991), but a clear association between loss of MHC diversity and susceptibility to disease has not been established (Hedrick and Kim 2000; Acevedo-Whitehouse and Cunningham 2006; Radwan et al. 2009; Reed 2010). For example, low MHC variation does not appear to influence the immunocompetence of free-ranging Namibian cheetahs (Castro-Prieto et al. 2011), but on the other hand, an increased susceptibility to devil facial tumor disease has been attributed to the loss of MHC variation in free-ranging Tasmanian devils (*Sarcophilus harrisii*; Siddle et al. 2007). So far, no major epizootics have been recorded for African leopards in contrast to other free-ranging African carnivores (Kat et al. 1995; Roelke-Parker et al. 1996), which might be considered as a sign of a robust immunocompetence in the species. The low MHC variation detected in African leopards from Namibia is not conclusive, and further research is required to assess the extent of MHC variation in this population. Also, further research should focus on MHC composition in relation to parasite load in different populations of African leopards. We also recommend expanding the MHC genotyping to critically endangered leopard populations such as the Far Eastern leopard (*P. p. orientalis*) that exhibits markedly reduced levels of neutral genetic variation (Uphyrkina et al. 2002) and may be at great potential risk of disease in the presence of a newly emerging pathogen.

Using next-generation sequencing technologies to investigate patterns of genome-wide variation, even on the population level, will become feasible in the near future. Such techniques will provide a much more complete picture on the evolutionary adaptive potential of leopard populations of different subspecies.

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Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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