Leibniz Institut für Zoo und Wildtierforschung
Forschungsgruppe Evolutionsgenetik

Immunogenetics of free-ranging felids on Namibian farmlands

Dissertation

Zur Erlangung des akademischen Grades

“doctor rerum naturalium”

(Dr. rer.nat.)

in der Wissenschaftsdisziplin “Evolutionsgenetik”

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Potsdam

von

Aines del Carmen Castro Prieto

Potsdam, Juni 2011

Published online at the
Institutional Repository of the University of Potsdam:
URL http://opus.kobv.de/ubp/volltexte/2011/5550/
URN urn:nbn:de:kobv:517-opus-55505
http://nbn-resolving.de/urn:nbn:de:kobv:517-opus-55505

In Loving Memory of my Mother

You inspire me to achieve everything I wish

Contents

Preface

The present study is part of The Cheetah Research Project from the Leibniz Institute for Zoo and Wildlife Research (IZW) established in 2002. The project focuses on the world's largest remaining free-ranging cheetah population in Namibia which is considered as the key population for the survival of the species. The aim of Cheetah Research Project is to shed light into the cheetah's biology, ecology and factors affecting the health status in order to provide a scientific basis for the sustainable protection of this population.

For my doctoral research, I conducted the first large-scale study on immune gene variation within the Major Histocompatibility Complex (MHC) in free-ranging Namibian cheetahs and assessed its relevance for the host's susceptibility to diseases. In addition, I investigated the genetic diversity and evolutionary patterns of the MHC in free-ranging leopards that were opportunistically collected on Namibian farmlands. Cheetahs and leopards coexist in Namibia being exposed to similar pathogenic pressures. On the other hand, they differ in their evolutionary and demographic histories resulting in contrasting levels of neutral genetic diversity.

My dissertation comprises three manuscripts that can be read independently. Two are published and the one remaining is submitted to international peer-reviewed scientific journals. All articles include co-authors and are therefore written in first person plural. Each article summary describes individual co-authors contributions in order to highlight my own contributions.

Acknowledgments

I would like to express my gratitude to everyone who has helped to accomplish this thesis. First of all, I am deeply thankful to my "Doktormutter" Simone Sommer, for trusting me in doing this job, mentoring me, and for continuous support and friendship during the four years of my study. I would like to thank Bettina Wachter, for introducing me to the cheetahs in Namibia and for great feedback during the development of my research as well as in every single manuscript; Members of the Cheetah Research Project during my stay in Namibia, for showing me the exciting fieldwork of trapping and handling cheetahs in the wild, specially to Annika Krengel for hosting me warmly in the farm and showing me nice places in Namibia. Thank you all for giving me the opportunity to join the fieldwork in Namibia; it was truly a great experience!

I am thankful to my colleagues from the "Evolutionary Genetics Research Group" at the Leibniz-Institut für Zoo und Wildtierforschung (IZW), for the ideas you have shared with me, fruitful discussions and all insights and comments on my work, especially to Jan Axtner, Yvonne Meyer-Lucht, Nina Schwensow, Julia Schad, Goetz Froeschke, Thomas Püttker and Francisca Robles. Guys, I need more than a couple of sentences to express my gratitude for the way you received me (us) when I first arrived in Berlin, not only at work but in your lives too. I can only say that I am really lucky for having met you and shared sometime with you during these years; Alexandra Weyrich and Camila Mazzoni for saying the right words at the right time, which is essential for surviving a PhD, and for having you on my side. Thanks a lot to all of you for continuous emotional and intellectual support. I also want to thank Jörns Fickel, Jürgen Streich, Arne Ludwig, Michael Cieslak, Andreas Wilting, Niko Balkenhol and Pablo Santos for advice and support in whatever way; Technical Assistants from our group Tanja Noventa, Dietmar Lieckfeldt, Birgit Bieber, and Ramona Taubert for never hesitate in offering help and for the nice atmosphere in the Lab. I am especially thankful to Anke Schmidt for continuous assistance in the Lab.

I appreciate the nice moments with my "doktoranden" colleagues and friends, thanks for sharing with me. I am also thankful to all of those that are not mentioned here but have contributed in whatever way to make my stay at the IZW a very pleasant one.

I would like to thank Michael Monaghan for data analyses assistance and helpful suggestions during my study. Also, Renita Danabalan for language advice in the mansucripts. Thank you guys for great support and friendship when most needed.

Finally, I want to thank the most important people in my life, my beautiful family.

Con esta tesis de doctorado, finalmente culmino mis estudios de posgrado!

Quiero expresar mi más profundo agradecimiento a mi hermosa familia. Especialmente a mis padres por darme las herramientas necesarias para poder alcanzar esta meta que tenía trazada desde hace ya muchos años. Mamá, gracias por enseñarme que las puertas se abren a quien gira el picaporte y por seguir siempre adelante ante la adversidad. Papá, gracias por confiar en mí y apoyarme siempre en mis decisiones, también por cuidar de Cosmo. Monty, sé que mis estudios me llevaron lejos de tu lado tal vez cuando más me necesitabas; gracias hermana por apoyarme siendo fuerte y dando lo mejor de ti todo este tiempo. A mi Tita y mis hermanas mayores Rommy y Janet porque siempre he contado con su cariño y apoyo incondicional. Tío Emir, gracias por motivarme a retomar el rumbo de la Biología; siempre supe que era lo mío! A mis sugeros Eli y Fallo por apoyarnos con la decisión de venir a Alemania, sé que extrañan mucho a su pollito mayor. Gracias David por ayudarnos con la Chata y el Gabino. Finalmente, y no menos importante, quiero agradecerle a mi marido. Rafa, no sabes cuánto aprecio el gran apoyo que he recibido de tu parte día tras día durante estos cuatro años, es realmente invaluable. Gracias por aventurarte conmigo dejando tu mundo marino de lado para que estuviéramos juntos y yo pudiera cumplir este anhelo; significa mucho para mí. Sé que la experiencia no ha sido fácil pero estoy segura de que lo que hemos aprendido juntos este tiempo, tan lejos de nuestras raíces, nos ha hecho valorar lo que somos, lo que tenemos y lo que queremos. Confío en que los frutos de nuestro esfuerzo pronto se verán reflejados.

Agradezco el apoyo otorgado a través de la beca Bicentenario de alta competencia para posgrado e investigación en el extranjero ciclo 2010, de la Secretaría de Educación Pública y el Gobierno Mexicano.

Summary

Genetic variation is crucial for the long-term survival of the species as it provides the potential for adaptive responses to environmental changes such as emerging diseases. The Major Histocompatibility Complex (MHC) is a gene family that plays a central role in the vertebrate's immune system by triggering the adaptive immune response after exposure to pathogens. MHC genes have become highly suitable molecular markers of adaptive significance. They synthesize two primary cell surface molecules namely MHC class I and class II that recognize short fragments of proteins derived respectively from intracellular (e.g. viruses) and extracellular (e.g. bacteria, protozoa, arthropods) origins and present them to immune cells. High levels of MHC polymorphism frequently observed in natural populations are interpreted as an adaptation to detect and present a wide array of rapidly evolving pathogens. This variation appears to be largely maintained by positive selection driven mainly by pathogenic selective pressures.

For my doctoral research I focused on MHC I and II variation in free-ranging cheetahs (*Acinonyx jubatus*) and leopards (*Panthera pardus*) on Namibian farmlands. Both felid species are sympatric thus subject to similar pathogenic pressures but differ in their evolutionary and demographic histories. The main aims were to investigate 1) the extent and patterns of MHC variation at the population level in both felids, 2) the association between levels of MHC variation and disease resistance in free-ranging cheetahs, and 3) the role of selection at different time scales in shaping MHC variation in both felids. Cheetahs and leopards represent the largest free-ranging carnivores in Namibia. They concentrate in unprotected areas on privately owned farmlands where domestic and other wild animals also occur and the risk of pathogen transmission is increased. Thus, knowledge on adaptive genetic variation involved in disease resistance may be pertinent to both felid species' conservation.

The cheetah has been used as a classic example in conservation genetics textbooks due to overall low levels of genetic variation. Reduced variation at MHC genes has been associated with high susceptibility to infectious diseases in cheetahs. However, increased disease susceptibility has only been observed in captive cheetahs whereas recent studies in free-ranging Namibian cheetahs revealed a good health status. This raised the question whether the diversity at MHC I and II genes in free-ranging cheetahs is higher than previously reported. In this study, a total of 10 MHC I alleles and four MHC II alleles were observed in 149 individuals throughout Namibia.

All alleles but one likely belong to functional MHC genes as their expression was confirmed. The observed alleles belong to four MHC I and three MHC II genes in the species as revealed by phylogenetic analyses. Signatures of historical positive selection acting on specific sites that interact directly with pathogen-derived proteins were detected in both MHC classes. Furthermore, a high genetic differentiation at MHC I was observed between Namibian cheetahs from east-central and north-central regions known to differ substantially in exposure to feline-specific viral pathogens. This suggests that the patterns of MHC I variation in the current population mirrors different pathogenic selective pressure imposed by viruses. Cheetahs showed low levels of MHC diversity compared with other mammalian species including felids, but this does not seem to influence the current immunocompetence of free-ranging cheetahs in Namibia and contradicts the previous conclusion that the cheetah is a paradigm species of disease susceptibility. However, it cannot be ruled out that the low MHC variation might limit a prosperous immunocompetence in the case of an emerging disease scenario because none of the remaining alleles might be able to recognize a novel pathogen.

In contrast to cheetahs, leopards occur in most parts of Africa being perhaps the most abundant big cat in the continent. Leopards seem to have escaped from large-scale declines due to epizootics in the past in contrast to some free-ranging large carnivore populations in Africa that have been afflicted by epizootics. Currently, no information about the MHC sequence variation and constitution in African leopards exists. In this study, I characterized genetic variation at MHC I and MHC II genes in free-ranging leopards from Namibia. A total of six MHC I and six MHC II sequences were detected in 25 individuals from the east-central region. The maximum number of sequences observed per individual suggests that they likely correspond to at least three MHC I and three MHC II genes. Hallmarks of MHC evolution were confirmed such as historical positive selection, recombination and trans-species polymorphism. The low MHC variation detected in Namibian leopards is not conclusive and further research is required to assess the extent of MHC variation in different areas of its geographic range.

Results from this thesis will contribute to better understanding the evolutionary significance of MHC and conservation implications in free-ranging felids. Translocation of wildlife is an increasingly used management tool for conservation purposes that should be conducted carefully as it may affect the ability of the translocated animals to cope with different pathogenic selective pressures.

Zusammenfassung

Genetische Variabilität ist entscheidend für das langfristige Überleben von Arten, denn es ermöglicht dem Organismus sich Umweltveränderungen, wie z.B. neu aufkommende Krankheiten, schneller anzupassen. Der Haupthistocompatibilitätskomplex (MHC) ist eine Familie von Genen, der eine zentrale Rolle im Immunsystem von Wirbeltieren zukommt, da sie nach Pathogenkontakt das adaptive Immunsystem aktivieren. Zudem sind MHC Gene geeignete molekulare Marker um Anpassungsfähigkeiten aufzuzeigen. MHC Gene kodieren primär für Zelloberflächenmoleküle, die kurze Peptidfragmente erkennen und den Immunzellen präsentieren, die im Falle der Klasse I Gene intrazellulären (z.B. von Viren) oder im Falle der Klasse II Gene extrazellulären (z.B. von Bakterien, Protozoen, Arthropoden) Ursprungs sein können. In der Regel wird in natürlich vorkommenden Populationen ein hoher Grad an Polymorphismus im MHC beobachtet, was als Anpassung an das Erkennen und Präsentieren einer großen Anzahl sich schnell entwickelnder Pathogene interpretiert wird. Das Bestehen vieler MHC Varianten über große Zeiträume hinweg wird hauptsächlich durch positive Selektion bewirkt, der ein pathogengetriebener Selektionsdruck zugrunde liegt.

In meiner Doktorarbeit habe ich mich mit der Variation von MHC I and MHC II in freilebenden Geparden (*Acinonyx jubatus*) und Leoparden (*Panthera pardus*) in Farmgebieten innerhalb Namibias beschäftigt. Beide Felidenarten leben sympatrisch und sind so demselben Pathogendruck ausgesetzt, sie unterscheiden sich allerdings in ihrem evolutionären und demographischen Hintergrund. Mein Hauptziel war es 1) das Ausmaß und Muster der MHC Variation auf Populationsebene beider Feliden zu untersuchen; 2) einen möglichen Zusammenhang zwischen dem Grad der MHC Variation und der Krankheitsresistenz in frei lebenden Geparden aufzudecken und 3) zu untersuchen, welche Rolle der Selektion auf die MHC Variabilität beider Arten in der Vergangenheit wie auch gegenwärtig zukommt. Geparden und Leoparden repräsentieren die größten frei lebenden Carnivoren Namibias. Beide Arten kommen hauptsächlich in Farmgebieten vor, die sich in Privatbesitz befinden, und können dort mit anderen Wild- aber auch Haustieren zusammentreffen und potentiell Krankheitserreger austauschen. Die Kenntnis über die adaptive genetische Variation, die für Krankheitsresistenzen mitverantwortlich ist, kann für den Schutz beider Felidenarten von Bedeutung sein.

Geparden werden häufig in Lehrbüchern als klassische Beispiele für eine Tierart mit einer generell geringen genetischen Diversität verwendet. Neben neutralen Markern ist bei Geparden auch eine geringe Variabilität der MHC Gene beschrieben worden, die als Ursache einer hohen Anfälligkeit für infektiöse Krankheiten gesehen wird. Bisher wurde allerdings eine erhöhte Krankheitsanfälligkeit nur bei Geparden aus Gefangenschaft beschrieben, wohingegen neuste Studien an frei lebenden Geparden diesen einen guten Gesundheitsstatus attestierten. Dadurch stellt sich die Frage, ob die MHC I und II Diversität in frei lebenden Geparden nicht höher sein könnte als bisher angenommen. In dieser Arbeit konnten insgesamt 10 MHC I und vier MHC II Allele in 149 frei lebenden Geparden aus ganz Namibia nachgewiesen werden. Die Zugehörigkeit zu funktionellen MHC Genen wurde durch Expressionsanalysen bei allen Allelen, außer einem, bestätigt. Durch phylogenetische Analysen konnten die Allele vier MHC I und drei MHC II Genen zu geordnet werden. Das Wirken von positiver Selektion in der Vergangenheit konnte an spezifischen Aminosäuren des Proteins, die in direktem Kontakt zu den pathogenen Antigenen stehen, festgestellt werden. Dies traf für beide MHC Klassen zu. Des Weiteren konnte eine starke genetische Differenzierung des MHC I zwischen Geparden aus einer nord-zentralen und einer ost-zentralen Region festgestellt werden, von denen auch bekannt ist, dass sie unterschiedlichen, felidenspezifischen, viralen Pathogenen ausgesetzt sind. Das lässt vermuten, dass die unterschiedlichen Muster der MHC I Variation in der gegenwärtigen Population den unterschiedlichen pathogengetriebenen Selektionsdruck durch Viren in den beiden Regionen widerspiegelt. Verglichen mit anderen Säugetierarten, insbesondere andere Feliden, zeigen Geparden einen geringen Grad an MHC Diversität, doch das scheint die derzeitige Immunkompetenz frei lebender Geparden in Namibia nicht einzuschränken und widerspricht der bisherigen Meinung dass Geparden ein typisches Beispiel für eine krankheitsanfällige Tierart sind. Es kann allerdings nicht ausgeschlossen werden, dass bei neu auftauchenden Krankheiten die geringe MHC Variation eine erfolgreiche Immunkompetenz verhindert, da möglicherweise keines der gegenwärtigen Allele die Fähigkeit besitzt neue Pathogene zu erkennen.

Im Gegensatz zu Geparden kommen Leoparden in allen Teilen Afrikas vor und sind wahrscheinlich die am weitverbreiteste Großkatze des afrikanischen Kontinents. Es scheint, dass Leoparden, im Gegensatz zu anderen afrikanischen Großkatzen, einer ausgedehnten Dezimierung durch Tierseuchen in der Vergangenheit, der einige Populationen afrikanischer Großkatzen ausgesetzt waren, entkommen sind. Bisher fehlten Information über die MHC Variabilität in afrikanischen Leoparden. In dieser Studie konnte ich die genetische Variation der MHC I und MHC II Gene frei lebender namibischer Leoparden charakterisieren. In 25 Tieren aus einer Population der ost-zentralen Region konnten sechs MHC I sowie sechs MHC II

Sequenzen nachgewiesen werden. Aus der maximalen Anzahl Allele pro Tier kann auf drei MHC I und auf drei MHC II Gene geschlossen werden. Außerdem konnten die typischen Kennzeichen einer variationserhaltenden MHC Evolution betätigt werden, wie positive Selektion in der Vergangenheit, Rekombination und über Artgrenzen hinaus bestehender Polymorphismus. Der geringe Grad an MHC Variation in namibischen Leoparden ist jedoch noch nicht endgültig und weitere Untersuchungen in unterschiedlichen Regionen aus der gesamten geographischen Verbreitung des Leoparden sind notwendig um die MHC Variation der Leoparden in Gänze einschätzen zu können.

Die Ergebnisse dieser Arbeit werden zu einem besseren Verständnis des evolutionären Stellenwerts des MHC und in Folge zu einem besseren Schutz von frei lebenden Feliden beitragen. Die Umsiedelung von Wildtieren ist ein zunehmend angewendetes Hilfsmittel im Natur- und Artenschutz, welches jedoch mit Sorgfalt eingesetzt werden sollte, da die umgesiedelten Tiere möglicherweise einem anderen pathogenen Selektionsdruck ausgesetzt sind, dem sie nichts entgegenzusetzen haben.

Introduction

Genomic variation and Diseases in free-ranging felids

Genomic variation is the clay of evolution, the raw material on which adaptation and speciation depend (Amos and Harwood 1998). The evolutionary potential of natural populations for ecologically important quantitative traits can be predicted by combining substantial information on neutral and adaptive genetic variation (Reed 2010). Neutral genetic variation provides information regarding the demographic and evolutionary history of natural populations, whereas adaptive genetic variation gives information on selective processes involving the interaction of individuals with their environment or on the capacity for future adaptive changes, as for example, an emerging disease scenario (Sommer 2005). Therefore, patterns of neutral and adaptive variation may differ markedly in natural populations (Miller et al. 2008). Particularly, the extent and patterns of adaptive (advantageous) variation is crucial for the long-term survival of the species, and even more so if they are endangered (Hedrick 2001). This is because most endangered populations are small and fragmented with low genetic variation (Altizer et al. 2000).

Within the cat family (Felidae) there are 36 extant species of wild felids, nearly all of them listed as threatened or nearly threatened with extinction (Johnson et al. 2006; O'Brien and Johnson 2007). Furthermore, most felid species offer numerous examples of reduced genetic variation in natural populations (e.g. lions *Panthera leo* from the Ngorongoro Crater in Tanzania, Florida panther *Puma concolor coryi*, and African cheetah *Acinonyx jubatus*) that have been associated with decreased fitness-related features such as poor reproduction, increased mortality, and increased susceptibility to opportunistic infectious diseases (O'Brien et al. 2006; Munson et al. 2010).

Pathogens are likely to be powerful selective agents in natural populations (Altizer et al. 2003) as they may cause infectious diseases leading to major declines in wildlife populations increasing their extinction risk (Pedersen et al. 2007). Carnivores, especially members of the cat and dog families belong to the most threatened taxa by pathogens within all mammalian species (Murray et al. 1999; Pedersen et al. 2007). This is partly because many carnivore populations are already seriously endangered by anthropogenic factors (e.g. human population expansion, alteration and loss of habitat, climate change) that simultaneously influence disease ecology by disrupting historically stable host-pathogen interactions and/or introducing highly virulent

pathogens leading to potential epizootic events (Smith et al. 2009; Munson et al. 2010). An alarming example of the impact of infectious diseases on natural populations of free-ranging felids is given by the canine distemper epizootics that swept through the Serengeti ecosystem, killing hundreds of lions and many other felids (Roelke-Parker et al. 1996). A summary of infectious diseases reported to date in captive and free-ranging felids, as well as case examples of felid diseases with population consequences can be found in Munson et al. (2010).

MHC: A paradigm of adaptively important genetic variation

Genomic variation of both host and infectious agent is of critical importance in the outcome of a disease outbreak (O'Brien et al. 2006). The Major Histocompatibility complex (MHC) is a multigene family responsible for the adaptive immune response in vertebrate hosts (Klein 1986). It has therefore become one of the most preferred markers to study patterns of adaptive genetic variation related to disease resistance in host natural populations. As such, the evolutionary dynamics of MHC is relevant in ecology, population biology and conservation (Piertney and Oliver 2006). MHC genes encode cell-surface glycoproteins involved in pathogen and T-cell receptor recognition. The MHC class I (MHC I) and class II (MHC II) proteins bind and present self and nonself peptides derived from intracellular (e.g. viruses, cancer infected cells) and extracellular (e.g. bacteria, protozoa, arthorpods) pathogens to cytotoxic and T-helper cells, respectively, thereby triggering a cascade of immune responses (Klein 1986). The ability of both MHC I and II genes to bind various pathogens is believed to be mainly related to sequence variation among MHC alleles in the antigen binding site or ABS (Potts and Wakeland, 1990). The ABS is comprised by the second and third exons that encode the alpha 1 and alpha 2 domains respectively of MHC I molecules (Bjorkman et al. 1987) whereas only by the second exon that encode the beta 1 domain of MHC II molecules (Brown et al. 1993). While the general architecture of multigene MHC families appears relatively conserved within each class of vertebrates, the number of either MHC I or II loci has been found to vary substantially among species (Bernatchez and Landry 2003; Kelley et al. 2005). Comparative MHC studies among mammalian orders indicated lack of MHC I orthology (Hughes and Nei 1989), while most MHC II genes are orthologs across mammals although deletions of some genes and expansions of others occur, due to recent duplications (Kumanovics et al. 2003).

A hallmark of the mammalian MHC genes is the relatively high level of polymorphism observed in most natural populations generally interpreted as an adaptation to detect and present a wide array of peptides from rapidly evolving pathogens (Yuhki and O'Brien 1990a). Selection

of varying nature is suggested to maintain overall levels of MHC variation in a population (Hedrick 1994). Positive selection acting on the evolutionary history of a species has been inferred from the patterns of nucleotide substitutions among MHC alleles, resulting in excess of non-synonymous over synonymous substitutions in the ABS, while the opposite pattern is observed in non-ABS (Takahata and Satta 1998). There are some other tests of selection and their evolutionary parameters for detecting selection on different timescales (i.e. current generation, recent and distant past; reviewed in Garrigan and Hedrick 2003). Pathogenic and sexual selective pressures are considered as potential scenarios that explain the patterns of MHC variation observed in natural populations (Hedrick 2002; Sommer 2005; Piertney and Oliver 2006). There is growing evidence for the association of MHC genotypes or individual alleles and disease susceptibility in wildlife (reviewed in Sommer 2005; Schwensow et al. 2007; Alcaide et al. 2010) confirming that the pressure from pathogens is a primary source of selection on MHC (Radwan et al. 2010; Alcaide et al. 2010). Pathogen mediated selection mechanisms have been proposed (i.e. heterozygote advantage, Doherty and Zinkernagel 1975; rare-allele advantage, Slade and McCallum 1992); and fluctuating selection in space and time, Hill 1991) and approaches to test and identify among them (i.e. proportions of genotypes within populations, patterns of population structure, and associations with pathogens) have been widely discussed (Spurgin and Richardson 2010). Despite selection shaping MHC variation on the long-term, MHC variation is often substantially reduced in species that have experienced severe population bottlenecks (e.g. Weber et al. 2004; Babik et al. 2005; Bollmer et al. 2007; Munguía-Vega et al. 2007; Radwan et al. 2007; Siddle et al. 2007; Miller et al. 2008). The loss of MHC variation has the potential to affect the ability to mount a protective immune response, namely immunocompetence (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. 2010; Reed 2010). Therefore, understanding the role of selection in maintaining MHC variation in bottlenecked populations has implications for the conservation of endangered species (Ejsmond and Radwan 2009).

Most comprehensive studies of MHC in felids namely Feline Leucocyte Antigen have been conducted in the domestic cat (*Felis catus*, O'Brien and Yuhki 1999; Kennedy et al. 2002; Yuhki et al. 2008). The feline MHC region is located on chromosome B2 and includes 19 MHC I genes and eight MHC II genes (for details on gene organization see Yuhki et al. 2008). The general organization of the feline MHC region is similar to that of primates with two main differences (1) MHC I region has been rearranged by distal inversion (Beck et al. 2005) and (2)

MHC II region lacks DQ and DP genes, and shows reorganized/multiplied DR genes (Beck et al. 2001; Yuhki et al. 2003). In comparison to all mammalian MHCs, cats have three versus one functional DRA within the MHC II region (O'Brien and Yuhki 1999). In wild cat species, recent MHC studies have focused on genotyping particularly MHC II-DRB (e.g. Wang et al. 2008, 2009; Pokorny et al. 2010; Wei et al. 2010) and to a lesser extent MHC I (e.g. Smith and Hoffman 2001; Pokorny et al. 2010) which is further limited by the small number of individuals included. Only a few studies have estimated MHC variation at the population level but again with low sample sizes (e.g. Drake et al. 2004; Sachdev et al. 2005).

Study species

In this study, I used two nonmodel species that are phylogenetically related and live in sympatry but differ in their evolutionary and demographic histories resulting in contrasting levels of genetic diversity.

Cheetahs (*Acinonyx jubatus*; Schreber 1775; Fig. 1) belong to the cat family and represent the single survivor from the genus *Acinonyx* comprising four potential African and one Asiatic subspecies (Wilson and Mittermeier 2009a). A recent study on phylogeography of African and Asiatic cheetahs showed that northern-east African, southern African and Asiatic cheetahs are long-term geographic isolates with independent evolutionary histories (Charruau et al. 2011). The cheetah's historic range has been reduced to few fragmented populations in Africa and southwestern Asia (Nowell and Jackson 1996; Durant et al. 2008), with the largest remaining population occurring in Namibia (Marker-Kraus et al. 1996). According to the IUCN criteria the cheetah is classified as 'Vulnerable' to extinction globally and 'Critically Endangered' in Iran and North Africa (Durant et al. 2008). The causes for the cheetah's current state of threat include habitat loss and fragmentation, conflict with humans, and depletion of their wild prey base (Marker 2002). Cheetahs exhibit remarkably low levels of genetic variation at neutral loci (e.g. minisatellites, microsatellites, mitochondrial DNA; Menotti-Raymond and O'Brien 1993, 1995; Freeman et al. 2001) and adaptive loci (e.g. allozymes, MHC I and II; O'Brien et al. 1983; Yuhki and O'Brien 1990b, 1994; Drake et al. 2004) in comparison to other felids. However, further studies have revealed that neutral genetic variation (e.g. microsatellites and mitochondrial DNA) in modern cheetahs is as high as in other outbred felid populations or species (Driscoll et al. 2008; Charruau et al. 2011). The cause(s) of the cheetah's poor genetic makeup and its significance to the long-term survival of the species has remained controversial

(Caughley 1994; Caro and Laurenson 1994; O'Brien 1994; Laurenson et al. 1995; May 1995). Potential scenarios explaining the reduced levels of genetic variation in cheetahs include 1) severe historical and more recent demographic reductions (O'Brien et al. 1983; Menotti-Raymond and O'Brien 1993; Driscoll et al. 2008), 2) metapopulation structure with small effective population sizes (Hedrick et al. 1996), and 3) historically low levels of genetic variation due to behavioral and ecological factors (Caro and Laurenson 1994; Merola 1994).

Reduced genetic variation particularly at adaptively important 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 disease 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). Whether the extent of MHC variation has an effect on the immunocompetence of free-ranging cheetahs has not been yet explored.

Figure 1 Cheetahs on Namibian farmlands © Bettina Wachter

Leopards (*Panthera pardus;* Linnaeus, 1758; Fig 2) belong to the cat family within the genus *Panthera* including all members of the big cats (Johnson et al. 2006; Davis et al. 2010). Leopards are highly adaptable to different habitats ranging from desert to rainforest and therefore have the largest geographic distribution among the wild cats (Henschel et al. 2008; Macdonald et al. 2010). The current leopard distribution includes most of their historic range, which covers most of Africa and large parts of Asia (Wilson and Mittermeier 2009b). However, anthropogenic pressures have resulted in heavily fragmented and isolated leopard populations (Uphyrkina et al. 2001), placing most leopard subspecies as 'Near Threatened' and few others as 'Critically endangered' (Henschel et al. 2008). The African leopard (*P. p. pardus*) is the most common of nine revised leopard subspecies (Miththapala et al. 1996; Uphyrkina et al. 2001), and probably the most abundant large felid in Africa. African leopards represent the most genetically diverse leopard subspecies based on mitochondrial DNA and nuclear microsatellite loci (Spong et al. 2000; Uphyrkina et al. 2001).

Leopard MHC-related studies have earlier been performed only in a small scale, for both MHC class I (southern blot analysis from one individual, Yuhki et al. 1989), and MHC class II (sequence analysis of clones from one individual, Wang et al. 2008; sequence analysis of clones from seven individuals, Wei et al 2010). Currently, no information about the MHC sequence variation and constitution in African leopards exists.

Figure 1 Leopard on Namibian farmlands © Oliver Höner

Study area

The study area was in Namibia, southwestern Africa. Namibia is a large country, covering an area of 823,680 km^2 encompassing many different landscapes and biomes (Mendelsohn et al. 2009). Although biological samples analyzed in this study are from several regions throughout Namibia, the core research area is primarily the farmland on the Khomas Hochland Plateau landscape. This is a large ridge of high ground (altitude range between 1,700 to 2,000 m above sea level) in the center of the country, characterized by Acacia tree-and-shrub Savanna biome. A detailed description of the study area can be found in Mendelsohn et al. (2009). In Namibia, the southern African cheetah (*A. j. jubatus*) and African leopard (*P. p. pardus*) are the most abundant large carnivores, widely distributed and mainly concentrated in the northern and central parts of the country. Estimated population sizes range, approximately, between 2,500-5,000 cheetahs and 5,000-10,500 leopards (Hanssen and Stander 2004). Although Namibia boasts a large extension of protected areas including nature reserves and national parks (14.1% of Namibia's surface area; Mendelsohn et al. 2009), a large proportion of free-ranging cheetahs and leopards inhabit unprotected areas encompassing several continuous privately owned farms often bordering towns and cities (Marker-Kraus and Kraus 1995). The Namibian farmland is characterized by commercial domestic stock as well as wild prey, namely game (mainly ungulates). Abundant wild prey and the lack of inter-specific competitors such as lions and hyenas *Crocuta crocuta* have resulted in favorable ecological conditions for cheetahs and leopards to flourish in this habitat (Marker 2000). On the other hand, the great overlap among humans, domestic and wildlife communities on farmlands have led to other situations that may jeopardize the long-term survival of the cheetah and leopard populations in Namibia (Marker et al. 2010). For example, free-ranging cheetahs and leopards on Namibian farmland get in close proximity with domestic carnivores (cats and dogs) that are often unvaccinated (Schneider 1994) and may be important vectors of diseases such as canine distemper, which has affected many other free-ranging carnivores in the Serengeti including cheetahs and leopards (Roelke-Parker et al. 1996).

Study Aims

Free-ranging cheetahs and leopards coexist throughout Namibia and are potentially exposed to the same selective pressures imposed by infectious agents (e.g. virus, bacteria, protozoa, fungi and arthropods). On the other hand, both felid species exhibit contrasting patterns of evolutionary and demographic histories which should be reflected in different patterns of neutral

and adaptive genetic variation. Therefore, free-ranging cheetahs and leopards on Namibian farmland represent an encouraging opportunity to investigate patterns of adaptive genetic variation related to disease resistance, particularly at the genes of the Major Histocompatibility Complex.

For my doctoral research I focused on MHC I and II variation in free-ranging cheetahs and leopards on Namibian farmlands. The general aims were (1) to describe the extent and patterns of MHC variation at the population level in both felids, (2) to examine the association between the extent of MHC variation and disease resistance in free-ranging cheetahs, and 3) to investigate the role of natural selection at different time scales in shaping MHC variation in both felids.

The specific questions for the Namibian cheetah population can be phrased as followed:

1) Is the extent of adaptive variation at MHC I and II loci in a large sample of free-ranging Namibian cheetahs higher than previously observed in the species? Does the observed MHC I and II variation appear to be functional?
2) Does the extent of MHC I and II variation has any effect on the observed immunocompetence of free-ranging Namibian cheetahs?
3) How does selection and demographic processes have contributed to the observed patterns of MHC I and II variation in cheetahs?
4) Does differential exposure to viral pathogens influence the patterns of variation and genetic differentiation at MHC on the current Namibian cheetah population?

The specific questions for the Namibian leopard population can be phrased as followed:

1) How is the MHC I and II composition of free-ranging leopards from Namibia?
2) What evolutionary forces generate and maintain MHC variation in African leopards?
3) Is the extent of MHC I and II variation in leopards higher than that observed for cheetahs across the Namibian farmlands?

Article Summaries

Article 1

AINES CASTRO-PRIETO, BETTINA WACHTER and SIMONE SOMMER

Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population

Molecular Biology and Evolution (2011), 28:1455–1468.

Cheetahs (*Acinonyx jubatus*) have been used as a classic example in numerous conservation genetics textbooks as well as in many related scientific publications highlighting their remarkably overall low levels of genetic diversity compared to other felid species. Moreover, the cheetah has been considered a paradigm of disease vulnerability associated with low genetic diversity, particularly at the immune genes of the major histocompatibility complex (MHC). However, earlier studies used methods with low resolution to quantify MHC diversity and/or small sample sizes. Furthermore, high disease susceptibility was reported only for captive cheetahs, whereas free-ranging cheetahs show no signs of infectious diseases and a good general health status. The extent of MHC variation in free-ranging cheetahs and its effect on disease vulnerability has not been yet explored. In Namibia, the world's largest remaining population of free-ranging cheetahs occurs. A large portion of the population inhabits privately owned livestock and game farmlands with domestic carnivores carrying infectious agents that cheetahs may be susceptible to. Yet, recent studies on seroprevalence, physical condition and necropsies have shown no clinical symptoms of acute viral diseases in cheetahs and a good general health status. 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.

We examined whether the diversity at MHC class I (MHC I) and class II-DRB (MHC II-DRB) loci in 149 Namibian cheetahs was higher than previously reported using single-strand conformation polymorphism analysis (SSCP), cloning and sequencing. MHC genes were examined at the genomic and transcriptomic levels. We also investigated if MHC alleles showed

evidence of positive selection acting during the evolutionary history the species. Finally, we conducted Bayesian phylogenetic analyses in order to assign the observed alleles into putative MHC loci.

We detected ten unique MHC I exon 2 (249 bp), intron 2 (141/200/202 bp) and exon 3 (263/265 bp) alleles and four MHC II-DRB exon 2 (243/246 bp) alleles, of which nine MHC I and all MHC II-DRB were expressed and thus, functional. Since the 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. Phylogenetic analyses and individual genotypes suggested that the observed alleles belong to four putative MHC I loci (two monomorphic and two polymorphic) and three putative MHC II-DRB loci (two monomorphic and one polymorphic) in the species. The number of functional putative MHC I and MHC II-DRB loci in cheetahs were consistent with the number of classical MHC I and MHC II-DRB loci found in domestic cats. Evidence of historical positive selection, as revealed by an excess of non-synonymous over synonymous substitutions on antigen binding sites (ABS) in contrast to non-ABS among the different alleles, was detected for both MHC classes. Positive selection appeared, however, stronger on MHC I than MHC II-DRB loci, suggesting different selection intensities between the two MHC classes. Our study indicated that the low number of MHC I alleles previously observed in cheetahs was due to a smaller sample size examined. On the other hand, the low number of MHC II-DRB alleles previously observed in cheetahs was further confirmed. Compared with other mammalian species including felids, cheetahs showed low but functional MHC diversity; this however, does not seem to influence the current immunocompetence of free-ranging cheetahs in Namibia and contradicts the previous conclusion that the cheetah is a paradigm species of disease vulnerability.

Authors' contributions:

I contributed on the development of the research questions, conducted the laboratory procedures (e.g. primers design, MHC genotyping, qualitative expression analyses), statistical analyses, data interpretation, and the manuscript writing. B. Wachter provided the cheetah samples as well as comments and suggestions on the manuscript. S. Sommer initiated the collaboration with The Cheetah Research Project, conceived the study design, supervised the research, and revised the manuscript.

Article 2

AINES CASTRO-PRIETO, BETTINA WACHTER, JOERG MELZHEIMER, SUSANNE THALWITZER and SIMONE SOMMER

Patterns of adaptive variation and genetic differentiation in free-ranging cheetahs on Namibian farmlands

Research paper (*in review*)

The world's largest remaining population of free-ranging cheetahs (*Acinonyx juabuts*) occurs in Namibia; it is therefore considered as the key population for the survival of the species. Compared with other mammalian species including felids, cheetahs show low levels of immune gene (MHC) diversity. In a recent study, a total of ten MHC class I alleles and four MHC class II-DRB alleles were detected. MHC class I codes for cell-surface glycoproteins important for the activation of the immune cascade against intracellular antigens (e.g. from viruses) whereas MHC class II plays an important role in the defence of extracellular antigens (e.g. from helminths). Pathogenic selective pressure is considered the main evolutionary force shaping the patterns of MHC variation observed in natural populations.

Free-ranging Namibian cheetahs concentrate in unprotected areas encompassing several continuous privately owned livestock or game farmlands where they get potentially in contact with feral and unvaccinated domestic carnivores. Serosurvey of common feline and canine viruses known to cause severe disease in captive cheetahs confirmed the exposure of free-ranging Namibian cheetahs to these viral pathogens but detected no evidence of clinical signs of infectious diseases in the examined individuals. However, substantial differences were revealed in the seroprevalences to viral pathogens between cheetahs from north-central and east-central Namibia. This raises the question whether differences in pathogen exposure influence the patterns of variation and genetic differentiation at MHC loci of free-ranging cheetahs on Namibian farmlands. Here, we compared patterns of variation at the second exon of MHC class I and class II-DRB loci in 88 individuals from north-central and east-central Namibia by using single-stranded conformation polymorphism analysis and direct sequencing.

An essential pre-requisite to conduct the corresponding analyses is the locus-specific assignment of MHC alleles in the species, which was previously conducted in Namibian

cheetahs by combining information on the evolutionary affinities between the observed MHC alleles, qualitative expression analysis, and the distribution of alleles among 149 individuals. A total of six alleles (229 bp) from three MHC class I loci and four alleles (246 bp) from three MHC class II-DRB loci were detected. No differences in the allelic diversity or in the expected heterozygosities for any MHC loci were observed. However, a high genetic differentiation was observed in MHC class I ($F_{ST} = 0.07$, $P < 0.0001$) in contrast to MHC class II-DRB and neutral microsatellite markers previously reported, between individuals from north-central and east-central Namibia. Our results suggest that patterns of MHC class I variation mirror different pathogenic selective pressure imposed by viruses in free-ranging cheetahs across the Namibian farmlands. Translocation of cheetahs within Namibia is an increasingly used management tool for conservation purposes that should be conducted carefully as it may affect the ability of translocated animals to cope with different pathogenic selective pressures. Therefore, further research should focus on MHC composition in relation to pathogen load in cheetahs from different African populations.

Authors' contributions:

I contributed on the development of the research questions, conducted the laboratory procedures (e.g. primers design, MHC genotyping), statistical analyses, data interpretation, and the manuscript writing. B. Wachter provided comments and suggestions on the manuscript. J. Melzheimer and S. Thalwitzer collected the cheetah samples. S. Sommer initiated the collaboration with The Cheetah Research Project, supervised the research, and revised the manuscript.

Article 3

AINES CASTRO-PRIETO, BETTINA WACHTER, JOERG MELZHEIMER, SUSANNE THALWITZER and SIMONE SOMMER

Diversity and evolutionary patterns of immune genes in free-ranging Namibian leopards (*Panthera pardus pardus*)

Journal of Heredity (2011), 102(6):653–665.

Diseases caused by pathogens are strong selective pressures that may result in major declines of wildlife populations, increasing their risk of extinction. Carnivores, mainly members of felid and canid families, represent the most threatened taxa by pathogens. Host genetic variation plays an important role in buffering natural populations against potential disease scenarios. 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. African leopards (*Panthera pardus pardus*) are highly adaptable carnivores to different habitats and occur in most parts of Africa; therefore are exposed to a great variety of pathogens. In contrast to some free-ranging large carnivore populations in Africa that have been afflicted by epizootics, African leopards seem to have escaped from large-scale declines due to epizootics in the past. This may suggest that free-ranging African leopards are not limited in their immunocompetence against infectious agents and therefore represent a potential candidate nonmodel species to understand evolutionary processes that shape the patterns of adaptive immune gene variation in free-ranging populations of felids. Currently, no information about the MHC sequence variation and constitution in African leopards exists.

In this study, we isolated and characterized for the first time genetic variation at the adaptively most important region of MHC class I (MHC I) and MHC class II-DRB (MHC II-DRB) genes in 25 free-ranging leopards from Namibia. We also investigated the evolution of MHC 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 were examined. Using single-stranded conformation polymorphism analysis (SSCP) and direct sequencing, we detected six MHC I exon 2 (229 bp) and six MHC II-DRB exon 2 (246 bp) nucleotide sequences which likely correspond to at least

three MHC I and three MHC II-DRB loci in the species. Sequences were distributed into six MHC I and ten MHC II-DRB haplotypes among the population. Our sampling coverage was limited to individuals from east-central Namibia therefore it may not reflect the extent of MHC variation of the whole population. Amino acid sequence variation in both MHC classes was higher or similar in comparison to other reported felids. An excess of non-synonymous over synonymous substitutions in putative antigen binding sites (ABS) indicates historical positive selection most likely driven by pathogens acting on the second exon of MHC I sequences of African leopards. Although such pattern was not observed in MHC II-DRB, high amino acid divergence especially at ABS suggests that selection has favoured amino acid changes in positions that are postulated to interact with pathogen-derived peptides at least in the past. A comparison of the leopard's MHC I and MHC II-DRB sequences to those of other felids suggests a trans-species mode of evolution. The presence of recombination was detected in sequences from both MHC classes. Evolutionary affinities among MHC II-DRB sequences of African and Asian leopard subspecies suggested that they share at least three putative gene copies originated before the divergence between both leopard subspecies. 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.

Authors' contributions:

I contributed on the development of the research questions, conducted the laboratory procedures (e.g. primers design, MHC genotyping), statistical analyses, data interpretation, and the manuscript writing. B. Wachter provided comments and suggestions on the manuscript. J. Melzheimer and S. Thalwitzer collected the leopard samples and provided comments on the manuscript. S. Sommer initiated the collaboration with The Cheetah Research Project, supervised the research, and revised the manuscript.

Discussion

In my doctoral research I investigated the patterns of adaptively important MHC I and II genetic variation in natural populations of African felids, mainly cheetahs and to a lesser extent leopards that range freely on the Namibian farmlands. The following discussion is organized in three major topics that deal with the general goals and specific questions intended for this study.

1) Patterns of MHC variation in free-ranging felids on the Namibian farmlands

I conducted a robust comprehensive approach to measure levels of functional MHC I and II-DRB diversity in a large sample of free-ranging Namibian cheetahs (Castro-Prieto et al. 2011). A total of 10 alleles from four MHC I loci in 108 individuals and four alleles from three MHC II-DRB loci in 139 individuals were detected. Since the individuals were sampled throughout the country it is likely that the number of alleles observed in this study represent largely the extent of MHC variation in Namibian cheetahs. Qualitative expression analyses indicated that all alleles but one are functional. Levels of MHC I variation in terms of number of alleles were higher than previously observed in cheetahs (Yuhki and O'Brien 1994), however, this difference is mainly due to the fact that a larger number of individuals were screened in the present study. On the other hand, low levels of MHC class II-DRB variation previously observed in cheetahs (Drake et al. 2004) were further confirmed. Results from this study therefore confirm the relatively low levels of MHC genetic variation, previously suggested for the species (Yuhki and O'Brien 1990b). Interestingly, the amount of DNA sequence variation among alleles especially in the functionally important antigen-binding sites of MHC I as well as MHC II-DRB loci was similar to other felids and relatively high compared with other canids that showed even more alleles (Castro-Prieto et al. 2011). Low levels of adaptive MHC diversity are consistent with low levels of neutral genetic diversity previously observed in the species (Menotti-Raymond and O'Brien 1993, 1995; Freeman et al. 2001). Nevertheless, more recent studies have shown that neutral microsatellite variation in modern cheetahs is as high as in outbred populations or species (Driscoll et al. 2008; Charruau et al. 2011) although the extent of neutral variation appears to be higher in cheetahs from southern and east African populations compared with northern east African and Asiatic populations (Charruau et al. 2011).

Additionally, I conducted a first effort to describe genetic variation at MHC I and II-DRB loci in free-ranging African leopards that were opportunistically collected on Namibian farmlands ([3]Castro-Prieto et al. 2011). For simplicity, I will refer to the sequences obtained in this study as alleles. A total of 6 alleles from at least three putative MHC I loci and six alleles from at least three putative MHC II-DRB loci in 25 individuals were detected. Our sampling coverage was limited to individuals from east-central Namibia therefore it may not reflect the extent of MHC variation of the whole population. I was not able to confirm the expression of the observed MHC alleles because RNA samples were not available. However, all sequences contain the conserved residues expected in functional alleles of MHC I and II (Kaufman et al. 1994), and no stop codons or frameshift mutations, suggesting that they likely correspond to functional alleles. Amino acid sequence variation in both MHC classes was similar to or higher than other felids, such as domestic cat (Yuhki and O'Brien 1990b; 1997), Eurasian lynx (Wang et al. 2009), African cheetah (Castro-Prieto et al. 2011); Asiatic lion (Sachdev et al. 2005), and Bengal tiger (Pokorny et al. 2010). The low MHC variation, in terms of number of alleles, in leopards from this study is not conclusive and further research is required to assess the extent of MHC variation in different areas of its geographic range.

The extent of variation, in terms of number of alleles, in both types of MHC classes observed in free-ranging cheetahs and leopards from Namibia is relatively low compared with other mammalian species including felids (Castro-Prieto et al. 2011). However, it is difficult to do an objective comparison on the levels of MHC variation between populations of different species, and so it must be interpreted carefully. This is mainly 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 unique demographic history, degree of admixture and selective factors. In addition to the latter, some other aspects that further difficult interspecific comparisons on MHC variation reside in that (1) most MHC-related studies conducted in natural populations of mammalian species focus on a single or few populations and low sample sizes due to sampling-related difficulties, (2) the total number of alleles reported in most of these studies correspond to different MHC loci/copies, relative to the primer set(s) used, and the corresponding locus cannot be identified due to the lack of knowledge on the MHC organization of these non-model species, and (3) the number of functional alleles is often assumed but not confirmed through expression analysis because of the extreme difficulty to collect RNA samples in the wild. According to the latter, the comparison of MHC I and II-DRB allelic variation between cheetahs and leopards from this study is not yet conclusive. In general, leopard subspecies exhibit comparable to or higher amounts of neutral genetic variation than

those reported for other felids, although it varies across their geographical range (Uphyrkina et al. 2001). Moreover, African leopards represent the most genetically diverse leopard subspecies as revealed by mitochondrial DNA and nuclear microsatellite loci (Spong et al. 2000; Uphyrkina et al. 2001). By contrast, cheetahs exhibit overall low mitochondrial DNA nucleotide diversity (0.66%; Charruau et al. (2011) compared with leopards (1.21%; Uphyrkina et al. 2001), although no significant differences were observed in the levels of nuclear microsatellite variation between the two felid species. Still, I would expect to observe a greater MHC variation in leopards compared with cheetahs. In this study, however, only minor differences were observed between cheetahs and leopards on the Namibian farmlands, which can be explained mainly by sampling-related artifacts. First, a six-fold difference in the sample size analyzed for each species, and second, different coverage of the samples; while cheetah samples are from throughout Namibia, leopard samples are restricted to the east-central region of the country. Because MHC variation (in terms of number of alleles) was similar between leopards from only east-central Namibia and cheetahs from throughout Namibia, it is likely that leopards may exhibit higher MHC variation than cheetahs, regardless the difference in the evolutionary history between these two felid species. Nevertheless, further analysis extending the sample size and sampling coverage in leopards is needed to confirm this scenario.

2) The extent of MHC variation and its association with disease susceptibility in Namibian cheetahs

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 1995; O'Brien and Evermann 1988). However, the effect of reduced MHC variation on the long-term viability of bottlenecked populations has remained unclear (Edwards and Potts 1996; Hedrick 2003; Radwan et al. 2010). During my doctoral research I investigated whether the extent of MHC variation has an effect on the immunocompetence of free-ranging cheetahs (Castro-Prieto et al. 2011). The Namibian cheetah population has been continuously monitored for its health status (e.g. physical examinations, nutritional status, serosurvey studies; Munson et al. 2004, 2005; Thalwitzer et al. 2010). This population tested seropositive for feline calcivirus (FCV), feline parvovirus (FPV), feline herpesvirus (FHV), 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, which suggests that this population is not compromised in its ability to respond effectively against challenges imposed by these infectious agents (Munson et al. 2004, 2005; Thalwitzer et al. 2010). Yet, results from the present study suggest that the extent of MHC variation in free-ranging Namibian cheetahs is still low compared to previous studies conducted in cheetahs (mentioned before). Therefore, low levels of MHC variation do not appear to have an effect on the immunocompetence of free-ranging cheetahs in Namibia. Some ecological traits that potentially reduce the pathogenic selective pressure acting on the MHC of free-ranging Namibian cheetahs may include their social structure (small group sizes or solitary individuals; Caro 1994) and low population density (0.7-1.5/100 Km^2; Hanssen and Stander 2004), resulting in a low rate of pathogen transmission and reinfection among individuals. However, transmission of, for example, FPV, FCoV and CDV is facilitated through indirect contact at marking trees where cheetahs deposit faeces (Marker-Kraus et al. 1996; Thalwitzer 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). Results from this study 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). Still, these findings do not dispute the importance of maintaining MHC variation in natural populations. The extent of MHC variation observed in the Namibian cheetahs may not guarantee 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). This may be of major concern for the critically endangered Iranian population of Asiatic cheetah (*A. j. venaticus*) as it is the smallest (70-110 individuals; Farhadinia 2004; Hunter et al. 2007; Durant et al. 2008) and less genetically diverse population among all different cheetah subspecies (Charruau et al. 2011) and may be consequently at great risk in the case of an emerging disease scenario.

No association between low MHC variation and disease susceptibility has also been observed in bottlenecked populations of Canadian mountain goat (*Oreamnos americanus;* Mainguy et al. 2007) and Gila topminnow (*Poeciliopsis occidentalis*; Giese and Hedrick 2003). Furthermore, there are some other studies demonstrating the long-term survival of species

despite having little or no detectable MHC variation (reviewed in Radwan et al 2010; Reed et al. 2010). These mentioned examples appear to challenge the classical view about the negative impact of MHC genetic depletion on populations. It's important, however, to notice that not only genetic but also environmental factors and their interaction, the strength of the pathogenic selective pressure, and the life history of the organisms determine the relative importance of genetic factors to disease susceptibility (Reed et al. 2010). Moreover, within the genetic factors, there are many other non MHC immune genes (e.g. cytokines, Toll-like receptors) that are also involved in modulating host resistance against pathogens (Jepson et al. 1997; Acevedo-Whitehouse and Cunningham 2006). Therefore, considering all aspects previously mentioned may offer a resolution to the discrepancies observed in the associations between the extent of MHC variation and the evolutionary potential for disease resistance in natural populations of wildlife species.

3) The role of selection at different time scales in shaping MHC variation patterns in free-ranging felids on the Namibian farmlands

The role of historical positive selection acting on the MHC I and II-DRB loci was revealed by an excess of nonsynonymous over synonymous substitutions retained at antigen binding sites of both MHC classes in cheetahs (Castro-Prieto et al. 2011) as well as in leopards ([3]Castro-Prieto et al. in review). High divergence observed in amino acid positions that are postulated to interact directly with pathogen-derived peptides supports the influence of pathogen-driven selection in shaping the MHC variation in both felid species for a long evolutionary time. These results are consistent with positive selection patterns observed in MHC I and MHC II-DRB molecules during Felidae evolution (Yuhki and O'Brien 1990b; Yuhki and O'Brien 1997). However, the intensity of selection appears to be much stronger in MHC I than MHC II-DRB loci for both felid species. This pattern was also observed in the domestic cat (Yuhki and O'Brien 1997) and Bengal tiger (Pokorny et al. 2010). According to the latter, selection could maintain functional variation more effectively at MHC I than MHC II loci in felid species. Different selection intensities have also been shown in humans where MHC I loci revealed higher selection coefficients than MHC II loci (Satta et al. 1994). Trans-species polymorphism is commonly interpreted as balancing selection acting on MHC during the evolutionary history of the species (Klein et al. 1990; 1998). Although the intensity of selection on MHC II-DRB loci appears to be weak in felid species, there is evidence of trans-species polymorphism among the major lineages of feline MHC II-DRB (Wei et al. 2010). In my study, 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 supporting trans-species polymorphism ([3]Castro-Prieto et al. 2011). This result is consistent with a trans-species mode of evolution of the 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.

The role of contemporary selection on the patterns of MHC I and MHC II-DRB variation observed on the Namibian cheetah population was also investigated. I expected that contemporary selection has a detectable effect on genotypic frequencies within the population (Hedrick et al. 2000). An essential prerequisite to test the latter is the locus-specific assignment of MHC alleles in the species, which was previously conducted in cheetahs by combining information on the evolutionary affinities between the observed alleles, qualitative expression analysis, and the distribution of alleles among 149 individuals (Castro-Prieto et al. 2011).

Balancing selection is expected to result in high levels of heterozygosity, leading to departures from Hardy-Weinberg expectations (Doherty and Zinkernagel 1975). Genotypic frequencies of two polymorphic MHC I loci in cheetahs, however, resulted in a deficit of heterozygotes compared to Hardy-Weinberg expectations ([2]Castro-Prieto et al. in review). This may be explained by non-random mating in the population (Frankham et al. 2002) or Whalund effect (Hartl and Clark 1989). However, these possibilities were discarded because no deviations from Hardy-Weinberg expectations were detected at any of 38 neutral microsatellite loci in 89 unrelated cheetahs from the same area as in this study, and a lack of population subdivision suggested the Namibian cheetahs to form a large panmictic population (Marker et al. 2008). The presence of null alleles due to genotyping errors is another potential cause of deficit in observed heterozygosity. Our primer design however aimed to amplify the maximum possible number of alleles by using different primer sets including intron-spanning primers that were tested in both genomic DNA and copy DNA (Castro-Prieto et al. 2011) but of course we cannot rule out this possibility until deep knowledge of MHC organization in the species is available. In contrast to MHC I, genotypic frequencies of a single polymorphic MHC II-DRB locus in cheetahs matched with Hardy-Weinberg expectations ([2]Castro-Prieto et al. in review). Other studies which also did not observe a heterozygote excess have suggested that selection varies spatially and temporally, or that it cannot be detected in a single generation (reviewed in Piertney and Oliver 2006). This pattern may be also due to low statistical power to detect selection because of low allelic

diversity or sample size and/or demographic processes masking any effects (Seddon and Ellegren 2004).

Substantial differences in the seroprevalences to common feline and canine viral pathogens (i.e. FCV, FPV, FHV, CDV, FCoV and rabies) between free-ranging Namibian cheetahs from north-central and east-central regions (Munson en et al. 2004; Thalwitzer et al. 2010) indicate different pathogenic selective pressures acting on the current population. This raises the question weather differences in pathogen exposure influence the patterns of variation and genetic differentiation at MHC loci of free-ranging cheetahs on Namibian farmlands ([2]Castro-Prieto et al. in review). Results from this study revealed that individuals from north-central and east-central regions were highly differentiated at MHC I loci whereas no difference was observed at MHC II-DRB locus. Also, no genetic differentiation at 38 neutral microsatellite markers was observed in 89 unrelated cheetahs from the same area as in this study (Marker et al. 2008). A more pronounced genetic differentiation at MHC loci than at neutral makers indicates that selective factors (e.g. pathogens) are of greater magnitude than non-selective factors (e.g. genetic drift, gene flow) (Garrigan & Hedrick 2003). Therefore, the genetic differentiation observed in MHC I but not in microsatellites between cheetahs from north-central and east-central Namibia is likely explained by differences in the selective pressure driven by viral pathogens rather than geographic barriers affecting gene flow. It is difficult, however, to draw conclusions based solely on empirical comparisons between MHC genes and microsatellites because the mutational processes and selective regimes of these genetic markers are different (Hedrick 2001). Thus, conclusions from this study should be interpreted as preliminary. Fluctuating selection (i.e. spatial and temporal heterogeneity in the type and abundance of pathogens may maintain diversity at the MHC; Hill 1991) has been proposed as the mechanism of pathogen-mediated selection that better explains stronger genetic differentiation at MHC compared with neutral loci across natural populations. Some other examples that have shown this pattern include the great snipe (*Gallinago media*, Ekblom et al. 2007), Atlantic salmon (*Salmo salar*, Landry and Bernatchez 2001), and San Nicolas Island fox (*Urocyon littoralis dickey*, Aguilar et al. 2004). It is difficult, however, to determine the relative roles of pathogen-mediated selection mechanisms in maintaining MHC variation as they are not mutually exclusive, may interact with one another, and may operate in concert with other selective and neutral forces (Spurgin and Richardson 2010).

On the other hand, no genetic differentiation in both MHC II-DRB and microsatellites (as mentioned before), suggests that selection on MHC II-DRB in the current Namibian cheetah population is not strong enough to counteract drift resulting from demographic processes (i.e. past bottleneck events or metapopulation structure with small effective population sizes) proposed for African cheetahs (Hedrick 1996). Selection on MHC faded by drift has been commonly observed in natural populations of species that have undergone fragmentation and bottleneck events in their evolutionary history. Some examples of mammalian species that exhibit this pattern include bighorn sheep (*Ovis Canadensis*, Boyce et al. 1997; Gutierrez-Espeleta 2001), spotted suslik (*Spermophilus suslicus*, Biedrzycka and Radwan 2008), Malagasy jumping rat (*Hypogeomys antimena*, Sommer 2003), and Australian bush rat (*Rattus fuscipes greyii*, Seddon & Baverstock, 1999).

Contrasting patterns of variation between MHC I and MHC II-DRB loci may further support that viral pathogens likely exert a stronger selection pressure than extracellular-derived pathogens (e.g. bacteria, protozoa, arthropods) in cheetahs across the Namibian farmlands. To understand the role of selection on MHC II-DRB variation of the Namibian cheetah population data on extracellular-derived pathogens an their diversity, abundance and distribution is essential.

Implications for conservation

- Adaptive genetic variation is crucial for the long-term survival of wildlife species and therefore of primary interest in conservation genetics (Hedrick 2001). As shown in the present study, low MHC variation does not appear to influence the immunocompetence of free-ranging Namibian cheetahs. However, it cannot be ruled out that low levels of MHC variation might limit a prosperous immunocompetence in the face of a newly emerged pathogen (Castro-Prieto et al. 2011). This may be of major concern particularly to “critically endangered” populations of free-ranging felids that exhibit remarkably reduced levels of neutral genetic variation such as the Asiatic cheetah in Iran (*A. j. venaticus*; Charruau et al. 2011) and the Far Eastern leopard (*P. p. orientalis*; Uphyrkina et al. 2002). I would therefore recommend extending the MHC genotyping to these particular populations.

- Translocation of cheetahs and leopards within Namibia is an increasingly used management tool for conservation purposes that should be conducted carefully as it may affect the ability of translocated animals to cope with different pathogenic selective pressures. Therefore, further research should focus on MHC composition in relation to pathogen load in cheetahs and leopards from different African populations.

- Using next-generation sequencing technologies to investigate 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 genetic basis underlying disease susceptibility to infectious diseases in free-ranging populations of felids and other wildlife species.

References

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, et al. 2004. High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. *Proc. Natl. Acad. Sci. USA* 101:3490-3494

Alcaide M, Lemus JA, Blanco G, et al. 2010. MHC diversity and differential exposure to pathogens in kestrels (Aves:Falconidae). *Mol. Ecol.* 19:691–705

Altizer S, Foufopoulos J, Gager A. 2000. Conservation and diseases. In: Levin SA (eds) Encyclopedia of Biodiversity. San Diego: Academic Press. pp. 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

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

Beck TW, Menninger J, Voigt G, et al. 2001. Comparative feline genomics: a BAC/PAC contig map of the major histocompatibility complex class II region. *Genomics* 71:282-295

Beck TW, Menninger J, Murphy WJ, et al. 2005. The feline major histocompatibility complex is rearranged by an inversion with a breakpoint in the distal class I region. *Immunogenetics* 56:702-709

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

Biedrzycka A, Radwan J. 2008. Population fragmentation and major histocompatibility complex variation in the spotted suslik, *Spermophilus suslicus*. *Mol. Ecol.* 17:4801-4811

Bjorkman PJ, Saper MA, Samraoui B, et al. 1987. The foreign antigen-binding site and T-Cell recognition regions of class-I histocompatibility antigens. *Nature* 329:512-518

Bollmer JL, Vargas FH, Parker PG. 2007. Low MHC variation in the endangered Galápagos penguin (*Spheniscus mendiculus*). *Immunogenetics* 59:593-602

Boyce WM, Hedrick PW, Muggli-Cockett NE, et al. 1997. Genetic variation of major histocompatibility complex and microsatellite loci: a comparison in bighorn sheep. *Genetics* 145:421-433

Brown JH, Jardetzky TS, Gorga JC, et al. 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* 263:485486

Castro-Prieto A, Wachter B, Sommer S. 2011. Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. *Mol. Biol. Evol.* 28:1455-1468

[2]Castro-Prieto A, Wachter B, Melzheimer J, Thalwitzer S, Sommer S. Patterns of adaptive variation and genetic differentiation in free-ranging cheetahs on Namibian farmlands. *(In review)*

[3]Castro-Prieto A, Wachter B, Melzheimer J, Thalwitzer S, Sommer S. 2011. Diversity and evolutionary patterns of immune genes in free-ranging Namibian leopards (*Panthera pardus pardus*). *J. Hered.*102(6):653-665

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

Charruau P, Fernandes C, Orozco-TerWengel P, et al. 2011. Phylogeography, genetic structure and population divergence time of cheetahs in Africa and Asia: evidence for long-term isolates. *Mol. Ecol.* 4:706-724

Davis BW, Li G, Murphy WJ. 2010. Supermatrix and species tree methods resolve phylogenetic relationships within the big cats, Panthera (Carnivora: Felidae). *Mol. Phylogenet. Evol.* 56:64-76

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

Drake GJC, Kennedy LJ, Auty HK, et al. 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, et al. 2002. Genomic microsatellites as evolutionary chronometers: a test in wild cats. *Genome Res.* 12:414-423

Durant S, Marker L, Purchase N, et al. 2008. *Acinonyx jubatus*. In: IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. <www.iucnredlist.org>. Downloaded on 04 April 2011

Edwards S, Potts W. 1996. Polymorphism of genes in the major histocompatibility complex: implications for conservation genetics of vertebrates. In: Smith T, Wayne R, (eds) Molecular Genetic Approaches in Conservation. New York and Oxford: Oxford University Press. pp. 214-237

Ejsmond MJ, Radwan J. 2009. Mhc diversity in bottlenecked populations: a simulation model. *Conserv. Genet.* 12:129-137

Ekblom R, Saether SA, Jacobsson P, et al. (2007). Spatial pattern of MHC class II variation in the great snipe (*Gallinago media*). *Mol. Ecol.* 16:1439-1451

Evermann JF, Heeney JL, Roelke ME, et al. 1988. Biological and pathological consequences of feline infectious peritonitis virus infection in the cheetah. *Arch. Virol.* 102:155-171

Farhadinia M. 2004. The last stronghold: cheetah in Iran. *Cat News* 40:11-14

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

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

Freeman AR, Machugh DE, McKeown S, et al. 2001. Sequence variation in the mitochondrial DNA control region of wild African cheetahs (*Acinonyx jubatus*). *Heredity* 86:355-362

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, et al. 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

Hartl DL, Clark AG. 1989. Principles of Population Genetics. Sunderland Massachusetts: Sinauer Associates Inc. Publishers

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

Hedrick PW. 1996. Bottleneck(s) or metapopulations in cheetahs. *Conserv. Biol.* 10:897-899

Hedrick PW. 2001. Conservation genetics: where are we now? *Trends Ecol. Evol.* 16:629-636

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

Hedrick PW. 2003. The major histocompatibility complex (MHC) in declining populations: an example of adaptive variation. In: Holt W, Pickard A, Rodger J, et al. (eds) Reproduction Science and Integrated Conservation. Cambridge, United Kingdom: Cambridge University Press. pp. 97-113

Hedrick PW, Kim T. 2000. Genetics of complex polymorphisms: parasites and maintenance of MHC variation. In: Singh R, Krimbas C (eds) Evolutionary Genetics: From Molecules to Morphology. Cambridge, United Kingdom: Cambridge University Press. pp. 204-234

Hedrick PW, Parker KM, Gutierrez-Espeleta GA, et al. 2000. Major histocompatibility complex variation in the Arabian oryx. *Evolution* 54:2145-2151

Heeney JL, Evermann JF, McKeirnan AJ, et al. 1990. Prevalence and implications of feline coronavirus infections of captive and free-ranging cheetahs (*Acinonyx jubatus*). *J. Virol.* 64:1964-1972

Henschel P, Hunter L, Breitenmoser U, et al. 2008. *Panthera pardus*. In: IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. <www.iucnredlist.org>. Downloaded on 04 April 2011

Hill AVS. 1991. HLA associations with malaria in Africa: some implications for MHC evolution. In: Klein J, Klein D (eds) Molecular evolution of the major histocompatibility complex. Berlin, Germany: Springer. pp. 403-419

Hughes AL. 1991. MHC polymorphisms and the design of captive breeding programs. *Conserv. Biol.* 5:249-251

Hughes AL, Nei M. 1989. Evolution of the major histocompatibility complex: independent origin of nonclassical class I genes in different groups of mammals. *Mol. Biol. Evol.* 6:559-579

Hunter L, Jowkar H, Ziai H, et al. 2007. Conserving the Asiatic cheetah in Iran: launching the first radio-telemetry study. *Cat News* 46:8-11

Jepson A, Banya W, SisayJoof F, et al. 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

Johnson WE, Eizirik E, Pecon-Slattery J, et al. 2006. The late Miocene radiation of modern Felidae: a genetic assessment. *Science* 311:73-77

Kaufman J, Salomonsen J, Flajnik M. 1994. Evolutionary conservation of MHC class I and class II molecules–Different yet the same. Semin Immunol 6:411–424.

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

Kennedy LJ, Ryvar R, Gaskell RM, et al. 2002. Sequence analysis of MHC DRB alleles in domestic cats from the United Kingdom. *Immunogenetics* 54:348-352

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

Klein J, Bontrop RE, Dawkins RL, et al. 1990. Nomenclature for the major histocompatibility complexes of different species - a proposal. *Immunogenetics* 31:217-219

Klein J, Sato A, Nagl S, et al. 1998. Molecular trans-species polymorphism. *Annu. Rev. Ecol. Syst.* 29:1-21

Kumanovics A, Takada T, Fischer Lindahl K. 2003. Genomic organization of the mammalian MHC. *Annu. Rev. Immunol.* 21:629-665

Landry C and Bernatchez L. 2001. Comparative analysis of population structure across environments and geographical scales at major histocompatibility complex and microsatellite loci in Atlantic salmon (*Salmo salar*). *Mol. Ecol.* 10:255-2539

Laurenson MK, Caro TM, Gros P, et al. 1995. Controversial cheetahs? *Nature* 377:392

Macdonald DW, Loveridge AJ, Nowell K. 2010. *Dramatis personae*: an introduction to the wild felids In: Macdonald DW, Loveridge AJ (eds) Biology and Conservation of wild felids. Great Britain: Oxford University Press. pp. 3-58

Mainguy J, Worley K, Cote SD, et al. 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 L. 2000. Aspects of the ecology of the cheetah (*Acinonyx jubatus*) on north central Namibian farmlands. *Nam. Sci. Soc. J.* 48:40-48

Marker L. 2002. International Cheetah Studbook. Otjiwarongo, Namibia: Cheetah Conservation Found.

Marker LL, Wilkerson AJP, Sarno RJ, et al. 2008. Molecular genetic insights on cheetah (*Acinonyx jubatus*) ecology and conservation in Namibia. *J. Hered.* 99:2-13

Marker L, Dickman AJ, Mills MGL, et al. 2010. Cheetahs and ranchers in Namibia: a case study. In: Macdonlad DW and Loveridge AJ (eds) Biology and Conservation of wild felids. Great Britain: Oxford University Press pp. 353-372

Marker-Kraus L, Kraus D. 1995. The Namibian free-ranging cheetah. *Environ. Conserv.* 21:369-370

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

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

Mendelsohn J, Jarvis A, Roberts C, et al. 2009. Atlas of Namibia, a portrait of the land and its people. Cape Town, South Africa: Sunbird Publishers

Menotti-Raymond M, O'Brien SJ. 1993. Dating the genetic bottleneck of the African cheetah. *Proc. Natl. Acad. Sci. USA* 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

Miller HC, Miller KA, Daugherty CH. 2008. Reduced MHC variation in a threatened tuatara species. *Anim. Conserv.* 11:206-214

Miththapala S, Seidensticker J, Phillips LG, et al. 1991. Genetic Variation in Sri Lankan Leopards. *Zoo Biol.* 10:139-146

Munguía-Vega A, Esquer-Garrigós Y, Rojas-Bracho L, et al. 2007. Genetic drift vs. natural selection in a long-term small isolated population: Major histocompatibility complex class II variation in the Gulf of California endemic porpoise (*Phocoena sinus). Mol. Ecol.* 16:4051-4065

Munson L, Marker L, Dubovi E, et al. 2004. Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*). *J. Wildl. Dis.* 40:23-31

Munson L, Terio KA, Worley M, et al. 2005. Extrinsic factors significantly affect patterns of disease in free-ranging and captive cheetah (*Acinonyx jubatus*) populations. *J. Wildl. Dis.* 41:542-548

Munson L, Terio KA, Ryser-Degiorgis MP, et al. 2010. Wild felid diseases: conservation implications and management strategies. In: Macdonlad DW and Loveridge AJ (eds) Biology and Conservation of wild felids. Great Britain: Oxford University Press pp. 237-259

Murray DL, Kapke CA, Evermann JF, et al. 1999. Infectious disease and the conservation of free-ranging large carnivores. *Anim. Conserv.* 2:241-254

Nowell K, Jackson P. 1996. Wild cats: Status survey and conservation action plan. IUCN/ SSC cat specialist group. Switzerland: Burlington Press.

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

O'Brien SJ, Wildt DE, Goldman D, et al. 1983. The cheetah is depauperate in genetic variation. *Science* 221:459-462

O'Brien SJ, Roelke ME, Marker L, et al. 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, Evermann JF. 1988. Interactive influence of infectious disease and genetic diversity in natural populations. *Trends Ecol. Evol.* 3:254-259

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

O'Brien SJ, Troyer JL, Roelke M, et al. 2006. Plagues and adaptation: Lessons from the Felidae models for SARS and AIDS. *Biol. Conserv.* 131:255-267

O'Brien SJ, Johnson W. 2007. The evolution of cats. *Sci. Am.* July:68-75

Pedersen AB, Jones KE, Nunn CL, et al. 2007. Infectious Diseases and Extinction Risk in Wild mammals. *Conserv. Biol.* 21:1269-1279

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

Pokorny I, Sharma R, Goyal SP, et al. 2010. MHC class I and MHC class II DRB gene variability in wild and captive Bengal tigers (*Panthera tigris tigris*). *Immunogenetics* 62:667-79

Potts WK, Wakeland EK. 1990. Evolution of diversity at the major histocompatibility complex. *Trends Ecol. Evol.* 5:181-187

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

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

Reed DH. 2010. Albatrosses, eagles and newts, Oh My!: exceptions to the prevailing paradigm concerning genetic diversity and population viability? *Anim. Conserv.* 13:448-457

Roelke-Parker ME, Munson L, Packer L, et al. 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature* 379:441-445

Sachdev M, Sankaranarayanan R, Reddanna P, et al. 2005. Major histocompatibility complex class I polymorphism in Asiatic lions. *Tissue Antigens* 66:9-18

Satta Y, Ohuigin C, Takahata N, et al. 1994. Intensity of natural selection at the major histocompatibility complex loci. *Proc. Natl. Acad. Sci. USA* 91:7184-7188

Schneider, HP. 1994. Animal health and veterinary medicine in Namibia. Windhoek, Namibia: AGRIVET

Schwensow N, Fietz J, Dausmann K, et al. 2007. Neutral versus adaptive genetic variation in parasite resistance: importance of MHC-supertypes in a free-ranging population. *Heredity* 99:265-277

Seddon JM, Baverstock PR. (1999) Variation on islands: major histocompatibility complex (Mhc) polymorphism in populations of the Australian bush rat. *Mol. Ecol.* 8:1971-2179

Seddon JM, Ellegren H. 2004. A temporal analysis shows major histocompatibility complex loci in Scandinavian wolf population are consistent with neutral evolution. *Proc. R. Soc. London Ser. B-Biol. Sci.* 271:2283-2291

Siddle HV, Kreiss A, Eldridge MDB, et al. 2007. Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. *Proc. Natl. Acad. Sci. USA* 104:16221-16226

Slade RW, McCallum HI. 1992. Overdominant vs. frequency-dependent selection at MHC loci. *Genetics* 132:861–862

Smith KF, Acevedo-Whitehouse K, Pedersen AB. 2009. The role of infectious diseases in biological conservation. *Anim. Conserv.* 12:1-12

Smith MM, Hoffman SMG. 2001. A class I MHC locus compared among felid species. *Mamm. Genome* 12:394-396

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

Spong G, Johansson M, Bjorklund M. 2000. High genetic variation in leopards indicates large and long-term stable effective population size. *Mol. Ecol.* 9:1773-1782

Spurgin LG, Richardson DS. 2010. How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proc. R. Soc. London Ser. B-Biol. Sci.* 277:979–988

Takahata N, Satta Y. 1998. Selection, convergence, and intragenic recombination in HLA diversity. *Genetica* 102/103:157-169

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, et al. 2010. Seroprevalences to viral pathogens in free-ranging and captive cheetahs (*Acinonyx jubatus*) on Namibian farmland. *Clin. Vaccine Immunol.* 17:232-238

Uphyrkina O, Johnson WE, Quigley H, et al. 2001 Phylogenetics, genome diversity and origin of modern leopard, *Panthera pardus*. *Mol. Ecol.* 10:2617-2633

Wang Q, Wu X, Yan P, et al. 2008. Sequence variability analysis on major histocompatibility complex class II DRB alleles in three felines. *Front. Biol. China* 3:55-62

Wang X, Wei K, Zhang Z, et al. 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, et al. 2004. Major histocompatibility complex variation at three class II loci in the northern elephant seal. *Mol. Epidemiol.* 13: 711-718

Wei K, Zhang Z, Wang X, et al. 2010. Lineage pattern, trans-species polymorphism, and selection pressure among the major lineages of feline Mhc-DRB peptide-binding region. *Immunogenetics* 62:307-317

Wilson DE, Mittermeier RA. 2009a. *Acinonyx jubatus*. Handbook of the Mammals of the World. Vol. 1. Carnivores. Barcelona, Spain: Lynx Edicions.

Wilson DE, Mittermeier RA. 2009b. *Panthera pardus*. Handbook of the Mammals of the World. Vol. 1. Carnivores. Barcelona, Spain: Lynx Edicions

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, O'Brien SJ. 1990a. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proc. Natl. Acad. Sci. USA* 87:836-840

Yuhki N, O'Brien SJ. 1990b. 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. 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

Yuhki N, Beck T, Stephens RM, et al. 2003. Comparative genome organization of human, murine, and feline MHC class II region. *Genome Res*. 13:1169-1179

Yuhki N, Mullikin JC, Beck T, et al. 2008. Sequences, annotation and single nucleotide polymorphism of major histocompatibility complex in the domestic cat. *PLoS ONE* 3:e2674

Article 1

This is a pre-copy-editing, author-produced PDF of an article accepted for publication in *Molecular Biology and Evolution* following peer review. The definitive publisher-authenticated version [Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. Aines Castro-Prieto, Bettina Wachter, and Simone Sommer. Molecular Biology and Evolution (2011) 28:1455–1468] is available on line at:

[http://mbe.oxfordjournals.org/content/28/4/1455.full.pdf?keytype=ref&ijkey=3PrfidNDAzefDp7].

Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population

Research Article

AINES CASTRO-PRIETO, BETTINA WACHTER and SIMONE SOMMER

Molecular Biology and Evolution (2011), 28:1455–1468.

Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany

Keywords: MHC class I, MHC class II-DRB, diversity, *Acinonyx jubatus*, free-ranging cheetahs, positive selection

Corresponding author: Simone Sommer, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany, Telephone: 0049-30-5168-315, Fax: 0049-30-5126-104, sommer@izw-berlin.de

Running title: Immune gene diversity in Namibian cheetahs

Abstract

For more than two decades the cheetah (*Acinonyx jubatus*) has been considered a paradigm of disease vulnerability associated with low genetic diversity, particularly at the immune genes of the major histocompatibility complex (MHC). Cheetahs have been used as a classic example in numerous conservation genetics textbooks as well as in many related scientific publications. However, earlier studies used methods with low resolution to quantify MHC diversity and/or small sample sizes. Furthermore, high disease susceptibility was reported only for captive cheetahs, whereas free-ranging cheetahs show no signs of infectious diseases and a good general health status. We examined whether the diversity at MHC class I and class II-DRB loci in 149 Namibian cheetahs was higher than previously reported using single-strand conformation polymorphism analysis, cloning and sequencing. MHC genes were examined at the genomic and transcriptomic levels. We detected 10 MHC class I and four class II-DRB alleles, of which nine MHC class I and all class II-DRB alleles were expressed. Phylogenetic analyses and individual genotypes suggested that the alleles belong to four MHC class I and three class II-DRB putative loci. Evidence of positive selection was detected in both MHC loci. Our study indicated that the low number of MHC class I alleles previously observed in cheetahs was due to a smaller sample size examined. On the other hand, the low number of MHC class II-DRB alleles previously observed in cheetahs was further confirmed. Compared with other mammalian species including felids, cheetahs showed low levels of MHC diversity, but this does not seem to influence the immunocompetence of free-ranging cheetahs in Namibia and contradicts the previous conclusion that the cheetah is a paradigm species of disease vulnerability.

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 1990a). 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, 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, MHC I and II; O'Brien et al. 1983; Yuhki and O'Brien 1990a, 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 1990a), 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 (RFLP) analysis of MHC I genes, Yuhki and O'Brien 1990a) 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 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 June 2002 and June 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, examined for their overall health status, sampled and released as described in Thalwitzer et al. (2010). EDTA-blood samples were collected and centrifuged, and the leucocyte pellets were stored in liquid nitrogen until later genomic DNA isolation. For expression analysis, PAXgene^{TM} 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.

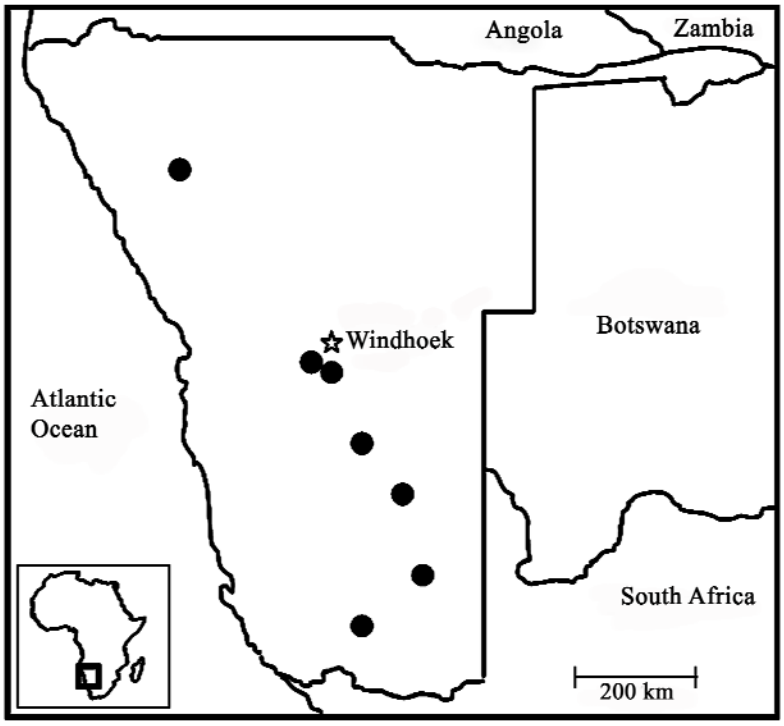


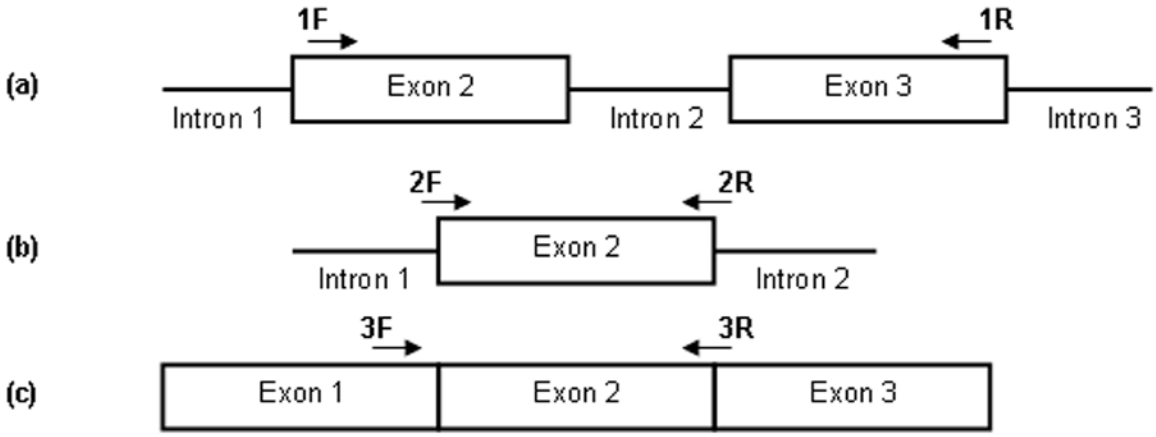
Fig 1 Schematic map showing the origin (dots) of the cheetah samples in Namibia.

Nucleic acid isolation and complementary DNA (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^{TM} 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). cDNA synthesis was obtained using 200 U of RevertAid^{TM} 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 μg/μl, Invitrogen, Karlsruhe, Germany), 0.5 μl ribonuclease inhibitor (20 U/μl), 2 μl 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), i.e. 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 are. 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). 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, USA). 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).



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.

MHC genotyping

Of the 149 cheetahs, 108 and 139 individuals, respectively, were genotyped for their MHC I and II-DRB constitution. Individual's 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% non-denaturing 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 h and 4:30 h 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, USA) 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 pCR®4-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 Clustal W 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 (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 non-synonymous (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 α=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 + γ (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 non-coding (intron 2) regions. A non-partition Bayesian analysis (all data in a single partition) was also conducted for comparison purposes. In both partition and non-

partition analyses, tree searches were conducted using a GTR + invariable + γ 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 –lnL 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/~guy/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 identified in 108 Namibian cheetahs (Supplementary Figure 1). 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.

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.

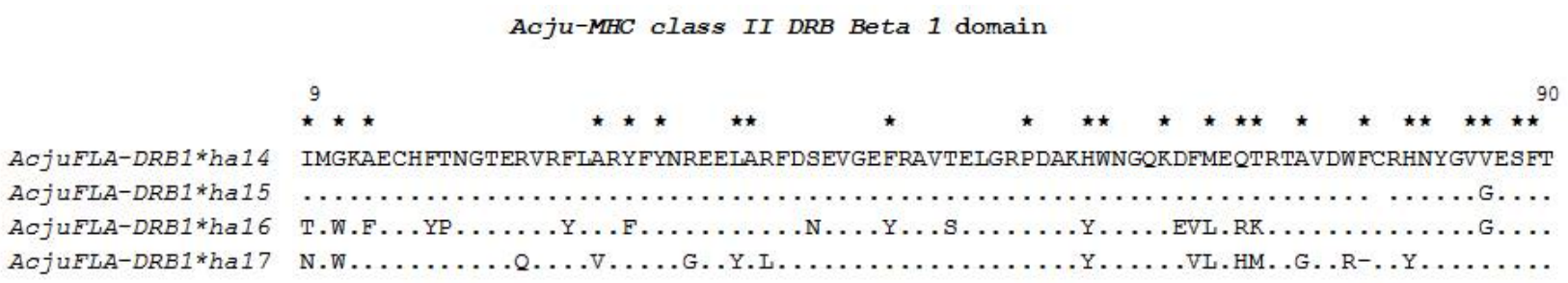


Fig. 4 Amino acid sequence alignment of expressed MHC II-DRB alleles from Namibian cheetahs aligned to *AcjuFLA-DRB1*ha14* (Drake et al. 2004, GenBank Accession number AY312960). Numbers indicate the amino acid positions of the β_1 domain according to the human MHC II-DRB sequence. Asterisks indicate putative ABS (Brown et al. 1993). Dots indicate identity to the top sequence and minuses indicate an amino acid deletion.

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 Figure 1). The corresponding amino acid sequence consisted of 83 amino acids in the α_1 domain (positions 8 to 90, Fig. 3) and 87 amino acids in the α_2 domain (positions 91 to 177, Fig. 3). Out of 22 and 17 variable sites along the α_1 and α_2 domains, respectively, 11 and eight 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 Figure 1). 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 Figure 2). 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 Figure 2). This corresponded to 81/82 amino acids of the β_1 domain (positions 9 to 90, Fig. 4). Out of 25 variable sites 15 were located in important positions for antigen binding (Fig. 4). The overall mean genetic 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).

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.

								Genetic distances		
MHC	Region	Length	V	C	Si	%Si	No. differences	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, antigen-binding sites (ABS) and non-antigen-binding sites (non-ABS).

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 non-coding (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 non-functional (*Acju-MHCI*12*, see above), this result suggests that the alleles observed in *A. jubatus* belong to three functional and one non-functional MHC I loci.

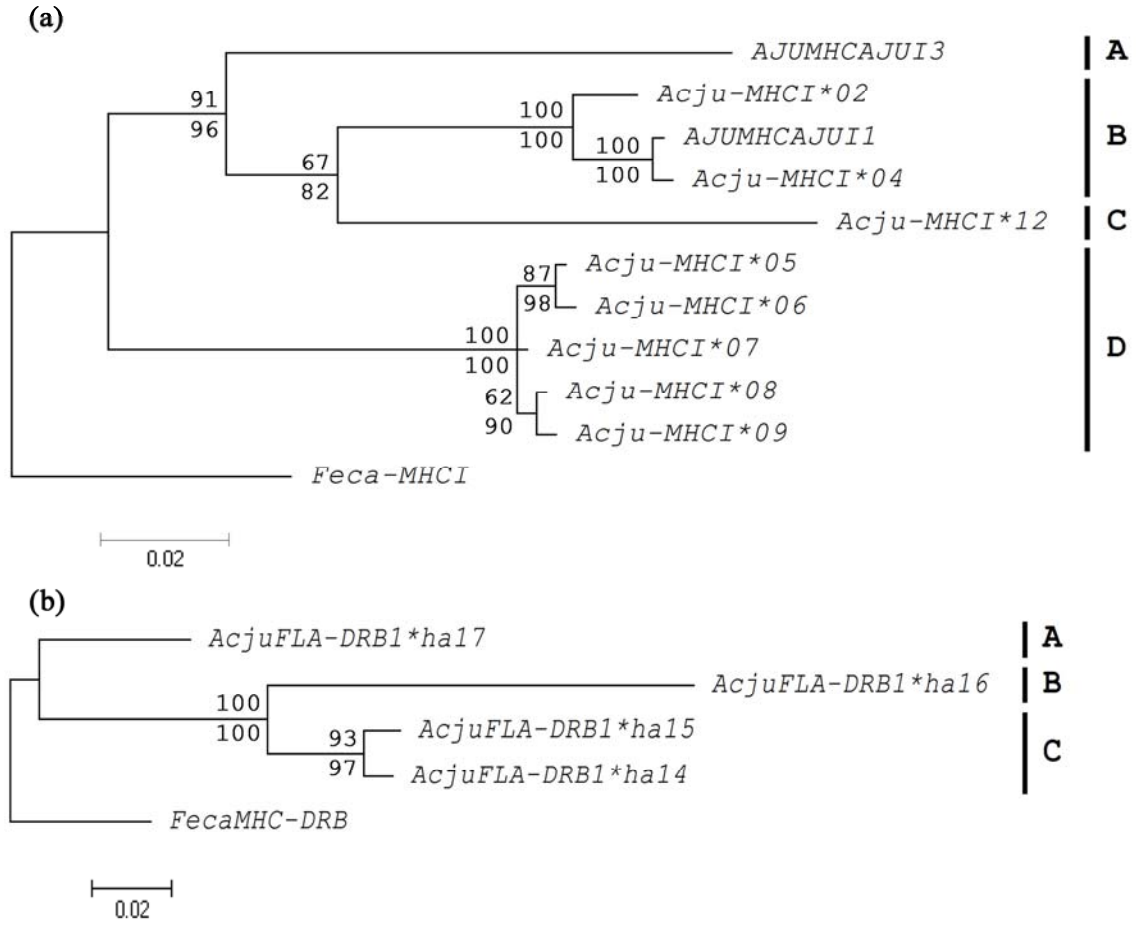


Fig. 5 Phylogenetic relationships among the MHC I (a) and MHC II-DRB (b) alleles detected in Namibian cheetahs. Numbers above and below the branches refer to bootstrap and posterior probabilities values, respectively. The trees were rooted using a MHC-annotated genomic sequence of the domestic cat (GenBank Accession number EU153401).

Pairwise sequence comparisons assigned the 10 cheetahs' MHC I alleles as the best hits to three classical (i.e. ubiquitously expressed, *FLAI-E*, *FLAI-H* and *FLAI-K*) and one non-classical (i.e. non-ubiquitously transcribed, *FLAI-J*) MHC I loci described for the domestic cat (Yuhki et al. 2008), thereby corresponding to the three functional and one non-functional 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*) out 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 non-synonymous (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 ($t = 3.40$, $df = 28$, $P < 0.01$) 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; $t = 3.96$, $df = 10$, $P < 0.01$).

Table 2 Non-synonymous (d_N) and synonymous (d_S) substitutions (± standard error) as well as their ratio in antigen-binding sites (ABS) and non-antigen-binding sites (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.

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. Since the 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 10 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 non-synonymous 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. 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 candis and other non-carnivore species (including rodents and primates), felids showed higher rates of synonymous over non-synonymous 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 to the homologous sequences of the domestic cat (12% and 12%, respectively, Yuhki and O'Brien 1990b), 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).

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] (*A. 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] (*P. leo*)	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] (*P. 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] *(L. 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] *(F. 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] (*C. 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] (*L. 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,i] (*C. 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
Non-carnivores									
Striped mouse[j] (*R. 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[k] (*A. 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[l] (*M. 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[m] (*H. s. 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 = non-synonymous substitutions rate; d_S = synonymous substitutions rate; NA = no available information; SSCP = single-strand conformation polymorphism analysis; RSCA = reference strand-mediated conformational analysis; 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,i]Hedrick et al. (2000b; 2002b); [j]Froeschke and Sommer (2005); [k]Meyer-Lucht and Sommer (2005); [l]Schad et al. (2005); [m]Trachtenberg et al. (2007).

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 1990b, 1994; Sachdev et al. 2005).

Non-coding regions evolving under neutral conditions can be expected to exhibit high genetic polymorphism. The non-coding intronic sequences between the second and third exons of MHC I loci, however, revealed a low diversity (3%, Table 1). This is because non-coding 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 non-classical 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 et al. 1998a), 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 non-synonymous 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 1990b). 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 non-synonymous and synonymous substitutions in the ABS of MHC II-DRB exon 2 was observed. Non-synonymous 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. While 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 (20^{th} 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 three 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 10 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 et al. 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 et al. 2000a; 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 1995b), 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 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 1995a; 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 (FHV), 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 faeces (Marker-Kraus et al. 1996; Thalwitzer 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 1990a) 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 figure S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgements

We would like to thank the Ministry of Environment and Tourism in Namibia for permission to conduct the study; S Thalwitzer, A Krengel, J Melzheimer, J Lonzer, B Förster and H Förster for collecting the samples; the Seeis and Hochfeld conservancies in Namibia and the AfriCat Foundation in Namibia for their continuous cooperation; M Monaghan, G Spudlich and J Fickel for data analysis assistance; A Schmidt and R Taubert for technical laboratory assistance; H Hofer, V Stefanski, J Axtner, N Schwensow, J Schad, Y Meyer-Lucht, G Froeschke, A Wilting, A Munguía-Vega, and R Ortiz-Rodríguez for fruitful discussions and/or helpful suggestions during the development of this project; R Danabalan for language advice. Comments from the associate editor, S Edwards, as well as two anonymous referees substantially improved the manuscript. This work is part of the cheetah research project of the Leibniz Institute for Zoo and Wildlife Research (IZW), Germany and was supported by the Messerli Foundation, Switzerland and the IZW.

References

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. USA* 101:3490-3494.

Altizer S, Foufopoulos J, Gager A. 2001. Conservation and diseases. In: Levin SA editors. Encyclopedia of Biodiversity. San Diego: 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, 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.

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

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.

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.

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, USA: 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, 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.

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

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 and Oxford: Oxford University Press. p. 214–237.

Ejsmond MJ and 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. USA* 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. Inbreeding and extinction - a threshold effect. *Conserv. Biol.* 9:792-799.

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

Frankham R, Ballou JD, Briscoe DA. 2002. Introduction to Conservation Genetics. United Kingdom: 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. 1996. Bottleneck(s) or metapopulations in cheetahs. *Conserv. Biol.* 10:897-899.

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

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

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

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

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.

Heeney J L, 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, 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 overdominant selection. *Proc. Natl. Acad. Sci.* USA 86:958-962.

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

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.

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

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.

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, O´hUigin C. 1998a. Evolution by gene duplication in the major histocompatibility complex. *Cytogenet. Cell Genet.* 80:123-127.

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

Kumar S, Tamura K, Nei M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinfor.* 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: 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. USA* 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. USA* 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. Evo.* 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, Wildt DE, Goldman D, Merril CR, Bush M. 1983. The cheetah is depauperate in genetic variation. *Science* 221:459-462.

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. USA* 84:508-511.

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. 1994. The cheetah's conservation controversy. *Conserv. Biol.* 8:1153-1155.

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. USA* 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* 62:667-79.

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

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.

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

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. USA* 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.* USA 104:16221-16226.

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.

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.

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 and 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, 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, O'Brien SJ. 1990a. DNA variation of the mammalian major histocompatibility complex reflects genomic diversity and population history. *Proc. Natl. Acad. Sci. USA* 87:836-840.

Yuhki N, O'Brien SJ. 1990b. 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. 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.

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, 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.

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.

Article 2

Patterns of adaptive variation and genetic differentiation in free-ranging cheetahs on Namibian farmlands

Research paper (*in review*)

AINES CASTRO-PRIETO, BETTINA WACHTER, JOERG MELZHEIMER, SUSANNE THALWITZER and SIMONE SOMMER

Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany

Key words: MHC, variation, genetic differentiation, *Acinonyx jubatus*, cheetahs

Corresponding author: Simone Sommer, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany, Telephone: 0049-30-5168-315, Fax: 0049-30-5126-104, sommer@izw-berlin.de

Running title: MHC variation and differentiation in Namibian cheetahs

Abstract

The world's largest remaining population of free-ranging cheetahs (*Acinonyx juabuts*) occurs in Namibia and is considered as the key population for the conservation of this species. Compared with other mammalian species including felids, cheetahs show low levels of immune gene diversity, particularly at the Major Histocompatibility Complex (MHC). In a recent study, a total of ten MHC class I alleles and four MHC class II-DRB alleles were detected (Castro-Prieto et al. 2011). MHC class I codes for cell-surface glycoproteins important for the activation of the immune cascade against intracellular antigens such as viruses whereas MHC class II plays an important role in the defence of extracellular antigens such as helminths. Serosurvey studies in Namibian cheetahs revealed higher exposure to specific feline and canine viruses in individuals from north-central than east-central Namibia (Thalwitzer et al. 2010). We examined whether the observed differences in exposure to viral pathogens influence the patterns of adaptive variation and genetic differentiation at the Major Histocompatibility Complex (MHC) in free-ranging cheetahs on Namibian farmland. Here, we compared patterns of variation at the second exon of MHC class I and class II-DRB loci in 88 individuals from north-central and east-central Namibia by using single-stranded conformation polymorphism analysis and direct sequencing. No differences between the regions in the allelic diversity or in the expected heterozygosities for any MHC loci were observed. However, a high genetic differentiation at MHC class I ($F_{ST} = 0.07$, $P < 0.0001$) was detected between cheetahs from north-central and east-central Namibia, whereas no such differentiation in MHC class II-DRB and neutral markers (Marker et al. 2008) existed. This suggests that MHC class I mirrors the different pathogenic selective pressure imposed by viruses in free-ranging cheetahs across Namibian farmland. These results are of high significance for future management and conservation programs of free-ranging cheetahs in Namibia and other African populations.

Introduction

Anthropogenic factors such as human population expansion and associated changes in natural resource use result in greater contact and overlap among humans, domestic animals and wildlife communities, thereby increasing the opportunities of pathogens to spread among species, sometimes with dramatic consequences for wildlife (Smith et al. 2009; Alexander et al. 2010). Alterations of the inherent ecological balance between pathogens and their human and animal hosts are particularly prevalent in rural Africa in the vicinity of remaining natural lands (Mazet et al. 2009).

The African cheetah (*Acinonyx jubatus*) is listed on Appendix I of the Convention on International Trade in Endangered Species (CITES) and is fully protected over most of its range. The world's largest remaining population of free-ranging cheetahs occurs in Namibia with an estimated population size of 3,100 to 5,800 individuals (Hanssen and Stander 2004) and is considered as the key population for the conservation of the species (Marker-Kraus et al. 1996). A large proportion of free-ranging Namibian cheetahs concentrates in the central and northern Namibia, where they inhabit unprotected areas that encompass continuous privately owned livestock or game farmland often bordering towns and cities (Marker-Kraus and Kraus 1995). Consequently, free-ranging cheetahs on Namibian farmland get potentially in close proximity with domestic dogs and cats that sometimes are unvaccinated (Schneider 1994). Such animals can be important vectors of diseases such as canine distemper, which has affected free-ranging cheetahs in the Serengeti (Roelke-Parker et al. 1996). Common feline and canine viruses such as feline herpesvirus 1 FHV1, feline calcivirus FCV, feline parvovirus FPV, feline coronavirus FCoV, canine distemper virus CDV and feline immunodeficeny virus FIV are known to cause severe disease in captive cheetahs (Munson et al. 2010). Serosurvey of these viruses confirmed the exposure of free-ranging Namibian cheetahs to these viral pathogens but detected no evidence of clinical signs of infectious diseases in the examined individuals (Munson et al. 2004; Thalwitzer et al. 2010). However, results from these studies showed substantial differences in the seroprevalences to viral pathogens between Namibian cheetahs from north-central (Munson en et al. 2004) and east-central (Thalwitzer et al. 2010) regions. A higher exposure to viruses in cheetahs from north-central than east-central Namibia was attributed to relatively high human population densities in the north-central region and its associated factors, particularly, the contact opportunities with (non-vaccinated) domestic and feral cats and dogs (Thalwitzer et al. 2010).

The ability of host populations to mount a protective immune response after exposure to pathogens is partly regulated by the genes of the Major Histocompatibility Complex (MHC).

MHC genes encode cell-surface glycoproteins involved in pathogen and T-cell receptor recognition. The MHC class I (MHC I) and class II (MHC II) proteins bind and present short peptides derived from intracellular (e.g. viruses) and extracellular (e.g. bacteria, helminths) pathogens, respectively, to cytotoxic and T-helper cells, thereby triggering a cascade of immune responses in all vertebrate species (Klein 1986). High levels of MHC polymorphism observed in most natural populations over generations (Hedrick 1994) are mainly driven by pathogenic selective pressures (Hedrick 2002; Piertney and Oliver 2006). Pathogen-mediated selection on MHC genes has been shown in a number of laboratory and in-situ studies (Garrigan and Hedrick 2003; Sommer 2005; Spurgin and Richardson 2010). In wildlife, an increasing number of studies have also found associations between the host MHC genetic composition and disease resistance (Bernatchez and Landry 2003; Sommer 2005; Alcaide et al. 2010).

Significant differences in the seroprevalences to viral pathogens observed between free-ranging Namibian cheetahs from east-central and north-central regions (Munson et al. 2004; Thalwitzer et al. 2010) raises the question whether differences in pathogen exposure influence the patterns of variation and genetic differentiation at MHC loci of free-ranging cheetahs on Namibian farmlands. Different levels of selection caused by varying pathogen pressure are expected to have a detectable effect on genotypic frequencies in the contemporary population (Hedrick et al. 2000). An essential pre-requisite to test this is the locus-specific assignment of MHC alleles in the species, which was previously conducted in Namibian cheetahs by combining information on the evolutionary affinities between the observed MHC alleles, qualitative expression analysis, and the distribution of alleles among 149 individuals (Castro-Prieto et al. 2011). In the latter study, a total of ten MHC I alleles and four MHC II-DRB alleles were identified in the population. Here, we compare patterns of MHC I and II-DRB variation in free-ranging Namibian cheetahs from regions that differ in viral pathogen exposure. We hypothesized that if there is any differential selective pressure imposed by viruses, we expect to detect significant differences in the allelic distribution of MHC I in contrast to MHC II-DRB and neutral markers (Marker et al. 2008) between free-ranging cheetahs from north-central and east-central central Namibia. Results from this study will contribute to a better understanding of the conservation implications of MHC on the Namibian cheetah population and may be of interest for the management and conservation programs of other free-ranging felid populations.

Methods

Study site and sample collection

We used blood samples from 88 wild-born cheetahs (67 males and 21 females) from east-central and north-central Namibia (Fig. 1). The animals were free-ranging individuals inhabiting commercial livestock or game farmland in Namibia (n = 49), wild-caught individuals kept in a large holding facility (n = 28) or dead individuals reported to us or found in the field (n = 11). Cheetahs were immobilized as described in Wachter et al. (2011). From related animals such as mother with cubs only one individual of the group was used for the analyses. Blood samples were centrifuged and the leucocyte pellets stored in liquid nitrogen until transport to Germany and further processing at the laboratory.

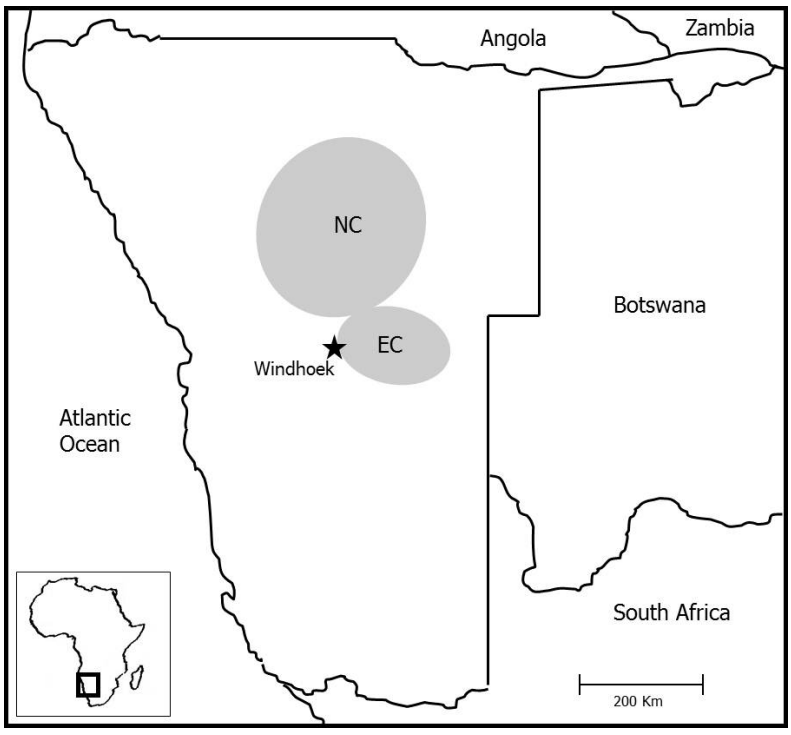


Fig. 1 Schematic map showing the origin (shaded) of the cheetah samples in Namibia. North central (NC) and east-central (EC) regions were devided as in Thalwitzer et al. (2010).

Molecular methods

All 88 samples were genotyped for both MHC class I and II-DRB loci. Sixty-two samples were already included in a previous study (Castro-Prieto et al. 2011). Total genomic DNA was isolated from blood buffy coat using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. We focused our analyses on both MHC classes, particularly in highly polymorphic regions that include functionally important antigen-binding sites (ABS), i.e. amino acid positions postulated to interact directly with the foreign antigens (Bjorkman et al. 1987; Bjorkman and Parham 1990; Brown et al. 1993). The second exon of MHC I alleles was amplified using primers Acju_Ex2MhcI_cF (5′-GCTCCCACTCCCTGAGGTAT-3′; Castro-Prieto et al. 2011) and Acju_Ex2MhcI_kR (5′-GGAKTCGCTCTGGTTGTAGT-3′) designed from class I transcript sequences available from felid species in GenBank. PCR amplification was 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, USA). 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°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). The second exon of MHC II-DRB alleles was amplified using primers and PCR conditions as described in Castro-Prieto et al. (2011). Genotyping of MHC I and II-DRB was conducted through SSCP analysis followed by sequence analysis of the distinctive single-strand bands as previously described (Castro-Prieto et al. 2011). To ensure that the sequences represented true alleles, the PCR-SSCP analysis was conducted twice per individual sample.

Data Analysis

To examine patterns of sequence variation, nucleotide sequences were edited manually 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). Standard diversity indices were estimated only for polymorphic functional MHC I and II-DRB loci (Castro-Prieto et al. 2011) using the software Arlequin 3.1 (Excoffier et al. 2005). Allele frequencies were estimated at all putative loci separately and as a whole haplotype for MHC I loci. In the latter case, the frequencies of co-occurring alleles (which presumably constitute a haplotype on a given chromosome) were

estimated as the number of individuals carrying a certain allele divided by the total count of alleles observed in the population. Expected heterozygosity (H_E) was estimated as a general indicator of the amount of genetic variation in the population (Nei 1987). Departures from Hardy-Weinberg (H-W) equilibrium were assessed by applying exact tests (Guo and Thompson 1992). For the analysis of variation and genetic differentiation at MHC I and II-DRB loci in Namibian cheetahs, all samples were classified into two main regions referred to north-central and east-central according to Thalwitzer et al (2010) (Fig. 1). These two regions lack physical barriers but differ substantially in viral pathogen exposure as revealed by seroprevalence studies conducted in Namibian cheetahs (Munson et al. 2004; Thalwitzer et al. 2010). The ratio of males and females was similar in the north-central (N males = 18, N females = 8) and the east-central region (N males = 49, N females = 13, $\chi^2 = 0.97$, df = 1, P = 0.33). The alternative hypothesis of heterogeneity between regions was tested by using F-statistics (Wright 1965) and exact tests of sample differentiation based on haplotype frequencies (Raymond and Rousset 1995) as implemented in Arlequin 3.1. To further test this hypothesis, we used the chi-square (χ^2) and Fisher's method of combined P values obtained by Fisher's exact test as implemented in CHIFISH (Ryman 2006) for MHC I loci. This was done because we combined information from multiple loci which may result in low statistical power preventing detection of true genetic divergence (Ryman and Jorde 2001). This problem occurs particularly in small contingency tables (few populations and few alleles per locus) as observed in our data set. Differences in heterozygosity between north-central and east-central Namibian cheetahs were tested with chi-square test in SPSS Version 16.0. We also conducted genetic differentiation analyses between male and female cheetahs, as previously mentioned, to control for any effect due to the male-biased sampling.

Results

In total, 88 unrelated cheetahs from Namibia have been genotyped at both MHC I and II-DRB loci. Samples genotyped in this study exhibited a total of six and four nucleotide sequences corresponding to the second exon of MHC I (229 bp) and MHC II-DRB (246 bp) alleles, respectively. MHC I sequences corresponded to the second exon of MHC I alleles previously described in the species (Genbank accession numbers AJU07665-66, GU971407, GU971409, GU971411, GU971414; Yuhki and O'Brien 1994, Castro-Prieto et al. 2011). MHC II-DRB sequences were also previously described (Genbank accession numbers AY312960-63, Drake et

al. 2004). We were not able to discriminate between MHC I alleles *AJUMHCAJUI1* and *Acju-MHCI*04* (differing in a single amino acid position) in the SSCP banding pattern and referred to them in this study as *Acju-MHCI*04*. The expression of all alleles except for one (GU971414) was previously determined as well as the locus-specific assignment of MHC alleles in *A. jubatus* in Castro-Prieto et al. (2011). All expressed alleles were distributed in seven and three genotypes at the MHC I and II-DRB loci, respectively (Table 1). Allele frequencies from polymorphic class I (MHC I-B, MHC I-D) and class II (MHC II DRB-C) loci observed in the population are presented in Table 2. Mean H_E was 0.35 ± 0.21 across both polymorphic MHC I loci and 0.50 for the polymorphic MHC II-DRB locus. This is lower than the mean expected heterozygosity observed at 38 microsatellite loci (mean H_E = 0.64-0.71; Marker et al. 2008). The null hypothesis of H-W equilibrium was not rejected at MHC II DRB-C locus but it deviated significantly (P = 0.0004) across MHC I loci, resulting in a deficit of heterozygotes (Table 2). This is due to the MHC I-B locus (P = 0.005) because the MHC I-D locus remained at H-W equilibrium (P = 0.30).

Table 1 MHC I (exon 2) and MHC II-DRB (exon2) genotypes detected in 88 free-ranging Namibian cheetahs.

Genotypes	Locus-specific MHC alleles					No. individuals
MHC I	Locus A	Locus B		Locus D		
	AJUMHCAJUI3	*Acju-MHCI*04*	*Acju-MHCI*02*	*Acju-MHC*05*	*Acju-MHC*07*	
I	X	X	X	X		28 (32%)
II	X	X	X	X	X	2 (2%)
III	X		X	X		14 (16%)
IV	X		X	X	X	10 (11%)
V	X	X		X		28 (32%)
VI	X	X		X	X	4 (5%)
VII	X	X			X	2 (2%)
MHC II-DRB	Locus A	Locus B	Locus C			
	*AcjuFLA-DRB1*ha17*	*AcjuFLA-DRB1*ha16*	*AcjuFLA-DRB1*ha15*	*AcjuFLA-DRB1*ha14*		
I	X	X		X		17 (19%)
II	X	X	X	X		50 (57%)
III	X	X	X			21 (24%)

Comparisons between cheetahs in north-central and east-central Namibia

Measures of MHC variation between north-central and east-central Namibian cheetahs are summarized in Table 2. The number of observed MHC I and II-DRB alleles did not differ between the regions and deviation from H-W equilibrium was observed across MHC I loci. Levels of heterozygosity across MHC I loci were comparable in east-central (mean $H_E = 0.33 \pm 0.20$) and north-central (mean $H_E = 0.36 \pm 0.17$) regions. Levels of heterozygosity at MHC II-DRB locus were also similar in both regions and no deviation from H-W equilibrium was detected. Contrasting patterns of genetic differentiation were observed between MHC I and MHC II-DRB loci within the Namibian cheetah population (Table 2). Namibian cheetahs from the north-central region were highly differentiated from those of the east-central region based on the allelic distribution across MHC I loci ($F_{ST} = 0.07$, $P < 0.0001$). In contrast, no differentiation between the regions was detected at MHC II-DRB locus ($F_{ST} = -0.01$, $P = 0.74$). Exact tests of differentiation also revealed significant differences between the regions based on the allelic distribution across MHC I loci ($P = 0.02$) but not at MHC II-DRB locus ($P = 0.61$). MHC I differentiation between both regions was further supported by combining a chi-square test ($\chi^2 = 9.19$, $df = 2$, $P = 0.01$) and Fisher's exact test ($P = 0.02$) in a locus-by-locus treatment.

Comparisons between male and female cheetahs in Namibia

Measures of MHC variation between male and female Namibian cheetahs are summarized in Table 2. The number of MHC I and II-DRB alleles did not differ between sexes. Levels of heterozygosity across MHC I loci in males (mean $H_E = 0.36 \pm 0.20$) were slightly higher than in females (mean $H_E = 0.31 \pm 0.25$) but the difference was not significant. Levels of heterozygosity at MHC II-DRB locus were similar between both sexes and no deviation from H-W equilibrium was detected. No genetic differentiation at any locus of MHC I or MHC II-DRB was detected between male and female Namibian cheetahs (Table 2).

Table 2 Estimates of genetic variation and differentiation at polymorphic MHC class I and MHC class II-DRB genes in free-ranging cheetahs from Namibia.

	All	Sex		Region	
		Males	Females	East-central	North-central
Sample size (*N*)	88	67	21	62	26
MHC I loci (Locus B & D)					
Haplotype frequencies					
*Acju-MHCI*04 / Acju-MHCI*05*	0.51	0.49	0.60	0.58	0.35
*Acju-MHCI*02 / Acju-MHCI*05*	0.38	0.39	0.33	0.32	0.52
*Acju-MHCI*02 / Acju-MHCI*07*	0.07	0.07	0.05	0.06	0.10
*Acju-MHCI*04 / Acju-MHCI*07*	0.05	0.05	0.02	0.05	0.04
Hardy-Weinberg equilibrium					
Mean H_O/H_E	0.26±0.11/ 0.35±0.21	0.26±0.09/ 0.36±0.20	0.26±0.20/ 0.31±0.25	0.25±0.10/ 0.33±0.20	0.29±0.14/ 0.36±0.17
Exact *P*	***	**	ns	*	ns
Genetic differentiation					
F_{ST} across loci		–0.001		0.07	
P (F_{ST})		ns		***	
Exact *P* (differentiation test)		ns		*	
χ^2		1.84		9.19	
df		2.00		2.00	
P (χ^2)		ns		**	
P (Fisher)		ns		*	
MHC II-DRB (Locus C)					
Allele frequencies					
*AcjuFLA-DRB1*ha14*	0.48	0.49	0.43	0.47	0.50
*AcjuFLA-DRB1*ha15*	0.52	0.51	0.57	0.53	0.50
Hardy-Weinberg equilibrium					
H_O/H_E	0.57/0.50	0.63/0.50	0.38/0.50	0.55/0.50	0.61/0.51
Exact *P*	ns	ns	ns	ns	ns
Genetic differentiation					
F_{ST}		–0.01		–0.01	
P (F_{ST})		ns		ns	
Exact *P* (differentiation test)		ns		ns	
P (Fisher)		ns		ns	

*P≤0.05, **P≤0.01, ***P≤0.001, ns not significant.

Discussion

Free-ranging Namibian cheetahs from the north-central and east-central regions were highly differentiated at MHC I loci whereas no difference was observed at MHC II-DRB locus (Table 2). This is true despite no difference in the number of alleles between the two regions. One might argue that the MHC I structure observed in the population is an artifact of the assignment of individual cheetahs to the regions compared. To reduce this possibility we tested the alternative hypothesis of heterogeneity by excluding samples originated from contact areas between northern and central regions (Fig. 1). Results from these tests indicated that genetic differentiation between north-central and east-central Namibian cheetahs still existed although a bit weaker (F_{ST} = 0.04; P = 0.03, Fisher's exact test: P= 0.05, chi-square test: χ^2 = 5.95, df = 2, P = 0.05). We also examined the influence of male-biased sampling on the patterns of MHC variation observed in the population. No detectable effect on the allelic distribution at any MHC loci between males and females was observed. This is in line with both sexes being similarly exposed to most viruses of concern for free-ranging cheetahs (Munson et al. 2004). Because of this, we discarded that the sex-biased sampling towards males in this study may temper any conclusions.

Demographic processes are predicted to affect all loci, whereas selective processes are not expected to influence neutrally evolving loci. Therefore, the effects of variation in population size and other historical processes become visible by comparing different types of molecular markers (Piertney and Oliver 2006). To control for the confounding effects of demographic processes on patterns of MHC variation in the Namibian cheetah population, we compared the extent of genetic differentiation at both MHC classes with the one estimated for neutral microsatellites by Marker et al. (2008). The latter study revealed 248 alleles in 38 microsatellite loci from 89 unrelated free-ranging Namibian cheetahs originated from the same regions as in the present study. A lack of genetic differentiation (mean F_{ST} = 0.02, P > 0.05) among regions indicated that free-ranging Namibian cheetahs represent a large panmictic population (Marker et al. 2008). According to this, the patterns of genetic differentiation at adaptive MHC I and neutral microsatellites differed markedly in the Namibian cheetah population. The more pronounced genetic differentiation at the MHC loci than at neutral makers indicates that selective factors (e.g. pathogens) are of greater magnitude than non-selective factors (e.g. genetic drift, gene flow) (Garrigan & Hedrick 2003). Therefore, the high genetic differentiation observed in MHC I but not in microsatellites between Namibian cheetahs from north-central and east-central regions is likely to be explained by differences in the selective pressure driven by viral pathogens rather

than geographic barriers affecting gene flow. It is difficult, however, to draw conclusions based solely on empirical comparisons between MHC genes and microsatellites because the mutational processes and selective regimes of these genetic markers are different (Hedrick 2001). Thus, conclusions from this study should be interpreted as preliminary. Nevertheless, significant differences in the seroprevalences to viral pathogens in Namibian cheetahs from north-central (where more and larger human settlements exist and a higher human and therefore domestic carnivore density on farmland can be expected) compared to individuals from east-central Namibia (Malan 1995; Munson en et al. 2004; Thalwitzer et al. 2010) are consistent with our results. In line with this argument, the contrasting levels of genetic variation between MHC I and MHC II-DRB loci may further suggest that intracellular-derived pathogens such as viruses exert a stronger selection pressure than extracellular-derived pathogens such as bacteria, protozoa and arthropods in cheetahs across Namibian farmland. However, data on these type of pathogens such as diversity, abundance and distribution is essential to understand the role of selection on MHC II-DRB variation of the Namibian cheetah population. To understand the role of selection on MHC II-DRB variation of the Namibian cheetah population data on extracellular-derived pathogens an their diversity, abundance and distribution is essential.

Conservation implications

Fitness-related genes of adaptive significance such as those of the MHC are crucial for the long-term conservation of a species in the wild, and therefore of primary interest in conservation genetics (Edwards and Potts 1996; Hedrick 2001). Conservation plans based solely on neutral genetic variation may not equally preserve adaptive genetic variation because both sources of genetic variation are not always positively correlated. Gene flow within a population might result in low differentiation in neutral markers, but there might still be high differentiation using adaptive markers in the population (Hedrick 2001). The Namibian cheetah population represents a large panmictic population as revealed by neutral microsatellite markers (Marker et al. 2008), however, our results show a high differentiation in adaptive MHC I loci between cheetahs from east-central and north-central regions. Translocation of cheetahs within Namibia is an increasingly used management tool for conservation purposes that should be conducted carefully as it may affect the ability of translocated animals to cope with different pathogenic selective pressures. Therefore, further research should focus on MHC composition in relation to pathogen load in cheetahs from different African populations.

Acknowledgements

We thank the Ministry of Environment and Tourism in Namibia for permission to conduct the study, A Krengel for collecting samples, the Seeis and Hochfeld conservancies, and the AfriCat Foundation for their continuous cooperation. N Balkenhol, J Schad and P Santos provided helpful comments on an earlier version of the manuscript. This work was supported by the Messerli Foundation in Switzerland, the Leibniz Institute for Zoo and Wildlife Research (IZW) in Germany, the Secretariat of Public Education (SEP) and the Mexican Government.

References

Alcaide, M., Lemus, J.A., Blanco, G., Tella, J.L., Serrano, D., Negro, J.J., Rodríguez, A. & García-Montijano, M. (2010). MHC diversity and differential exposure to pathogens in kestrels (Aves, Falconidae). *Mol. Ecol.* **19**, 691-705.

Alexander, K.A., McNutt, J.W., Briggs, M.B., Standers, P.E., Funston, P., Hemson, G., Keet, D. & van Vuuren, M. (2010). Multi-host pathogens and carnivore management in southern Africa. *Comp. Immun. Microbiol. Infect. Dis.* **33**, 249-265.

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, P.J., Saper, M.A., Samraoui, B., Bennett, W.S, Strominger, J.L. & Wiley, D.C. (1987). The foreign antigen-binding site and T-Cell recognition regions of class-I histocompatibility antigens. *Nature* **329**, 512-518.

Bjorkman, P.J. & Parham, P. (1990). Structure, function, and diversity of class-I major histocompatibility complex-molecules. *Annu. Rev. Biochem.* **59**, 253-288.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. & Wiley, D.C. (1993). Three-Dimensional structure of the human class-II histocompatibility antigen HLA-DR1. *Nature* **364**, 33-39.

Castro-Prieto, A., Wachter, B. & Sommer, S. (2011). Cheetah paradigm revisited: MHC diversity in the world's largest free-ranging population. *Mol. Biol. Evol.* **28**, 1455-1468.

Charruau, P., Fernandes, C., Orozco-$_{TER}$Wengel, P., Peters, J., Hunter, L., Ziaie, H., Jourabchian, A., Jowkar, H., Schaller, G., Ostrowski, S., Vercammen, P., Grange, T., Schlötterer., Kotze, A., Geigl, E.M., Walzer, C. & Burger, P.A. (2011). Phylogeography, genetic structure and population divergence time of cheetahs in Africa and Asia, evidence for long-term isolates. *Mol. Ecol.* **4**, 706-724.

Drake, G.J.C., Kennedy, L.J., Auty, H.K., Ryvar, R., Ollier, W.E.R., Kitchener, A.C., Freeman, A.R. & Radford, A.D. (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.

Edwards, S. & Potts, W. (1996). Polymorphism of genes in the major histocompatibility complex, implications for conservation genetics of vertebrates. In *Molecular Genetic Approaches in Conservation*: 214–237. Smith, T. & Wayne, R., (Ed.). New York and Oxford: Oxford University Press.

Ejsmond, M.J. & Radwan, J. (2009). Mhc diversity in bottlenecked populations, a simulation model. *Conserv. Genet.* **1**, 129-137.

Excoffier, L., Laval, L.G. & Schneider, S. (2005). Arlequin ver. 3.0, An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47-50.

Garrigan, D. & Hedrick, P.W. (2003). Perspective, Detecting adaptive molecular polymorphism, Lessons from the MHC. *Evolution* **57**, 1707-1722.

Guo, S. & Thompson, E. (1992). Performing the exact test of Hardy-Weinberg proportion fro multiple alleles. *Biometrics* **48**, 361-372.

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, P.W. (1994). Evolutionary Genetics of the Major Histocompatibility Complex. *Am. Nat.* **143**, 945-964.

Hedrick, P.W. (2001). Conservation genetics, where are we now? *TREE* **16**, 629-636.

Hedrick, P.W. (2002). Pathogen resistance and genetic variation at MHC loci. *Evolution* **56**, 1902-1908.

Hedrick, P.W., Parker, K.M., Gutierrez-Espeleta, G.A., Rattink, A. & Lievers, K. (2000). Major histocompatibility complex variation in the Arabian oryx. *Evolution* **54**, 2145–2151.

Hedrick, P. (2003). The major histocompatibility complex (MHC) in declining populations, an example of adaptive variation. In *Reproduction science and integrated conservation*: 97–113. Holt, W.V., Pickard, A.R., Rodger, J.C. & Wildt, D.E. (Eds). Cambridge: Cambridge University Press.

Klein, J. (1986). Natural history of the major histocompatibility complex. Wiley, New York.

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3, Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinfor.* **5**, 150-163.

Malan, J.S. (1995). Peoples of Namibia. Department of Anthropology, University of the North, Rhino Publisher, Pretoria, South Africa.

Marker-Kraus, L. & Kraus, D. (1995). The Namibian free-ranging cheetah. *Environ. Conserv.* **21**, 369-370.

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

Marker, L.L., Dickman, A.J., Jeo, R.M., Mills, M.G.L. & Macdonald, D.W. (2003) Demography of the Namibian cheetah, *Acinonyx jubatus jubatus*. *Biol. Conserv*. **114**, 413-425.

Marker, L.L., Wilkerson, A.J.P., Sarno, R.J., Martenson, J., Breitenmoser-Würsten, C., O'Brien, S.J. & Warren, E.J. (2008). Molecular genetic insights on cheetah (*Acinonyx jubatus*) ecology and conservation in Namibia. *J. Hered.* **99**, 2-13.

Mazet, J.A.K., Clifford, D.L., Coppolillo, P.B., Deolalikar, A.B., Erickson, J.D. & Kazwala, R.R. (2009). A "One Health" approach to address emerging zoonoses, The HALI Project in Tanzania. *PLoS Med.* **6**(12): e1000190. doi:10.1371/journal.pmed.1000190.

Mendelsohn, J., Jarvis, A., Roberts, C. & Robertson, T. (2009). Atlas of Namibia, a portrait of the land and its people. Cape Town, South Africa, Sunbird Publishers.

Munson, L., Marker, L., Dubovi, E., Spencer, J.A., Evermann, J.F. & O'Brien, S.J. (2004). Serosurvey of viral infections in free-ranging Namibian cheetahs (*Acinonyx jubatus*). *J. Wildl. Dis.* **40**, 23-31.

Munson, L., Terio, K.A., Ryser-Degiorgis, M.P., Lane, E.P., Courchamp & F. (2010). Wild felid diseases: conservation implications and management strategies. In: *Biology and Conservation of wild felids*: 237-259. Macdonlad, D.W. & Loveridge, .A.J. (eds). Great Britain: Oxford University Press.

O'Brien, S.J., Wildt, D.E., Goldman, D., Merril, C.R. & Bush, M. (1983). The cheetah is depauperate in genetic variation. *Science* **221**, 459-462.

O'Brien, S.J., Roelke, M.E., Marker, L., Newman, A., Winkler, C.A., Meltzer, D., Colly, L., Evermann, J.F., Bush, M. &Wildt, D.E. (1985). Genetic basis for species vulnerability in the cheetah. *Science* **227**, 1428-1434.

Piertney, S.B. & Oliver, M.K. (2006). The evolutionary ecology of the major histocompatibility complex. *Heredity* **96**, 7-21

Raymond, M. & Rousset, F. (1995). An exact test for population differentiation. *Evolution* **49**, 1280-1283.

Roelke-Parker, M.E., Munson, L., Packer, C., Kock, R., Cleaveland, S., Carpenter, M., O'Brien, S.J., Popischil, A., Hofmann-Lehmann, R., Lutz, H., Mwamengele, G.L, Mgasa, M.N., Machange, G.A., Summer, B.A. & Appel, M.J. (1996). A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). *Nature* **379**, 441-445.

Ryman, N. (2006). CHIFISH, a computer program testing for genetic heterogeneity at multiple loci using chi-square and Fisher's exact test. *Mol Ecol Notes* **6**, 285-287.

Ryman, N., Jorde, P.E. (2001). Statistical power when testing for genetic heterogeneity. *Mol. Ecol.* **10**, 2361-2373.

Schneider, H.P. (1994). Animal health and veterinary medicine in Namibia. Windhoek, Namibia: AGRIVET.

Seddon, J.M. & Baverstock, P.R. (1999). Variation on islands, major histocompatibility complex (Mhc) polymorphism in populations of the Australian bush rat. *Mol. Ecol.* **8**, 1971-2179.

Smith,K.F., Acevedo-Whitehouse, K. & Pedersen, A.B. (2009). The role of infectious diseases in biological conservation. *Anim. Conserv.* **12**, 1-12.

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

Spurgin, L.G. & Richardson, D.S. (2010). How pathogens drive genetic diversity, MHC, mechanisms and misunderstandings. *Proc. R. Soc. B* **277**, 979-988.

Thalwitzer, S., Wachter, B., Robert, N., Wibbelt, G., Muller, T., Lonzer, J., Meli, M.L., 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.

Wachter, B., Thalwitzer, S., Hofer, H., Lonzer, J., Hildebrandt, T.B. & Hermes, R. (2011). Reproductive history and absence of predators are important determinants of reproductive fitness: the cheetah controversy revisited. *Conserv. Lett.* **4**, 47-54.

Wright, S. (1965). The interpretation of population structure by F-statistics with special regard to systems of mating. *Evolution* **19**, 395-420.

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

Article 3

This is a pre-copy-editing, author-produced PDF of an article accepted for publication in *Journal of Heredity* following peer review. The definitive publisher-authenticated version [Diversity and evolutionary patterns of immune genes in free-ranging Namibian leopards (*Panthera pardus pardus*). Aines Castro-Prieto, Bettina Wachter, Joerg Melzheimer, Susanne Thalwitzer and Simone Sommer. *Journal of Heredity* (2011) 102(6):653–665] is available online at:
[http://jhered.oxfordjournals.org/content/102/6/653.full.pdf?keytype=ref&ijkey=5lKzKU1bgzFTngk].

Diversity and evolutionary patterns of immune genes in free-ranging Namibian leopards (*Panthera pardus pardus*)

Research Article

AINES CASTRO-PRIETO, BETTINA WACHTER, JOERG MELZHEIMER, SUSANNE THALWITZER and SIMONE SOMMER

Journal of Heredity (2011), 102(6):653–665.

Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany.

Key words: MHC class I; MHC class II; African leopard; *Panthera pardus*; Positive selection

Corresponding author: Simone Sommer, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, D-10315 Berlin, Germany, Telephone: 0049-30-5168-315, Fax: 0049-30-5126-104, sommer@izw-berlin.de

Running title: MHC diversity in African leopards

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 six MHC class I and six MHC class II-DRB sequences which likely correspond to at least three MHC class I and three 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.

Introduction

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 south-east 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 categorised as 'near threatened' or 'critically endangered' according to the IUCN Red List of Threatened Species (Henschel et al. 2008). The African leopard (*P. p. pardus*) is the most common of nine 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 5,000-10,500 individuals (Hanssen and Stander 2004). They are widely distributed but mainly concentrated in the northern and central parts of the country (Mendelsohn et al. 2002; 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 (*Panthera 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 life style (Stander et al. 1997; Bailey 1993). However, a solitary life style 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 non-vaccinated domestic dogs (*Canis familiaris*) can emerge in highly virulent forms resulting in major epizootics (Roelke-Parker-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 one individual, Wang et al. 2008; sequence analysis of clones of seven 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 (Fig. 1). All leopard samples were collected from wild-born free-ranging individuals. The relatedness of the individuals was not known except for one female with her two 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 eight 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.

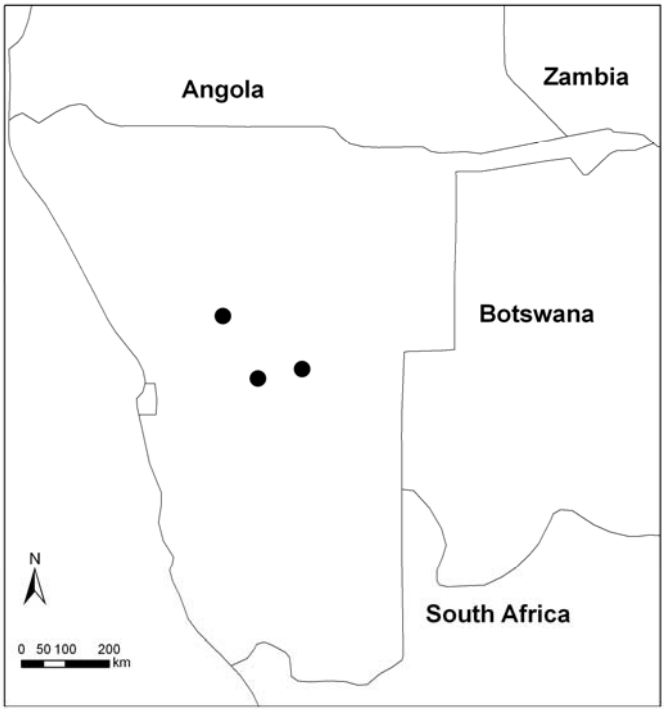


Fig. 1 Schematic map showing the origin (dots) of the African leopard samples in Namibia.

PCR and SSCP 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), i.e. amino acids positions postulated to interact directly with foreign peptides. The second and third exons of MHC I genes 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 *Acinonyx jubatus*, ocelot *Leopardus pardalis*, Asiatic lion *P. l. persica*, Bengal tiger *Panthera 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′-GCTCCCACTCCCTGAGGTAT-3′; Castro-Prieto et al. 2011) and Papa_Ex2MhcI_kR (5′-GGAKTCGCTCTGGTTGTAGT-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 AJDRBaIn1Ex2_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 dNTP mix,

2.5 μl buffer 10× and 0.5 U Taq polymerase (MP Biomedicals, Irvine, CA, USA). 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 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 two 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 two-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 two different approaches. First, we calculated by pairwise comparison the relative rates of non-synonymous (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 two-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 two negative log-likelihoods: D = 2[Lb – La]) was compared to the χ^2–distribution with the degrees of freedom 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 Naïve 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 1999). 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 (10,0000 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 outgroups 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 (AIC) 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 transvertional substitution with gamma shape distribution (TVM + Γ, $\alpha = 0.46$) for MHC I sequences and the general time reversible 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 zmodel 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 $5x10^6$ generations with a random starting

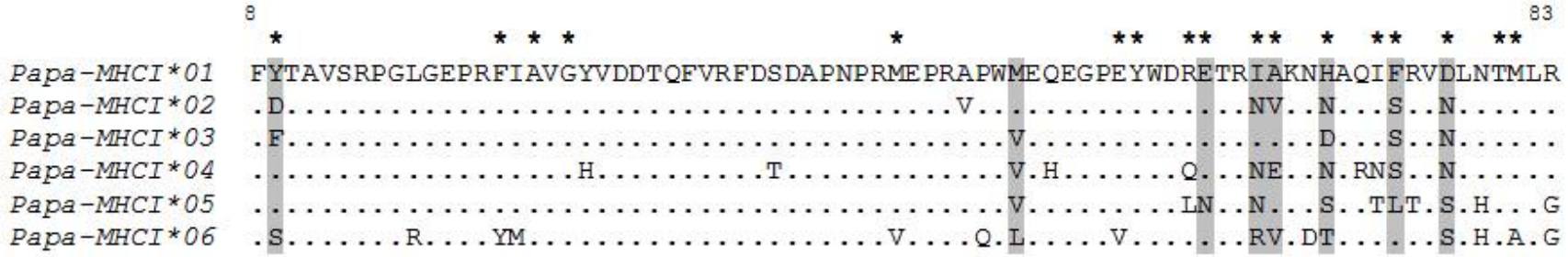
tree and two runs of four heated and one cold Markov chains (heating = 0.20) sampled every 1000 generations. Burn-in corresponded to the first 20% of sampled trees based on the average standard deviation of split frequencies as well as by plotting the likelihood scores against generation time. The presence 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 six 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 six haplotypes (Table 1a). Between two and six sequences were detected per individual (Table 1a), indicating that our primers amplified at least three 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 one (0.4%) of the 25 sampled individuals.

(a) *Papa-MHC class I* Alpha 1 domain



(b) *Papa-MHC class II DRB* Beta 1 domain

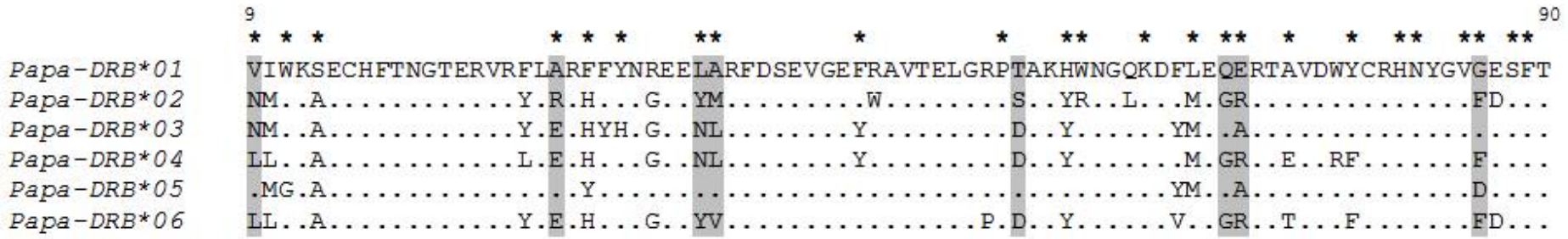


Fig. 2 Deduced amino acid alignment of (a) MHC I and (b) MHC II-DRB sequences detected in 25 free-ranging African leopards. Dots indicate identity to the top sequence. Putative antigen binding sites (indicated with asterisks) in the alpha 1 (α1) and beta 1 (β1) domains were inferred from human MHC I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

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

(a)

MHC haplotypes	No. of sequences	No. of individuals	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 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 (position 8 to 83; Fig. 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 (Fig. 2a). The mean number of pairwise nucleotide differences between pairs of sequences was 19.73 ± 2.56 ranging from seven (*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 five (*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).

Table 2 Overall mean genetic distances (± standard errors) of six MHC I and six MHC II-DRB nucleotide and amino acid sequences detected in 25 African leopards. Distances are presented for all sites as well as only for the putative antigen binding sites (ABS) and non-ABS

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

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 seven 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 upon 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 the null model M7 (neutral selection) (P = 0.02; 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 two 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 1993) (Fig. 2a).

Table 3 The average rates of non-synonymous substitutions (d_N) and synonymous substitutions (d_S) with standard errors and their ratio in antigen binding sites (ABS) and non-ABS assuming concordance with the human MHC I (Bjorkman and Parham 1993) 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

[a]Number of codons in each category

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

GENECONV detected two 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 to 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 to 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 canid outgroup (grey wolf *Canis lupus*) sequences (Fig. 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 PaleAY909873; 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; Fig. 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 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).

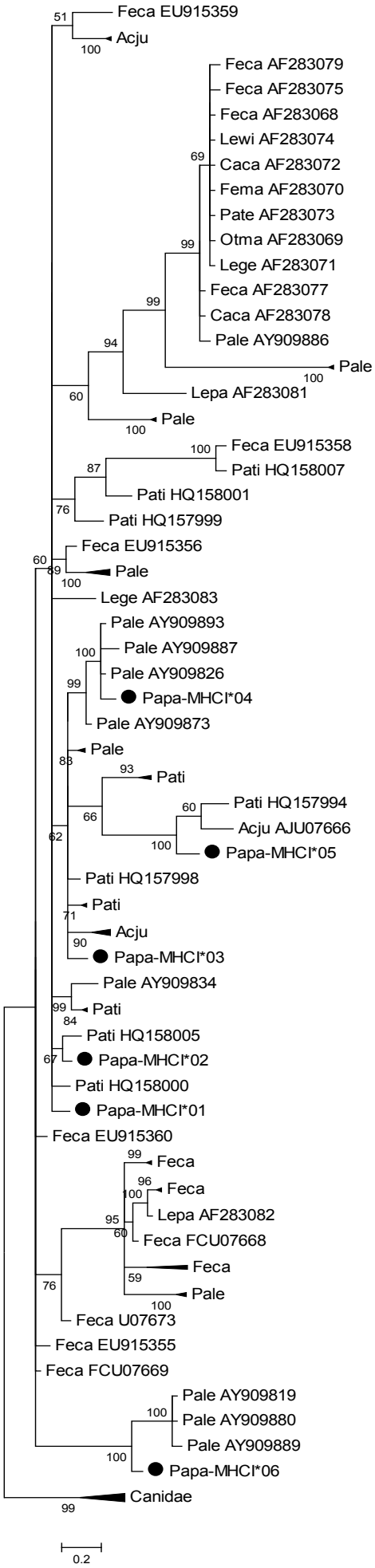


Fig. 3 Phylogenetic relationships of the African leopard (*Papa: Panthera pardus pardus*) MHC I exon 2 sequences (indicated by circles) with a representative set of other felid sequences (*Acju: Acinonyx jubatus, Caca: Caracal caracal, Feca: Felis catus, Fema: Felis margarita, Lege: Leopardus geoffroyi,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

MHC II-DRB

African leopards also showed a total of six distinct MHC II-DRB exon 2 nucleotide sequences (GenBank accession numbers HQ318099-104). Sequence *Papa-DRB*02* was previously observed in two 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 ten haplotypes (Table 1b). Between two and five sequences were observed in a single individual (Table 1b), indicating that our primers amplified at least three 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 (position 9 to 90; Fig. 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 (Fig. 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 eight (*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 six 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 upon 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 significantly better than the null models M1a and M7 (neutral selection) ($P < 0.001$; 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) (Fig. 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 to 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 outgroup (domestic dog, African wild dog, grey wolf, coyote *Canis 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 (Fig. 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 (Fig. 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* 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 (Fig. 5). Nonetheless, as the three sequences Papa DQ189262-64 were isolated from a single individual, they belong to two different loci rather than one locus (Wang et al. 2008).

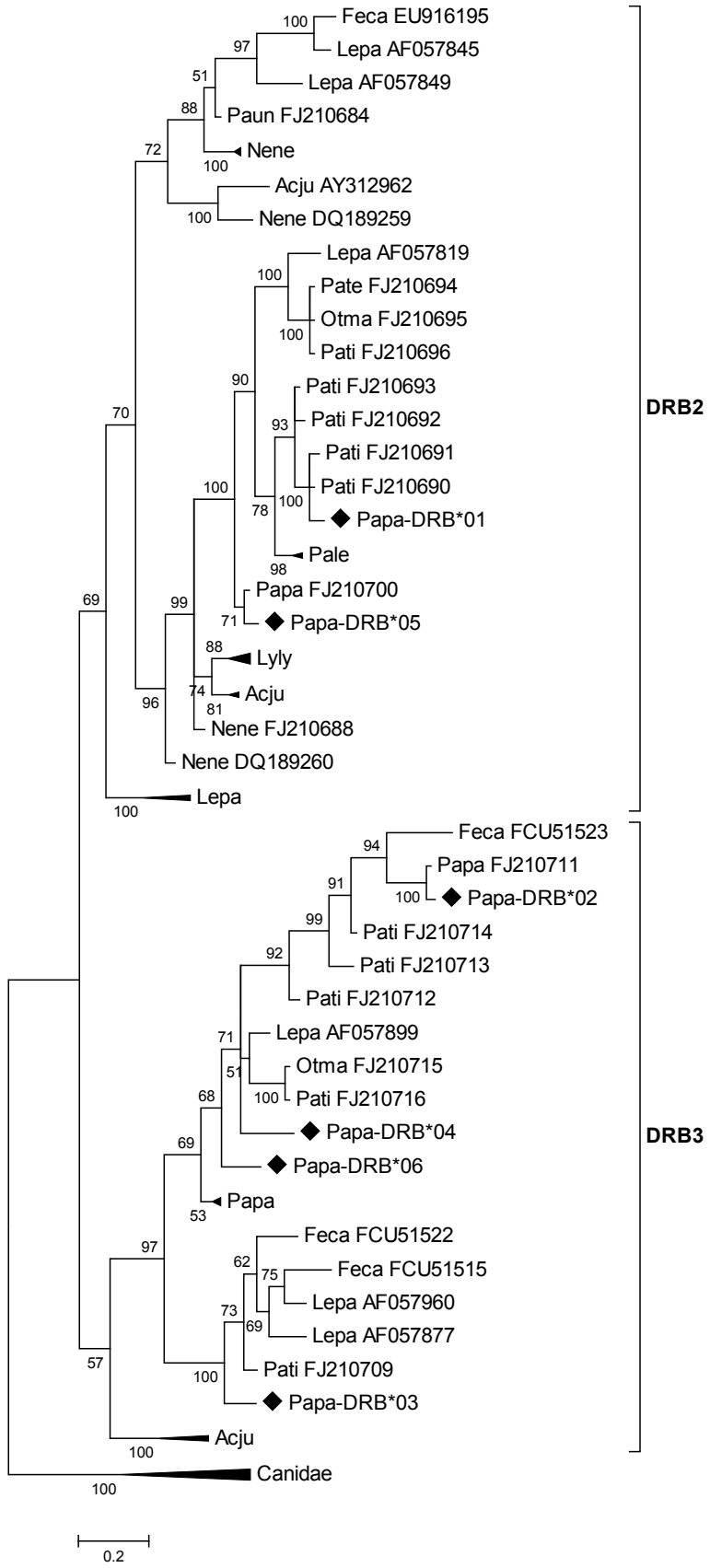


Fig. 4 Phylogenetic relationships of the African leopard (*Papa: Panthera pardus pardus*) MHC II-DRB exon 2 sequences (indicated by diamonds) with a representative set of other felid sequences (*Acju: Acinonyx jubatus, Feca: Felis catus, Lepa: Leopardus pardalis, Lyly: Lynx lynx, Nene: Neofilis nebulosa, Otma: Otocolobus manul, Pale: Panthera leo, Pati: Panthera tigris, Paun: Panthera uncia, Pate: Pardofelis temminckii*) followed by their corresponding Genbank accession numbers. We used canid sequences (*Cafa: canis familiaris, Cala: Canis latrans, Calu: canis lupus, Lypi: Lycaon pictus*) as outgroup to root the tree. DRB2 and DRB3 label two out of five 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.

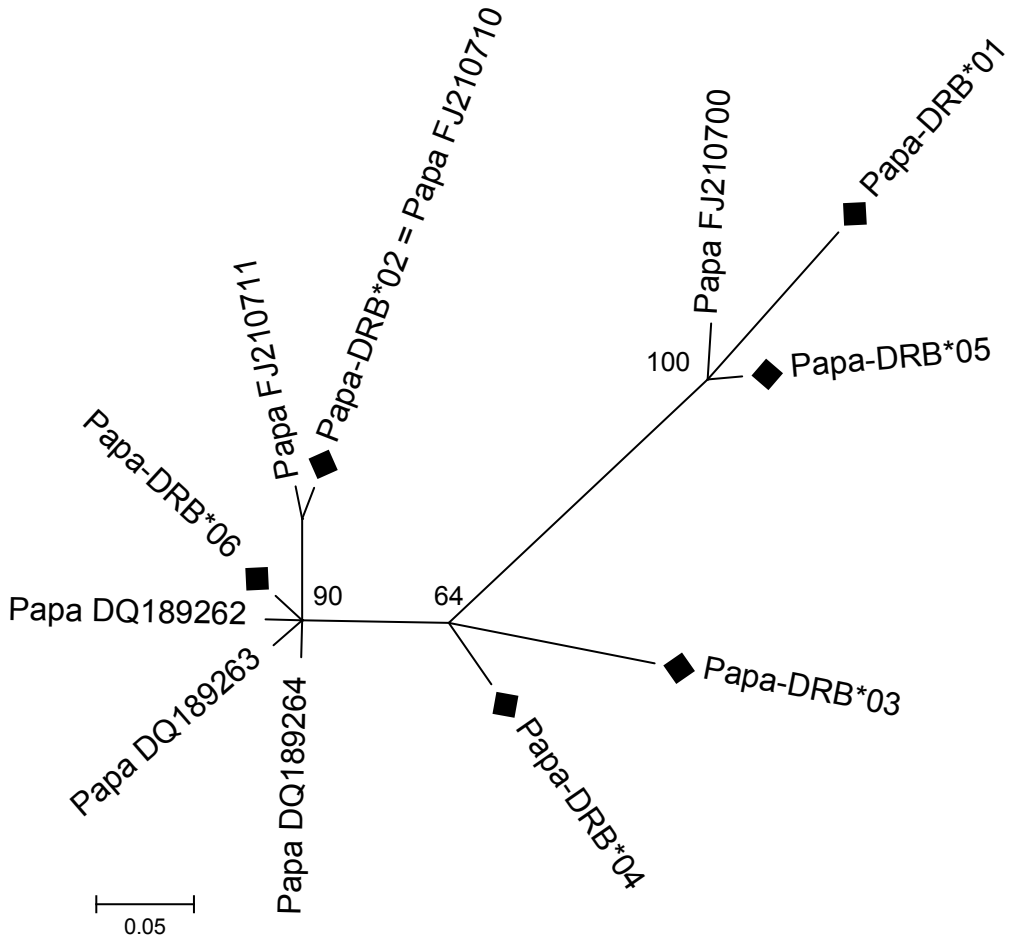


Fig. 5 Unrooted phylogenetic tree including only third codon positions of the six MHC II-DRB exon 2 sequences detected in African leopards (indicated by diamonds, this study) and six Asian leopard sequences (Wang et al. 2008; Wei et al. 2010). Sequence *Papa-DRB*02* was observed in both subspecies. The 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.

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 six sequences from at least three 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 four MHC I loci and four sequences from at least two 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 five 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 artefacts 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 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 three 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), while 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), while 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 six and five sequences, respectively, in a single leopard individual (Table 1a and 1b) indicating the occurrence of at least three gene copies in the species. This is consistent with three 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 2009). Most MHC-related studies in non-model 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 discuss 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 non-synonymous 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 non-synonymous 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 favoured 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 eight 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, 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 six sequences isolated are highly divergent (12%; Table 2) and correspond to at least three 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 non-classical MHC genes (i.e. limited polymorphism and non-ubiquitously expressed)

or even pseudogenes (i.e. monomorphic and non-expressed thus, non-functional) 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 and O'Brien 1997; Yuhki et al. 1989).

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 two independent amplifications per individual sample. The occurrence of common sequence motifs between MHC I sequences of African leopards and other divergent felid species (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 MHCII-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 two major clusters that were not species or lineage specific (Fig. 4). These clusters were consistent with two (DRB2 and DRB3) of five 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 among these two 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 MYA (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 eleven individuals with at least three of the four 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 (Fig. 3 and Fig. 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 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 2000) 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 four putative DRB loci in African and Asian leopards but only three of those loci are likely to be shared between both subspecies (Fig 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 MYA) migrations from Asian-derived Panthera species towards 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 analysed 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*, *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 four and one amino acid, respectively, from FJ21700, and all three 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 suggest 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.

Funding

This work was supported by the Messerli Foundation, Switzerland; the Leibniz Institute for Zoo and Wildlife Research (IZW), Germany; Secretariat of Public Education (SEP) and the Mexican Government.

Acknowledgments

We would like to thank the Ministry of Environment and Tourism in Namibia for permission to conduct the study; J Lonzer, N de Woronin, U Tubessing, A von Hacht, K Killian, J Vaatz, and Trophäendienste CC for collecting the samples; the Seeis and Hochfeld conservancies in Namibia for their continuous cooperation; A Schmidt for technical laboratory assistance; N Balkenhol for data analysis assistance; J Axtner, N Schwensow and C Mazzoni, four anonymous reviewers and the corresponding editor W. Johnson for helpful comments and suggestions on the manuscript.

References

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

Anisimova M, Nielsen R, Yang Z. 2003. Effect of recombination on the accuracy of the likelihood method for detecting positive selection at amino acid sites. Genetics. 164:1229–1236.

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

Babik W, Taberlet P, Ejsmond MJ, Radwan J. 2009. New generation sequences as a tool for genotyping of highly polymorphic multilocus MHC system. Mol Ecol Resour. 9:713–719.

Bailey TN. 1993. The African leopard. Ecology and behaviour of a solitary felid. Columbia University Press, New York.

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, 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.

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

Bollmer JL, Dunn PO, Whittingham LA, Wimpee C. 2010. Extensive MHC class II B gene duplication in a Passarine, the common yellowthroat (*Geothlypis trichas*). J Hered. 4:448–460.

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.

Carpenter MA, Appel MJ, Roelke-Parker ME, Munson L, Hofer H, East M, O'Brien SJ. 1998. Genetic characterization of canine distemper virus in Serengeti carnivores. Vet Immunol Immunopathol. 65:259–266.

Castro-Prieto A, Wachter B, Sommer S. 2010. Cheetah paradigm revisited: MHC diversity in the largest free-ranging population. Mol Biol Evol. 28:1455–1468.

Doherty PC, Zinkernagel RM. 1975. Biological Role for Major Histocompatibility Antigens. Lancet. 1:1406–1409.

Edwards SV, Chesnut K, Satta Y, Wakeland EK. 1995. Ancestral polymorphism of Mhc class II genes in mice: implications for balancing selection and mammalian molecular clock. Genetics. 146:665–668.

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

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. 2001. Conservation genetics: where are we now? Trends Ecol Evol. 16:629–636.

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

Hedrick PW, Kim T. 2000. Genetics of complex polymorphisms: parasites and maintenance of MHC variation. In: Singh R, Krimbas C (eds). Evolutionary Genetics: From Molecules to Morphology. (Cambridge) United Kingdom: Cambridge University Press. p. 204–234.

Henschel P, Hunter L, Breitenmoser U, et al. 2008. *Panthera pardus*. In: IUCN 2010. IUCN Red List of Threatened Species. Version 2010.4. <www.iucnredlist.org>. Downloaded on 04 April 2011.

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

Hughes, A.L. 1991. MHC polymorphism and the design of captive breeding programs. Conserv Biol. 5: 249–251.

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 overdominant selection. Proc Natl Acad Sci USA. 86:958–962.

Johnson WE, Eizirik E, Pecon-Slattery, Murphy J, Antunes WJ, Teeling E, O'Brien SJ. 2006. The late Miocene radiation of modern Felidae: a genetic assessment. Science. 311:73–77.

Jukes TH, Cantor CR. 1969. Evolution of protein molecules. In: Munro HN (ed) Mammalian Protein Metabolism. Academic Press, New York, pp 21–132.

Kat PW, Alexander KA, Smith JS, Munson L. 1995. Rabies and African wild dogs in Kenya. Proc Roy Soc Lond ser B. 262:229–233.

Kaufman J, Salomonsen J, Flajnik M. 1994. Evolutionary conservation of MHC class I and class II molecules–Different yet the same. Semin Immunol 6:411–424.

Klein J. 1986. Natural history of the major histocompatibility complex. Wiley, New York.

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'hUigiín. 1998. Molecular trans-species polymorphism. Annu Rev Ecol Syst. 29:1–21.

Kumanovics A, Takada T, Lindahl KF. 2003. Genomic organization of the mammalian MHC. Annu Rev Immunol. 21:629–657.

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

Macdonald DW, Loveridge AJ, Nowell K. 2010. *Dramatis personae*: an introduction to the wild felids In: Macdonald DW and Loveridge AJ (eds). Biology and Conservation of wild felids. Oxford (Great Britain): Oxford University Press. p. 3–58.

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

Mendelsohn J, Jarvis A, Roberts C, Robertson T. 2009. Atlas of Namibia, a portrait of the land and its people. Cape Town (South Africa): Sunbird Publishers.

Meyer-Lucht Y, Otten C, Püttker T, Sommer S. 2008. Selection, diversity and evolutionary patterns of the MHC class II DAB in free-ranging Neotropical marsupials. BMC Genetics. 9:39.

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.

Miller HC, Andrews-Cookson M, Daugherty CH. 2007. Two patterns of variation among MHC class I loci in Tuatara (*Sphenodon punctatus*). J Hered. 98:666–677.

Miththapala S, Seidensticker J, O'Brien SJ. 1996. Phylogeographic subspecies recognition in leopards (*Panthera pardus*): molecular genetic variation. Conserv Biol. 10:1115–1132.

Munson L, Terio KA, Ryser-Degiorgis MP, Lane EP, Courchamp F. 2010. Wild felid diseases: conservation implications and management strategies. In: Macdonald DW and Loveridge AJ (ed) Biology and conservation of wild felids. Oxford University press, New York, pp 238–259.

Murray DL, Kapke CA, Evermann JF, Fuller TK. 1999. Infectious disease and the conservation of free-ranging large carnivores. Animal Conserv. 2:241–254.

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

Nei M. 2005. Selectionism and neutralism in molecular evolution. Mol Biol Evol. 22:2318–2342.

Nielsen R, Yang Z. 1998. Likelihood models for detecting positively selected amino acid sites and applications to the HIV-1 envelope gene. Genetics. 148:929–936.

Nowell K, Jackson P. 1995. New red list categories for wilds cats. Cat News. 23:21–27.

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

O'hUigiín C. 1995. Quantifying the degree of convergence in primate Mhc-DRB genes. Immunol Rev. 143:123–140.

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 USA. 86:2766–2770.

Pedersen AB, Jones KE, Nunn CL, Altizer S. 2007. Infectious Diseases and Extinction Risk in Wild mammals. Conserv Biol. 21:1269–1279.

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. 62:667–79.

Posada D. 2002. Evaluation of methods for detecting recombination from DNA sequences: empirical data. Mol Biol Evol. 19:708–719.

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

14:817–818.

Promerová M, Albrecht T, Bryja. 2009. Extremely high MHC class I variation in a population of a long-distance migrant, the scarlet rosefinch (*Carpodacus erythrinus*). Immunogenetics. 61:451–461.

Radwan J, Biedrzycka A, Babik W. 2009. Does reduced MHC diversity decrease viability of vertebrate populations? Biol Conserv. 143:537–544.

Reed DH. 2010. Albatrosses, eagles and newts, Oh My!: exceptions to the prevailing paradigm concerning genetic diversity and population viability? Anim Conserv. 13:448–457.

Richman AD, Gerardo-Herrera L, Nash D. 2003. Evolution of MHC class II E beta diversity within the genus Peromyscus. Genetics. 164:289–297.

Roelke-Parker ME, Munson L, Packer L et al. 1996. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). Nature. 379:441–445.

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.

Sawyer SA. 1989. Statistical tests for detecting gene conversion. Mol Biol Evol. 6:526–538.

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 USA 104:16221–16226.

Smith MM, Hoffman SMG. 2001. A class I MHC locus compared among felid species. Mamm Genome. 12:394–396.

Smith KF, Acevedo-Whitehouse K, Pedersen AB. 2009. The role of infectious diseases in biological conservation. Anim Conserv. 12:1–12.

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.

Spong G, Johansson M, Björklund M. 2000. High genetic variation in leopards indicates large and long-term stable effective population size. Mol Ecol. 9:1773–1782.

Stander PE, Haden PJ, Kaqece A, Ghau A. 1997. The ecology of asociality in Namibian leopards. J Zool. 242:343–364.

Sunnuks P, Wilson ACC, Beheregary LB, Zenger K, French J, Taylor AC. 2002. 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. 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

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.

Uphyrkina O, Johnson WE, Quigley H et al. 2001. Phylogenetics, genome diversity and origin of modern leopard, *Panthera pardus*. Mol Ecol. 10:2617–2633.

Uphyrkina O, Miquelle D, Quigley H, Discroll C, O'Brien SJ. 2002. Conservation genetics of the far Eastern leopard (*Panthera pardus orientalis*). J Hered. 93:303–311.

Wang Q, Wu X, Yan P, Zheng S. 2008. Sequence variability analysis on major histocompatibility complex class II DRB alleles in three felines. Front Biol China. 3:55–62.

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

Wei K, Zhang Z, Wang X et al. 2010 Lineage pattern, trans-species polymorphism, and selection pressure among the major lineages of feline Mhc-DRB peptide-binding region. Immunogenetics. 62:307–317.

Yang, Z. 2007. PAML 4: a program package for phylogenetic analysis by maximum likelihood. Mol Biol Evol. 24: 1586–1591.

Yang Z, Wong WSW, Nielsen R. 2005. Bayes empirical Bayes inference of amino acid sites under positive selection. Mol Biol Evol. 22:1107–1118.

Yeager M and Hughes AL. 1999. Evolution of the mammalian MHC: natural selection, recombination, and convergent evolution. Immunol Rev. 167:45–58.

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, O'Brien SJ. 1990. 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. 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.

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.

Zhang J, Nielsen R, Yang Z. 2005. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol Biol Evol. 22:2472–2479.