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Evolution of Major Histocompatibility Complex genes in New World bats and their functional importance in parasite resistance and life-history decisions in the lesser bulldog bat (*Noctilio albiventris*)

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Für Sarah und Hanna

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Preface

In my doctoral thesis I studied evolution and diversity of functionally important major histocompatibility complex (MHC) genes in New World bats, one of the least studied mammalian order in this regard. Further, I assessed the relevance of immune gene variation in parasite resistance and potential benefits in individual life-history decisions, taking an appropriate bat species, the lesser bulldog bat (*Noctilio albiventris*), as an example. My thesis comprises three article manuscripts, which can be read independently. All three are published in international peer-reviewed scientific journals. Because all articles include co-authors, they are written in the first person plural. Each article chapter contains a declaration of the author's individual inputs to highlight my own independent contributions. A general introduction and discussion combine the ideas of the independent manuscripts and aim to deepen the scientific comprehension in considering aspects not provided in the single articles. At the end perspectives on future work are given which will be or have already been initiated as a result of the thesis in hand. Cited literature of single chapters was in parts redundant; therefore references are combined and presented at the end of the main chapters. Associated electronically online supplemental material of articles is given in the appendix.

General Introduction

Bats (Chiroptera) constitute the second largest mammalian order and exhibit an amazing ecological and evolutionary diversity. They contribute to the maintenance of ecosystem stability as pollinators, seed dispersers and insect predators in all continents except Antarctica and have thus a high relevance in ecosystem services worldwide (Kunz et al. 2011). For many decades, research efforts on bats focused on questions in evolutionary ecology. The past decade highlighted also the importance of bats as potential reservoir hosts of newly emerging disease pathogens such as SARS-like coronarvirus, henipaviruses, Ebola and Marburg viruses, and they have long been known as vectors of rabies. Thereby, pathogens are maintained by bats which show few or no signs of pathology and the transmission of pathogens to humans, domestic livestock and other wildlife seems to be an unidirectional spill-over event (e.g. Calisher et al. 2006; Field 2009). This raises the questions why certain pathogens can infect and persist in apparently healthy bats whereas the same pathogens may cause severe zoonoses in other vertebrates (Dobson 2004, 2005). Dobson and others have argued that bats may have important qualitative and quantitative differences in their initiate and acquired immune responses compared to other mammals (Dobson 2005; Calisher et al. 2006; Wibbelt et al. 2010a). On the other hand, a few pathogens may cause periodic outbreaks of infectious diseases and severe population declines (Calisher et al. 2006; Blehert et al. 2009). One of the recently recognized most fatal pathogenic agents is a fungal disease, called white-nose syndrome (WNS, Blehert et al. 2009). The disease's name derives from a visually apparent white growth of the fungus *Geomyces destructans* on the bats skin predominantly around the muzzles (Gargas et al. 2009). In contrast to North American bats where the fungus causes mass mortality, bats in Europe appear to coexist with *G. destructans* without being seriously affected (Wibbelt et al. 2010b). One possible explanation is that European bats may be immunologically more resistant to *G. destructans* because of having coevolved with the fungus over longer periods of time (Wibbelt et al. 2010b).

Despite being of central relevance for understanding bat-related newly emerging zoonoses in humans and domestic livestock, as well as dealing with the devastating effects of the emerging WNS among North American bats, surprisingly little is known about bat immunology. In recent years increased effort has been done to fill this gap. Several studies suggest that immune responses of bats have similarities with those of other mammals, even though there is evidence that antibody responses in bats may be

both qualitatively and quantitatively different (Baker et al. 2010; Virtue et al. 2011). Studies on bat immunology mainly focused on immunoglobulin (Wellehan et al. 2009; Baker et al. 2010), interferon (Virtue et al. 2011; Zhou et al. 2011) and genes of Toll-like receptors (Cowled et al. 2011), which recognize conserved structures broadly shared by pathogens in the innate immune pathway (Aderem and Ulevitch 2000). But only a few studies focused on genes involved in the acquired immune response (Mayer and Brunner 2007; Richmann et al. 2010).

The functionally most important genes of the adaptive immune system for parasite and pathogen resistance are those of the major histocompatibility complex (MHC). MHC molecules, cell-surface glycoproteins, are responsible for the recognition of specific pathogenic[1] agents and their presentation to T-cells, which subsequently trigger the cell-mediated immune response and the development of the immunological memory. Consistent with their function in recognizing different pathogenic antigens, MHC genes are among the most variable genes in vertebrates (Klein 1986). This polymorphism enables the immune system to recognise a wide range of intracellular (MHC class I) and extracellular (MHC class II) derived antigens (Sommer 2005). The polymorphism in MHC genes is concentrated in exons which code for the antigen binding region. There, certain amino acid residues are responsible for antigen adhesion (Brown et al. 1993; Stern et al. 1994). Characteristically, the encoding nucleotide codons exhibit a higher rate of non-synonymous over synonymous substitutions than other parts of the molecule and are thus subject to diversifying selection (e.g. positive Darwinian selection, Hughes 2007). In addition, variability at the MHC might also be generated by frequent gene duplication (birth) and gene loss (death) ('birth-and-death' model of molecular evolution, e.g. Nei and Hughes 1992; Nei and Rooney 2005) as well as by recombination and gene conversion between alleles[2] of the same or between different loci (e.g. Otha 1999; Reusch and Langefors 2005).

Since the landmark discovery of MHC restricted T-cell recognition by Zinkernagel and Doherty in 1974, research on MHC diversity focused not only on the evolutionary adaptation to infectious diseases, initially mainly in humans but subsequently also in other vertebrates, but also on the behavioural and ecological potential of species to respond to a changing environment. The fundamental role of the MHC in combating immune insults from pathogens implies that the MHC polymorphism is maintained by pathogen-driven selection mechanisms. Thereby, different forms of balancing selection

[1] I use the term 'pathogen' for both microparasites (viruses, fungi, bacteria, protozoa) and macroparasites (helminths, arthropods) which can cause a reduction in host fitness.

[2] For simplicity, I use the term 'allele' also for sequences of duplicated loci, knowing that this term accurately is used only for sequence-variants of the same locus.

have been discussed. The 'heterozygosity advantage hypothesis' presumes that heterozygote individuals are able to respond to a greater range of pathogenic agents than homozygote individuals and, consequently, benefit from increased resistance to pathogens. This higher relative fitness of heterozygote individuals result in the persistence of divergent alleles in the population (Doherty and Zinkernagel 1975; Hughes and Nei 1988). The 'rare allele advantage hypothesis' (also called 'negative-frequency-dependent selection hypothesis') proposes a co-evolutionary arms race between pathogen and host, where specific alleles are associated with certain pathogens and are selected in a cyclical, frequency-dependent manner (Slade and McCallum 1992). In this dynamic process, a new or rare allele may gain a selective advantage over a common frequent allele. This advantageous allele would subsequently increase in frequency. When it becomes more common itself selection will favour novel pathogenic strains not recognized by this allele and selection will favour another new or rare allele in the population (Takahata and Nei 1990). Finally, the 'fluctuating selection hypothesis' proposes that spatial and temporal heterogeneity in the type and abundance of pathogens may maintain diversity at MHC across subpopulations (Hill 1991). Thereby, fluctuation in pathogenic strains is directional rather than cyclical and determined externally by the biotic or abiotic environment or by chance dispersal and extinction events (Hedrick 2002). These three different forms of balancing selection appear not mutually exclusive and may also operate together with other selective and neutral forces (reviewed in e.g. Spurgin and Richardson 2010).

Besides the obvious fact of pathogen-mediated selection, there is also evidence that sexual selection facilitate the variability observed at MHC loci (Penn and Potts 1999). Several studies have highlighted that reproduction among MHC-dissimilar mates is favoured (Chaix et al. 2008; Schwensow et al. 2008; Ilmonen et al. 2009) presumably to generate a genetically diverse offspring genotype, which is advantageous in the defence against different pathogens (e.g. Parham and Ohta, 1996). Sexual selection processes may not solely act in direct mate choice situations, but also through cryptic pre- and post-copulatory mechanisms including myotic drive, gametic selection and maternal fetal interactions (e.g. Wedekind et al. 1996; Ober and Van der Ven 1997; Fernandez et al. 1999; Ziegler et al. 2005). In all these processes the selective forces might act due to MHC-linked genes, e.g. olfactory receptor genes rather than by the MHC itself (Ho et al. 1990; Gill 1992; Ziegler et al. 2002, 2005; Eisenach and Giojalas, 2006). This is supported by the fact that the MHC is in physical linkage with olfactory receptor genes in most vertebrates (Santos et al. 2010) as well as by experimental

analyses, which showed that the MHC might alter the individual body odour (e.g. Wedekind 1995, Penn and Potts 1998; Milinski 2006; Kwak et al. 2009).

Bats have remarkably diverse and exclusive social and mating systems, where olfactory cues are used to a great extent in social communication (Altringham and Fenton 2003; Dechmann and Safi 2005). Thus, they are interesting study subjects to understand the role of olfaction in mate choice decisions and its associated fitness benefits. In concert, it becomes evident that the study of bat MHC diversity offers the possibility to understand not only the mechanisms underlying the zoonotic potential of bats, but also to shed light on the link between MHC constitution, immunocompetence and aspects of the social and ecological peculiarities (e.g. olfaction-based mate choice) of this amazing mammalian order.

Thesis outline

Study Aim. Due to the zoonotic and ecological potential of bats, the overall aim of my thesis was first to gain general information on the immune gene evolution and diversity in selected bat species in comparison to other mammals. My further intention was then to investigate the functional importance of the individual MHC constitution in parasite resistance and life-history decisions as well as the underlying pathogen-driven and sexual selection pattern.

Study Species. I was fortunate to cooperate with bat ecologists, who are working with natural populations of New World bat species in Central America.

The greater sac-winged bat, ***Saccopteryx bilineata*** Temmick 1838 (Emballonuridae), is a harem-polygynous, 8 g, insectivorous bat (Yancey et al. 1998). Its common name derives from a sac-like organ situated in the antebrachial wing membranes of males and is used for storage of odours involved in social displays like courtship behaviours, mate choice and species recognition. In addition, both sexes scent mark their territory with secretions produced by specialized facial glands. Colonies are subdivided in smaller social units, called harems, each including one to three females which are defended by an adult territorial male. Groups are stable throughout the year, but harem males are not able to monopolize females, rathery they attract and retain females by various visual, acoustic and olfactory displays (Voigt et al. 2008).

Saccopteryx bilineata is known to be parasitized by few bat fly species (Streblidae), by an intestinal tapeworm (*Hymenolepis mazanensis*) and by a fungus (*Histoplasma capsulatum*). So far, individuals examined for rabies were negative for that virus (Yancey et al. 1998).

The short-tailed fruit bat, ***Carollia perspicillata*** Linnaeus 1758 (Phyllostomidae), is a 18 g, mainly fruit- and nectar-eating, also harem-polygynous bat (Cloutier and Thomas 1992). In general colonies are larger than those of *S. bilineata* and are also structured in harems. Harem sites are used by a single territorial male and one to five females. Males defend roosting sites and actively recruit females by hovering and vocalizing, but contrary to *S. bilineata,* without the display of olfactory cues. Females change harems frequently and choice depends presumably on the territorial males quality (Porter 1979), but also roosting site quality has been discussed (Fleming 1988). A large diversity of ectoparasites (mites, ticks, bat flies) and endoparasites (nematodes, trematodes protozoans) infest this species as well as several fungi. Rabies and other virus infections are reported only occasionally (Cloutier and Thomas 1992).

The lesser bulldog bat, ***Noctilio albiventris*** Desmarest 1818 (Noctilionidae) is a 25-30 g, insectivorous bat (Hood and Pitocchelli 1983). *Noctilio albiventris* forms small and stable female groups within their colony roost. Males usually roost together with females but presumably spatially segregated. Oily secretions in the sub-axial region are used for olfactory communication in females and arise when group-members rub their faces under each others arms depositing the secretions of their facial glands there, most probably to stabilize the social affiliation. In addition, males implement specific odours produced by glandular cells in inguinal pockets of the scrotum when they are reproductively active most likely for sexual displays (Dechmann et al. 2009, and personal observation; Voigt-Heucke et al. 2010). This species is faced with the infestation by ectoparasites including mites, bat flies, ticks and bugs. Infestations with endoparasites or fungi as well as viral infections are not described so far (Hood and Pitocchelli 1983).

The greater bulldog bat, ***Noctilio leporinus*** Linnaeus 1758 (Noctilionidae) is with 50 – 70 g larger as *N. albiventris* and predominantly fish-eating. Activity patterns diver from *N. albiventris* and the species show no peaks of activity, instead being active throughout the night. Colonies may consist of several hundred individuals. Female *N. leporinus* congregate at day roosts in harem groups of one male and three to ten females. Bachelor males roost individually or in small groups. Female groups stay together over years regardless of turnover in resident males (approximately after two years) and movements of the group to different roosts. Night roosts are occupied only when bats are between hunting bouts. Also in this species females scent-mark themselves with sub-axial secretions of other females from the same group. Analyses of the sub-axial secretions indicate that individuals have a distinct mixture and quantity of lipid compounds (Brooke 1997, Brooke and Decker 1996). As in the congeneric species males produce a musk odour by glandular cells in inguinal pocket of the scrotum. Ectoparasites (mites, ticks, batbugs, bat flies), endoparasites (nematodes, trematodes protozoans) and the fungus *Histoplasma capsulatum* are described to infest this species, whereas rabies has not been found in wild populations (Hood and Jones 1984).

MHC class II *DRB*. Within the multigene family of the MHC, I focused my research on MHC class II genes which are responsible for the recognition of extracellular derived antigens (e.g. arthropods, helminths, bacteria and fungi) – the predominant threats to all of my study species. Within the class II, I focused on *DRB* genes because parts of these loci (exon 2) code for the functionally important antigen binding region and are the most variable genes within the MHC family (Klein 1986). The study of MHC genes in bats is still in its infancy, mainly as a result of missing molecular knowledge of sequence data for primer design. Thus, a pivotal task was to establish reliable genetic methods to detect functional MHC diversity in bats. I proved the functionality of the investigated loci by using RNA samples and I designed primers located in conserved exons outside the highly variable exon 2 in order to avoid the likely loss of alleles due to mismatches in primer sites. For genomic DNA samples, I designed species specific intron primers using a genome-walking-approach (modified from Ko et al. 2003), which allows the amplification of specific DNA fragments in situations where only limited or highly variable sequence data are present.

Study questions. The central chapters (Chapter 2 - 4) of my thesis consists each of one published article and include the specific introduction to the study questions, outline the obtained results and discuss the findings in relation to the available literature.

Chapter 2 comprises the study on the evolutionary pattern of MHC class II *DRB* variation in four New World bat species from three different bat families. The specific study questions were:

- Are there differences in MHC evolution and diversity patterns in bats compared to other mammals?
- Do the investigated bat species and families vary in their adaptive genetic variation at the examined *DRB* loci and if so to what extend?
- Which molecular mechanisms generate sequence polymorphism: (1) is there evidence for positive Darwinian selection in the substitution pattern, (2) is selection favouring specific codons potentially involved in antigen binding and (3) does recombination or gene conversion events contribute to the observed sequence diversity?
- What are the phylogenetic relationships among bat species with respect to the underlying different functional parts of the *DRB* molecule? Do we get hints about the mode of evolution? Here I extended my analyses to all other published chiropteran *DRB* sequences.

In chapter 3 and 4 I investigated the evolutionary relevance of the adaptive MHC constitution to fitness related traits, such as parasite resistance and reproductive effort. I chose the lesser bulldog bat (*Noctilio albiventris*) because my pilot examinations showed that the MHC constitution of this species offers all prerequisites for in-depth studies of pathogen-driven as well as sexual selection patterns. First, in contrast to the other investigated bat species, this species has a single expressed *DRB* locus which allows testing predictions of the 'heterozygosity advantage hypothesis'. Second, its allelic diversity was within a range that allows allele-specific statistical analyses testing predictions of the 'frequency-dependent selection hypothesis'. Third, the free-living population of *N. albiventris* in Panama consisted of two subpopulations and moreover was structured by roosting colonies, so that it was promising to test for possible local adaptation events as a sign for the presence of 'fluctuating selection'. The specific questions of Chapter 3 and 4 were the following:

- What is the population-wide genetic variation at the MHC-*DRB* locus in a natural population of *N. albiventris*?
- Is the population structure, e.g. subpopulations and roosting colonies, reflected in the MHC, which would indicate local adaptation events or demographic processes?
- Is there indication for pathogen-mediated selection mechanisms on the MHC-*DRB* gene? Are there associations between heterozygosity or specific MHC variants and parasite loads (ticks and bat flies)?
- Is the infestation intensity in parasite load associated with life history traits? And if this is so:
- Does a males MHC constitution contribute to the individual fitness in the sense of the 'good genes model' which predicts that genetically well adapted males resistant to prevailing parasites have more resources to invest in reproduction?
- And, are there sex-related differences in the MHC constitution, which could indicate that selection pressure varies gender-specific?

In chapter 5 the results of all study questions are linked together and discussed and a perspective for future work is given.

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Independent evolution of functional MHC class II *DRB* genes in New World bat species

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Abstract

Genes of the major histocompatibility complex (MHC) play a pivotal role in the vertebrate immune system and are attractive markers for functional, fitness related, genetic variation. Although bats (Chiroptera) represent the second largest mammalian order and are prone to various emerging infectious diseases, little is known about MHC evolution in bats. In the present study we examined expressed MHC class II *DRB* sequences (exons 1 to 4) of New World bat species, *Saccopteryx bilineata*, *Carollia perspicillata, Noctilio albiventris* and *N. leporinus* (only exon 2). We found a wide range of copy number variation of *DRB* loci with one locus detected in the genus *Noctilio* and up to ten functional loci observed in *S. bilineata*. Sequence variation between alleles of the same taxa was high with evidence for positive selection. We found statistical support for recombination or gene conversion events among sequences within the same, but not between bat species. Phylogenetic relationships among *DRB* alleles provided strong evidence for an independent evolution of the functional MHC class II *DRB* genes in the three investigated species, either by recent gene duplication, or homogenization of duplicated loci by frequent gene conversion events. Phylogenetic analysis of all available chiropteran *DRB* exon 2 sequences confirmed their monophyletic origin within families, but revealed a possible trans-species mode of evolution pattern in congeneric bat species, e.g. within the genera *Noctilio* and *Myotis*. This is the first study investigating phylogenetic relationships of MHC genes within bats and therefore contributes to a better understanding of MHC evolution in one of the most dominant mammalian order.

[1] *Authors' contributions: I conceived the study, carried out laboratory procedures, performed the statistics, interpreted the data and drafted the manuscript. S. Sommer initiated the collaboration, supervised the research, and revised the manuscript. C. Voigt, S. Greiner and D. Dechmann provided the bat samples as well as comments and suggestions on the manuscript.*

Introduction

Bats (Chiroptera) belong to one of the most species rich, ecologically diverse, and geographically distributed orders within the class mammalia. They play a major role in pollination, seed dispersal and act as insect predators, providing substantial ecosystem services worldwide (Kunz et al. 2011). Recently, it has become evident that bats can also be connotative reservoir hosts and potentially important vectors of many newly emerging infectious diseases affecting other wildlife and humans (Calisher et al. 2006; Field 2009; Wibbelt et al. 2010). Although bats harbour a large number of emerging pathogens, it appears that they coexist without seriously affecting their host species (Dobson 2005). Even though, immune responses of bats are expected to have similarities with those of mammals, there is evidence that antibody responses in bats might be both qualitatively and quantitatively different (e.g. Baker et al. 2010; Virtue et al. 2011). However, little is known about bat immunology and the possible effect of genetic variation influencing their pathogen resistance. Given the combination of these facts, it becomes apparent that the study of bat immunogenetics is of fundamental relevance, not only for understanding the mechanisms influencing the zoonotic potential of bats, but also to shed light on the evolution of genes involved in the immune system of this remarkable mammalian order.

The immune genes most commonly studied with respect to the evolutionary adaptation to infectious diseases are those of the major histocompatibility complex (MHC). The MHC is a highly polymorphic genomic region that encodes cell-surface glycoproteins that are involved in immune responses in vertebrates (Klein 1986). Levels of MHC diversity in populations have been increasingly assessed in evolutionary, ecological and conservation studies because they are associated with factors likely related to the individual fitness, population viability and evolutionary potential in a changing environment (reviewed in Sommer 2005; Piertney and Olivier 2006). The immune relevant function of MHC class I and II proteins is to bind foreign antigens derived from pathogens (e.g. viruses, bacteria, parasites), and consecutively presenting them to T-cell receptors, which initiate the appropriate immune response in the adaptive immune pathway (Klein 1986). The extensive polymorphism in MHC genes is especially pronounced in exons which code for the antigen binding region where certain amino acids are directly involved in antigen adhesion (Brown et al. 1993; Stern et al. 1994). Typically, the encoding nucleotide codons exhibit a higher rate of non-synonymous over synonymous substitutions than other parts of the molecule and are thus under

diversifying selection, also known as positive Darwinian selection (Hughes 2007). Variability within a species might also be generated by frequent gene duplication (birth) and deletion (loss) especially in MHC class II genes ('birth-and-death model' of molecular evolution; Nei and Hughes 1992; Nei and Rooney 2005) as well as by recombination and gene conversion (e.g. Otha 1999; Reusch and Langefors 2005).
The evolution of mammalian MHC genes across species barriers appears to be characterized by ancient duplication followed by divergent evolution (e.g. Hughes and Yeager 1998). Thus, more distantly related species might share orthologous loci explained by their common ancestry (Figuroa et al. 1988; Trowsdale 1995; primates: Mayer et al. 1988; rodents: Musolf et al. 2004; Brya et al. 2006; cetaceans: Xu et al. 2009; felids: Wei et al. 2010). The persistence of allelic lineages through speciation and their passage from species to species is known as trans-species polymorphism and a result of balancing pathogen-driven selection mechanisms (Klein et al. 1987). But there is also evidence for a contrary pattern, more commonly observed in birds, where MHC genes usually cluster by species, which indicates recent duplication or elevated rates of concerted evolution that is homogenization by frequent gene conversion events among genes of probably older origin (Hess and Edwards 2002). Even though, recent studies on avian MHC evolution annotate this general conclusion and suggest rather a birth-and-death scenario also in birds with an ancient duplication event followed by independent gene losses (Burri et al. 2010). Alternatively to a direct descent, similarity in the antigen binding region may also arise by convergence most probable due to similar environmental or ecological selection pressures (O'hUiguin 1995; Kriener et al. 2000, 2001; Xu et al. 2008; Srithayakumar et al. 2012). Thus, the relationships among MHC alleles might be more accurately revealed by introns or by exons that are not subject of balancing selection (Kupfermann et al. 1999; Kriener et al. 2001). These observations suggest that complex evolutionary mechanisms account for the substantial variation reported in the MHC organization of vertebrate groups (Hess and Edwards 2002; Kumanovics et al. 2003; Yuhki et al. 2007) and sometimes even within the same species (e.g. Doxiadis et al. 2010).

Here we studied in several bat species the evolution of one of the functionally most important genes of the MHC with the highest levels of adaptive polymorphism in most vertebrates (Klein 1986). We analysed the variability pattern of MHC class II *DRB* genes, specifically within the antigen-binding β1-domain encoded by exon 2, as well as within the more conserved β2-domain encoded by exon 3 and 4 derived from complementary DNA (cDNA) from species of three different New World bat families, *Saccopteryx bilineata* (Emballonuridae), *Carollia perspicillata* (Phyllostomidae) and

Noctilio albiventris (Noctilionidae). In addition, we examined genomic DNA (gDNA, exon 2) of another species in the Noctilionidae, *N. leporinus.* We were interested (1) in the copy number of *DRB* loci and in possible molecular mechanisms generating sequence polymorphism: (2) evidence for positive selection by analysing the non-synonymous versus synonymous substitution pattern within the functionally different domains of the β-chain, (3) in specific codons potentially involved in antigen binding and (4) whether gene conversion events have lead to shared motifs in *DRB* sequences within and between the investigated species. Furthermore (5) we reconstructed for the first time the phylogenetic relationship among bat species with respect to the underlying different functional parts of the *DRB* molecule and conducted phylogenetic analyses based on exon 2 including also all other published chiropteran *DRB* sequences (GenBank, Mayer and Brunner 2007; Richmann et al. 2010; Schad et al. 2011).

Material and Methods

Source of RNA and DNA

RNA was isolated from liver samples of one greater sac winged bat, *Saccopteryx bilineata*, captured close to La Selva Biological Station in Costa Rica (10°25'N, 84°00'W), one Seba's short-tailed bat, *Carollia perspicillata*, from a breeding colony held at the University of Veterinary Medicine, Hannover, Germany and one lesser bulldog bat, *Noctilio albiventris*, from a roost in the village Gamboa, Panama (9°07'N, 79°41'W). The sample from *N. albiventris* was also included in a previous population genetics study (Schad et al. 2011). Animals were euthanized in accordance with appropriate guidelines (Gannon and Sikes 2007) and under the license of national authorities. Liver tissues were directly preserved in RNA-Later (Sigma-Aldrich, Steinheim, Germany) and stored at -20°C until RNA extraction. We focussed on liver tissue as liver is known to comprise a large reservoir of antigen presenting cells including cells carrying MHC class II *DRB* molecules (Nemeth et al. 2009; Axtner and Sommer 2011).

Genomic DNA was derived from tissue samples of the same *S. bilineata* individual, two additional *C. perspicillata* individuals captured on the property of La Selva Biological Station (Costa Rica), four additional *N. albiventris* captured close to Tiputini Biodiversity Station (Ecuador, 0°39.311'S, 76°8.92'W) and seven samples of *N. leporinus* from Barro Colorado Island National Monument (Panama, 09.10°N, 079.51° W). All bats were captured with mist nets and a small biopsy of the wing membrane was taken

(Worthington-Wilmer and Baratt 1996) and stored in 96 % ethanol until DNA isolation. All animals were released afterwards at the capture side.

Molecular Techniques

We isolated RNA from liver using the RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions including a DNase treatment. Up to 5 µg of total RNA were used to construct a first-strand cDNA library applying the Revert Aid^{TM} H Minus First Strand cDNASynthesis Kit (Fermentas, St. Leon-Rot, Germany). For reverse transcription we included 5 µg Oligo(dT)12-18 Primer, 200 U of reverse transcriptase (Revert Aid^{TM} H-Minus M-MuLV, Fermentas) and 20 U of a RNase inhibitor (RiboLock^{TM}, Fermentas) as instructed in the manufacturer's protocol. Reproducibility was ensured by two independent repeats of the whole procedure. We extracted gDNA from tissue samples using DNeasy Tissue Kit (Qiagen).

We used primers situated in conserved parts of exon1 (*JSex1*, Schad et al. 2011), exon 3 (*JSex3*, Schad et al. 2011), and exon 4 (*L729*, Bowen et al. 2004) to amplify functional MHC class II *DRB* alleles from the cDNA of the three bat species (Table 1). In a second step these sequences were used to design specific primers situated in exon1 (*JS ex1.4*) and exon 2 (*JSex2N*, *JSex2C*, *JSex2S*) which we used together with *JSex3* and *L729* to screen the cDNA for additional sequences possibly not amplified by the former used primer set (Table 1). In the case of the numerous *DRB* loci in *S. bilineata,* allele specific primers were also designed to receive the complete exon1 to 4 sequences (primer sequences are available on request).

Table 1. PCR primers used to amplify MHC class II *DRB* genes in the investigated bat species.

Primer	Position	Sequence 5' to 3'	Designed for	Reference
JSex1-DRB	Exon 1	GCTCCYGGATGRCAGCTCTGA	Mammals	Schad et al. 2011
JSex3-DRB	Exon 3	AGAGCAGACCAGGAGGTTGTG	Mammals	Schad et al. 2011
DRB-L729	Exon 4	ACTCAMCATCTTGCTCTG	Mammals	Bowen et al. 2004
JSex1.5-DRB	Exon 1	KGGGCCAGRGRCACACCA	Bats	this study
JSN2R-DRB	Exon 2	GTGCGCTTCGACAGCRACGT	Bats	Schad et al. 2011
JS2Sac-DRB	Exon 2	GAGTGTCATTTCTMCAAYGGGAC	*S. bilineata*	this study
JS2Cape-DRB	Exon 2	AGTGTCAKTWCTCCAACSGGAC	*C. perspicillata*	this study
JSi1Cape-DRB	Intron 1	GGTCGTYSCTGTCCCCACAG	*C. perspicillata*	this study
JSi2Cape-DRB	Intron 2	CCGCCGSRCTCACTTTGC	*C. perspicillata*	this study
JS2Noc-DRB	Exon 2	TTCWCCAACGGGACGGAGCGCGT	*Noctilio spec*	this study
JSi1N-DRB	Intron 1	GGCGCCCGGCCTGGCCGACGTC	*Noctilio spec*	Schad et al. 2011
JSi2N-DRB	Intron 2	CACACGCACGTACACAAGTACACA	*Noctilio spec*	Schad et al. 2011

To reduce misincorporation errors, PCR amplicons were generated with a proofreading polymerase (Hotstar Hifidelity polymerase, Qiagen, Hilden, Germany). Amplification was carried out in a total reaction volume of 25 µl including 20 ng cDNA, 1 U of proofreading polymerase, 1x Hotstar Hifidelity PCR buffer, 0.3 mM dNTPs and 0.4 µM of each primer (Sigma-Aldrich, Steinheim, Germany). To reduce the probability of artificial chimeric molecules we limited number of cycles to 32 and prolonged the extension time to 1.30 min in the PCR protocol. Thermocycling started with an initial denaturation step at 95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 60 s and elongation at 72°C for 1.30 min. An additional 15 min elongation step followed the last cycle. PCR products were purified (Cycle pure Kit, Peqlab, Erlangen, Germany) and cloned using the pCR®4-TOPO® TA cloning Kit (Invitrogen, Karlsruhe, Germany). Clones were amplified using the vector-primers *T7*for and *M13*rev. They were sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit (ABI) and analysed on ABI PRISM 310 Automated Genetic Analyser (Applied Biosystems, Foster City, Ca USA). Sequences were considered as unique alleles if the following criteria were met: (1) amplification by two different primer pairs, or (2) incidence in at least two independent PCR reactions and (3) identified by at least three identical clones (Kennedy et al. 2002).

The gDNA of the additional samples of *C. perspicillata* and *N. leporinus* were examined for their MHC class II *DRB* exon 2 variability. We used the vectorette PCR approach as described in detail by Schad et al. (2011) to design species specific intron primers to amplify the whole exon 2 sequences (*C. perspicillata*:*JSi1C* and *JSi2C;* Table 1). PCR, cloning and sequencing was performed as described for cDNA analyses. As in *N. albiventris,* a single *DRB* locus in *N. leporinus* was assured through the vectorette PCR approach and intron primers designed for the one expressed *DRB* locus in *N. albiventris* (Schad et al. 2011) fitted to the same conserved regions of intron sequences in *N. leporinus* (*JSi1N*, *JSi2N*; Table1). Thus, additional individuals of the genus *Noctilio* were genotyped by single strand confirmation polymorphism (SSCP) and direct sequencing as described in previous studies (Schad et al. 2004, 2011). For simplicity we refer in all investigated species to MHC alleles, even though the loci assignment was only possible in the genus *Noctilio*.

Data analyses

We edited and aligned nucleotide sequences manually based on their forward and reverse consensus chromatograms in the program MEGA 4.0 (Tamura et al. 2007). The MHC-*DRB*-like nature of the sequences was verified through a homology analysis

using BLAST search (http://blast.ncbi.nlm.nih.gov). The numbers of nucleotide and amino acid substitutions between alleles were calculated with MEGA 4.0. Under historical positive selection a relative excess of non-synonymous over synonymous substitutions is expected (Hughes and Nei 1988). We analysed the relative rate (ω) of non-synonymous (d_N) and synonymous (d_S) base pair substitutions according to Nei and Gojobori (1986) applying the Jukes-Cantor correction for multiple hits (Jukes and Cantor 1969), as implemented in MEGA 4.0 for each bat species separately. The d_N/d_S ratios of all sites, as well as for the β1- and β2-domain, were compared with an implemented Z-test based on a significance level of α=0.05 (Nei and Kumar 2000).

Further evidence for positive selection in the past was assessed using maximum likelihood analyses to examine heterogeneity in ω (d_N/d_S) among site partitions using CODEML (included in the software packages PAML 3.15; Yang 2007) for each bat species respectively. The program estimates ω among sites applying different models of codon evolution (neutral, purifying, or positive selection), where ω is used as a measure of the natural selection acting on the protein (Yang and Bielawski, 2000; Yang et al. 2005). We used two nested model pairs in our analysis M1a (nearly neutral) and M2a (positive selection) as well as models M7 (β) and M8 (β&ω) which assume a beta distribution for ω. Null model M1a assumes two site classes with $0<\omega_0<1$ and $\omega_1=1$ as fixed. The alternative model M2a incorporates an additional site class with $\omega_2 >1$. In the second model pair, model M7 served as a null model where the ω ratio varies according to the beta distribution and does not allow for positively selected sites ($0<\omega<1$). M8 adds a class of sites to account for the possible occurrence of positively selected sites ($\omega>1$) assuming positive selection (Yang et al. 2005). The models were compared and evaluated using a likelihood ratio test (LRT) calculating the likelihood difference $2\Delta l = 2(l_1-l_0)$, which was then compared to a χ^2-distribution (α=95%) with the degrees of freedom equal to the difference in the number of parameters between the compared models (Yang et al. 2005). Potential positively selected sites (PSS) were calculated by the Bayes empirical Bayes method (BEB; Yang et al. 2005) integrated in CODEML. Posterior probabilities for site classes with mean $\omega>1$ were identified at the 95% confidence level according to model M2a and M8 respectively. Whereas PSS of model comparison M1a and M2a seem to be more robust against misclassifying sites under weak purifying selection, because of the insistence of a site class $\omega_1=1$ in M2a (Yang et al. 2005), PSS detected under the M7 and M8 model pair seem to be more robust against the impact of recombination which can also potentially generate false positives (Anisimova et al. 2003).

We tested for presence of recombination or gene conversion events using the program GENECONV vers 1.81 (Sawyer 1999). Providing an aligned file of sequences it computes a prediction of what sequence fragments have the highest unique similarity between a pair of sequences. P-values are determined both globally and pairwise. We considered recombination/gene conversion events as significant when simulated global p-values (corrected for multiple comparisons by Blast-like global scores with 10,000 replicates) were less than 0.05, using the most conservative approach not allowing for any mismatches between fragments (gscale value of 0). GENECONV has been validated to identify intra- and interlocus recombination/gene conversion between homologous sequences when it is present, with a low rate of false positives (Posada 2002). Sequences spanning exon 1 to 4 obtained from *S. bilineata, C. perspicillata* and *N. albiventris* were analysed together to obtain possible signs for former recombination events and independently for each bat species respectively. Concerning *N. albiventris* and *N. leporinus* we restricted analyses to the second exon to reach a sufficient number of alleles including all known *DRB* sequences so far, *NoalDRB**01-*24 and *NoleDRB**01-06 (this study and Schad et al. 2011 GenBank accession numbers: HM347941-HM347958). Finally, we combined exon 2 sequences of these two sister groups to detect possible gene conversion events which may have occurred before speciation.

To examine phylogenetic relationships and to test for probable trans-species polymorphism between MHC class II *DRB* alleles we performed model-based likelihood tree searches using a Bayesian inference approach implemented in MRBAYES 3.1.2 (Ronquist and Huelsenbeck 2003). The human *HLADRB1**0101 sequence (GenBank accession number: HM067843) was used as outgroup to root the trees. Prior to the model based tree searches we performed statistical selection of different nucleotide substitutions models implemented in the program jMODELTEST 0.1.1 (Posada 2008) to evaluate the best-fitting models of DNA evolution represented by the data. Model selection was done using Akaike's information criterion (AIC). If the suggested best-fitting model by jMODELTEST was not implemented in MRBAYES we used the next more complex model provided. Based on the selected models and their estimated parameter values Bayesian inference trees were constructed. Analyses were run simultaneously for two independent estimations with randomly chosen starting trees. The runs consisted of four heated and one cold Markov chains (heating= 0.20) which were sampled every 1000 generations. Analyses was continued as long as the two runs converged onto stationary distribution indicated by an average of the standard deviation of split frequencies < 0.01. The first 25% of trees were discarded as burn-in.

To calculate the posterior probability of each bipartition, the majority-rule consensus tree was computed from the remaining trees.

Results

The nucleotide and deduced amino acid sequence of the 628 bp products (without primers) from cDNA from the bat species *S. bilineata, C. perspicillata* and *N. albiventris* covered a MHC class II *DRB*-like fragment starting in exon 1 and ending in exon 4 (electronic supplemental material (S)[2]; Fig. S1, Fig. S2). Transcripts showed a high BLAST homology with *DRB* sequences from humans (> 88 %) and other mammalian species and encoded parts of *DRB* molecules (208 aa), which included a fraction of the leader sequence (13 aa), the β1domain (95 aa) and the β2 domain (100 aa) (Fig. S2). None of our sequences showed BLAST homologies to other MHC class II loci than *DRB* and all contained several *DRB*-specific amino acids in the β2 domain, which differentiate them from *DQB*-genes (Table S1, Fig. S2; see also Bowen et al. 2002). Consequently, we assigned transcripts as *MhcSabi-DRB* (*S. bilineata*), *MhcCape-DRB* (*C. perspicillata*) and *MhcNoal*-DRB (*N. albiventris*, Schad et al. 2011) according to the established MHC nomenclature (Ellis et al. 2006). No insertions/deletions, frame-shifting mutations or stop codons were present in any of the detected sequences. Whereas in *N. albiventris* and *N. leporinus* (only gDNA, *MhcNole-DRB*) a single MHC class II *DRB* locus was detected (Schad et al. 2011, this study), we found evidence for several expressed *DRB* loci in *S. bilineata* and *C. perspicillata*.

Saccopteryx bilineata

In the cDNA of one single *S. bilineata* individual, taking common strict cloning rules into account (Kennedy et al. 2002), we verified 20 expressed *SabiDRB*-sequences (alleles *SabiDRB**01-20; GenBank accession numbers: JQ388810-JQ388829), indicating the presence of at least 10 functional *DRB* loci in this individual. The number of alleles might even be higher, as we detected additional unconfirmed sequences, which did not match the strict criteria we employed for uniqueness of alleles. Analyses of gDNA using primers *JSex2S* and *JSi2C* (Table 1) confirmed some of the transcribed alleles, but revealed also evidence for more sequences, with several of them showing signs of pseudogenes (Schad et al. unpublished).

The variation between *Sabi-DRB* alleles was high with 48.63 ± 3.71 nucleotide differences and ranged from 8 to 82 nucleotide positions (Fig. S1). All nucleotide

[2] see Appendix I

sequences could be translated into unique amino acid sequences (Fig. S2) with an average number of 29.0 ± 3.15 amino acid substitutions (range: 5 - 59). Polymorphic positions were mainly located in the antigen-binding β1-domain encoded by exon 2 (Table 2). We observed no significant difference between non-synonymous and synonymous substitutions considering either the whole sequence ($Z = 1.04$, $p = 0.29$), or the β1-domain ($Z = 1.24$, $p = 0.21$) and the β2-domain ($Z = -1.25$, $p = 0.21$) separately. The maximum likelihood analyses indicated positive selection on specific codon sites. We observed significantly higher log likelihood estimates for model M2a compared to model M1a ($2\Delta l = 191.2$, $df = 2$, $p < 0.0001$), as well as for model M8 compared to model M7 ($2\Delta l = 218.5$, $df = 2$, $p < 0.0001$) indicating positive selection acting on the *Sabi-DR* β-chains. Bayesian inference detected 19 (model M2a) and 21 (model M8) significant sites under positive selection (Fig. S2). The additional PSS of M8 concerned amino acid positions 9 and 88. Sixteen of these sites were congruent with polymorphic sites of the human *HLA-DR1* β-chain (Brown et al. 1993); two were located in close proximity and three were situated in the β2-domain (Fig. S2).

Three significant gene conversion events between *Sabi-DRB* alleles with lengths ranging from 213 to 297 bp were detected using GENECONV global scores (Table 3; Fig. S1). All three fragments were situated at the beginning of exon 3 coding mainly for the β2-domain. Additionally, gene conversion events with unknown parental origin were detected by global scores (Bonferroni corrected significant values) in two alleles. One fragment was situated in exon 2 of *Sabi-DRB**14 (12 bp) and the other in exon 3 of *Sabi-DRB**07 (35 bp).

Carollia perspicillata

We detected six different *Cape-DRB* alleles (*Cape-DRB**01-06; GenBank accession numbers: JQ388830-JQ388835) from cDNA of a single *C. perspicillata* specimen, indicating the presence of at least three functional *DRB* loci in this individual. We observed all six *DRB*-alleles also in gDNA, and no additional alleles were found when intron primers were applied (Fig. S1, Fig S2). We observed four and five *CapeDRB* alleles, respectively, in the gDNA of two additional individuals from Costa Rica (*Cape-DRB**07-15; GenBank accession numbers: JQ388789-JQ388797; Fig. S1, Fig. S2). None of the alleles showed signs of a pseudogene. Pairwise nucleotide differences ranged from 46 to 68 with an average of 53.40 ± 4.69. The deduced six unique amino acid sequences varied at 30.53 ± 3.52 positions (range: 13 - 44). Also here, most of the polymorphic residues were situated in the β1-domain (Table 2). Non-synonymous

Table 2. Mean number (± standard deviation) of synonymous (syn) and non-synonymous (non-syn) nucleotide substitutions among alleles at the functionally different domains of the MHC class II *DRB* molecule within the investigated bat species according to Nei and Gojobori (1986, 1000 replicates).

Species	#Alleles	β1-domain			β2-domain		
		Syn	non-syn	bp	syn	non-syn	bp
S. bilineata	*NA, 20 (1)*	8.19 ± 1.29	33.34 ± 4.05	284	1.87 ± 0.45	3.19 ± 0.72	300
C. perspicillata	*15, 6 (3,1)*	3.71 ± 1.09	39.00 ± 4.71	284	3.93 ± 1.25	5.20 ± 1.35	300
N. albiventris	*24, 2 (219,1)*	3.54 ± 1.04	23.69 ± 3.73	270	*NA*	*NA*	
N. leporinus	*6, NA (7)*	2.90 ± 1.04	21.30 ± 3.44	270	*NA*	*NA*	

bp: fragment length in base pairs; *NA:* not available; # Alleles: number of alleles (number of individuals analysed) for gDNA, cDNA respectively; *Saccopteryx bilineata*, *Carollia perspicillata*, *Noctilio albiventris*, *Noctilio leporinus*

substitutions occurred at a significantly higher rate than synonymous ones over the whole sequence ($Z = 3.10$, $p = 0.002$) and especially in the β1-domain ($Z = 5.38$, $p = 0.0001$), but not in the β2-domain ($Z = -1.65$, $p = 0.10$). The maximum likelihood analyses indicated positive selection on specific codon sites. Significantly higher log likelihood estimates were calculated for model M2a compared with model M1a ($2\Delta l = 65.06$, $df = 2$, $p < 0.0001$) as well as for model M8 compared with model M7 ($2\Delta l = 47.4$, $df = 2$, $p < 0.0001$). Thirteen (M2a) and 15 (M8) PSS were identified by Bayesian inference to be under positive selection (Fig. S2). The additional PSS of M8 concerned amino acid positions 13 and 84. Not all amino acid positions which are predicted to be involved in antigen binding in human *DR* β-molecules (Brown et al. 1993; Stern et al. 1993) were polymorphic in *Cape-DRB* alleles. Of the 15 PSS ten were congruent with polymorphic sites of the human *HLA-DR1* β-chain (Brown et al. 1993) and the remaining five were located in proximity (< 4 nucleotides; Fig. S2).

In *C. perspicillata* evidence for one gene conversion event was found by GENECONV global scores between *Cape-DRB**01 and *Cape-DRB**04 concerning a fragment situated in exon 2, 31 bp in length (Table 3; Fig. S1).

Noctilio albiventris and N. leporinus

MHC class II *DRB* exon 2 polymorphism of a natural *N. albiventris* population in Panama was previously described (Schad et al. 2011), indicating a single *DRB*-locus with moderate allelic variation (18 alleles in 215 individuals; GenBank accession numbers: HM347941-HM347958) and clear signs of balancing selection. Accordingly, only two sequences spanning exon 1 to 4 were detected in the cDNA of a single

individual (Fig. S2; this study). The four individuals from Tiputini Biodiversity Station (Ecuador) revealed six new exon 2 alleles (270 bp) assigned as *Noal-DRB**19-24 (GenBank accession numbers: JQ388804-JQ388809; Fig. S1, Fig S2). These six alleles were analysed together with the 18 *Noal*-alleles previously described (Schad et al. 2011). In exon 2, we observed an average nucleotide difference of 27.23 ± 3.00 within a range from 2 to 44. The 24 deduced amino acid sequences differed in 18.08 ± 2.59 amino acid positions (range: 1-28; Table 2). The PSS identified by Bayesian inference were consistent with previous analyses (Schad et al. 2011; Fig. S2). In exon 2, two statistically significant gene conversion events were found by global scores with fragment length of 79 and 105 bp respectively (Table 3, Fig. S1).

Furthermore, we analysed MHC class II *DRB* exon 2 variability (270 bp) in seven individuals from the congeneric *N. leporinus*. As in *N. albiventris* a single *DRB*-locus was amplified (Table 1). Six different *DRB* alleles were detected and assigned as *Nole-DRB**01-06 (GenBank accession numbers: JQ388798-JQ388803; Fig. S1, Fig S2). None of the individuals were homozygous. Average substitution rate between alleles was 25.53 ± 3.21 (range: 7-38). The six deduced, unique amino acid sequences varied at 17.33 ± 2.64 amino acid positions (range: 4-26). Non-synonymous substitutions

Table 3. Predicted gene conversion events between MHC class II *DRB* sequences of the investigated bat species identified by GENECONV (Sawyer 1999).

Species	MHC class II *DRB* sequences		Global	Fragment		
	Sequence 1	Sequence 2	Sim P	Position	Length (bp)	Source
S. bilineata	*Sabi-DRB**01	*Sabi-DRB**08	0.009	Exon 3; 303-541	239	inner
	*Sabi-DRB**01	*Sabi-DRB**16	0.017	Exon 3; 330-626	297	inner
	*Sabi-DRB**09	*Sabi-DRB**14	0.005	Exon 3; 354-566	213	inner
	*Sabi-DRB**07		0.013	Exon 3; 445-479	35	outer
	*Sabi-DRB**14		0.002	Exon 2; 276-287	12	outer
C. perspicillata	*Cape-DRB**01	*Cape-DRB**04	0.032	Exon 2; 208-239	31	inner
N. albiventris	*Noal-DRB**03	*Noal-DRB**07	0.009	Exon 2; 238-316	79	inner
	*Noal-DRB**09	*Noal-DRB**23	0.050	Exon 2; 133-238	105	inner
N. leporinus	*Nole-DRB**01	*Nole-DRB**02	0.002	Exon 2; 54-260	206	inner
	*Nole-DRB**02		0.0001	Exon 2; 262-281	20	outer

Length: length of the converted region in base pair (bp); Position: nucleotide position according to Fig. S1; Sim P: simulated P-values corrected for multiple comparisons by Blast like global scores with 10,000 replicates; Source: known pair of sequences (inner) and unknown parental sequence (outer); Species: *Saccopteryx bilineata*, *Carollia perspicillata*, *Noctilio albiventris*, *Noctilio leporinus*

occurred at a significantly higher rate than synonymous ones over the whole exon 2 sequence (Z = 3.16; p = 0.002; Table 2). We observed identical significantly higher log likelihood estimates for model M2a versus M1a and for model M8 versus M7 ($2\Delta l$ = 18.0, df = 2, $p < 0.001$) indicating positive selection acting on the *Nole-DR*-β1 domains encoded by exon 2. Bayesian inference under model M2a and M8 revealed the same six positively selected sites (Fig. S2). All except one were congruent with polymorphic sites of the human *HLA-DR1* β-chain (Brown et al. 1993), and the latter was in close proximity.

We also detected intra-locus recombination events by GENECONV. One statistically significant fragment between *Nole-DRB**01 and *NoleDRB**02 and one fragment with unknown parental origin were identified with global scores (Table 3; Fig. S1). When *NoleDRB* sequences were analysed together with the *Noal-DRB* sequences no gene conversion event was detected between sequences of the two sister species.

Phylogenetic Relationships

Phylogenetic relationship between bat MHC class II *DRB* sequences were reconstructed for exon 1 to 4, exon 1, 3 and 4 (excluding exon 2) and for exon 2 separately to detect differences with respect to the evolutionary histories of these functionally distinct regions. All phylogenetic reconstructions of the chiropteran *DRB* sequences showed strong posterior probabilities for a species specific distinct clustering (Fig. 1). This was true when the whole sequence was analyzed (exon 1 to 4, Fig. 1a) and for exon 1, 3 and 4 separately (Fig. 1b) with high posterior probabilities (100) for each species. The clusters also remained distinct in trees based on exon 2 alone (Fig. 1c), but with lower posterior probability support for *C. perspicillata* (85) and *S. bilineata* (55).

Extended phylogenetic analyses (Fig. 2) including all published chiropteran *DRB* exon 2 sequences (207 bp, nucleotide position 63-269 according to Fig. S1) from this study, from Schad et al. 2011 (GenBank accession numbers: HM347941-HM347958), as well as from *Myotis sp*. (*M. velifer, M. vivesi,* GenBank accession numbers: GU012449-80; Richman et al. 2010) and *S. bilineata* (GenBank accession numbers: EF533888-EF53390; Mayer and Brunner 2007) reached still high posterior probabilities for *C. perspicillata* (100) and the genus *Noctilio* (100), with intermingled clustering of *N. albiventris* and *N. leporinus DRB* alleles, indicating probable trans-species mode of evolution within the congeneric species. The sequences of the genus *Myotis* clustered with low posterior probabilities and both *Myotis* species showed an intermingled

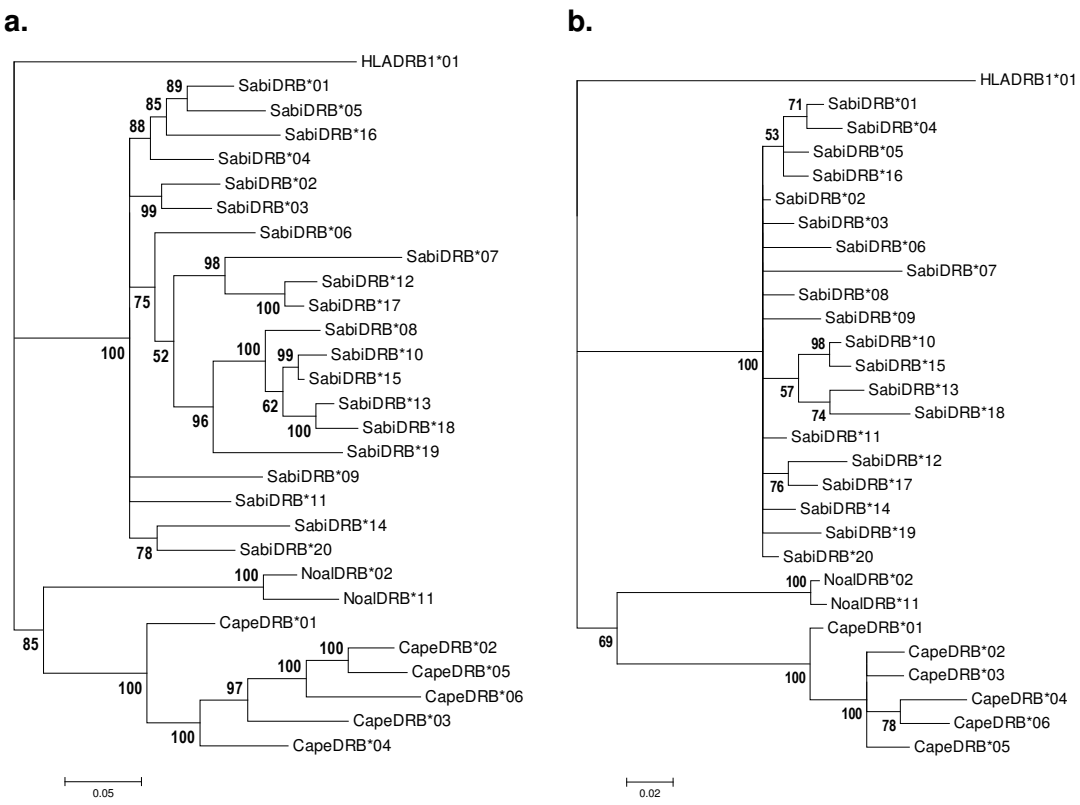

Figure 2. Consensus Bayesian trees (50%-majority-rule) for MHC class II *DRB* sequences based on **a.** exon 1 to 4 (GTR + Γ + I), **b.** exon 1, 3 and 4 (GTR + Γ) and **c.** exon 2 exclusively (GTR + Γ) of the bat species *Saccopteryx bilineata* (*Sabi-DRB**01-20), *Carollia perspicillata* (*Cape-DRB**01-06) and *Noctilio albiventris* (*Noal-DRB**02 and *11). The human *HLA-DRB*1*0101 (GenBank accession number: HM067843) allele was used as outgroup to root the trees. Two runs and 4 chains per run were run for 5 x 10^6 generations. Numbers under nodes signify clades that are received by Bayesian posterior probabilities. Distances are adjusted using best fit models indicated by jMODELTEST (Posada 2008).

clustering of sequences, too. Similarly, we did not detect any distinct clustering in the *S. bilineata* sequences, and posterior probabilities were low. Branches reached higher support for *S. bilineata* (96) when complete exon 2 sequences (this study) were analysed (Fig. S3).We did not find evidence for recombination between sequences of different bat species using GENECONV global scores, indicating that recent duplication events between loci most probably occurred after speciation.

Discussion

The study of MHC genes in bats is still in its infancy. In fact this is the first study of phylogenetic relationships of MHC class II *DRB* genes in Chiroptera. In order to gain a better picture about MHC-*DRB* polymorphism in several bat families, we analysed sequence variation, gene duplication and potential recombination or gene conversion events in transcribed *DRB* loci. We found a heterogeneous pattern of MHC variability among the investigated taxa. Positive Darwinian selection maintained polymorphism especially in specific codons probably involved in antigen binding in all investigated bat species. Phylogenetic relationships of *DRB* sequences provided evidence of an independent evolution by recent duplication events or concerted evolution, because *DRB* sequences of different loci cluster in a family specific manner.

Copy number variation in bats

We found a wide range of variability concerning number of functional *DRB* loci with one *DRB* locus present in both *Noctilio* species, and up to 10 *DRB* loci in *S. bilineata*. An intermediate number of at least three *DRB* loci were found in the cDNA of *C. perspicillata.* Analysis of gDNA revealed no indication for further alleles or pseudogenes in this species. The high number of different alleles detected in the four *N. albiventris* individuals of Ecuador could support the suggestion that more than one species might be present within *N. albiventris* (Vilamiu et al. 2011; Lewis-Oritt et al. 2001). In the only other bat genus from yet another family genotyped for MHC variability so far, *Myotis* (Vespertilionidae), two *DRB* loci were found in *M. velifer* and *M. vivesi* respectively (Richmann et al. 2010). Our findings of extreme high *DRB* polymorphism in *S. bilineata* are in contrast to a previous study of Mayer and Brunner (2007), who detected only two *DRB* loci in this species sampled at the same location but using species-unspecific primers, which failed to amplify all loci. These dissimilar results point to the fact that carefully designed species-specific primers are of major

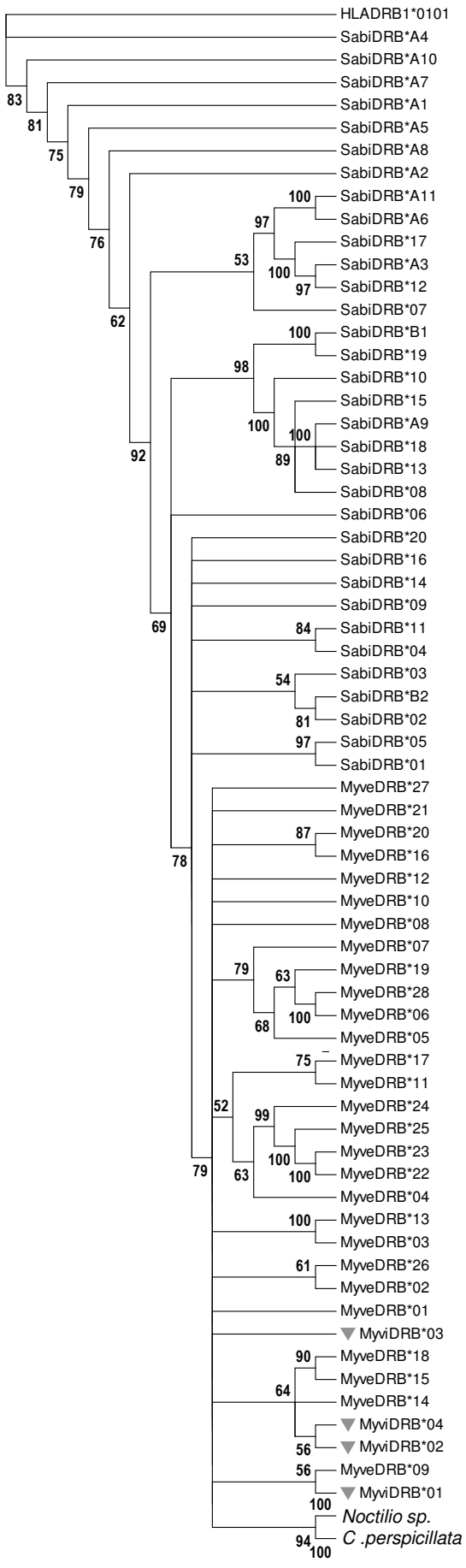

Figure 3. Consensus Bayesian tree (50%-majority-rule) for MHC class II *DRB* sequences based on parts of exon 2 (207 bp; nucleotide position 63-269 according to Fig. S1) of the bat species *Saccopteryx bilineata* (*Sabi-DRB**01-20, this study; *Sabi-DRB**A1-A11, *Sabi-DRB**B1-*B2 (Gen Bank accession numbers: EF533888-90, Mayer and Brunner 2007), *Carollia perspicillata* (*Cape-DRB**01-15, this study) *Noctilio albiventris* (*Noal-DRB**01-*24, this study and *Schad* et al. 2011, GenBank accession numbers: HM347941-58), *N. leporinus* (◆) (*Nole-DRB** 01-07, this study) and *Myotis vivesi* (▼) (*Myvi-DRB**01-04, GenBank accession numbers: GU012449-52) and *M. velifer* (*Myve-DRB**01-28, GenBank accession numbers: GU012453-80, Richman et al. 2010). The human *HLA-DRB*1*0101 allele (GenBank accession number: HM067843) was used as outgroup to root the trees. Two runs and 4 chains per run were run for 4 x 10^7 generations (SYM + Γ). Numbers under nodes signify clades that are received by Bayesian posterior probabilities. Distances are adjusted using best fit models indicated by jMODELTEST (Posada 2008).

importance, especially when primers are located in highly variable regions, such as exon 2. The risk of using cross- amplifying primers has also been pointed out for other taxa (Goüy de Bellocq et al. 2009; Kikkawa et al. 2009; Bollmer et al. 2010).

The MHC is characterized by gene duplication and deletion resulting in a variable gene number between closely related taxa and may even vary within a species (e.g. primates: Dioxidis et al. 2010; ruminants: Mikko et al. 1999; birds: Bollmer et al. 2010). Thus, it is not surprising to find different numbers of *DRB* loci in the investigated bat species from different families. But the high number of at least ten functional *DRB* loci in *S. bilineata* is remarkable and to

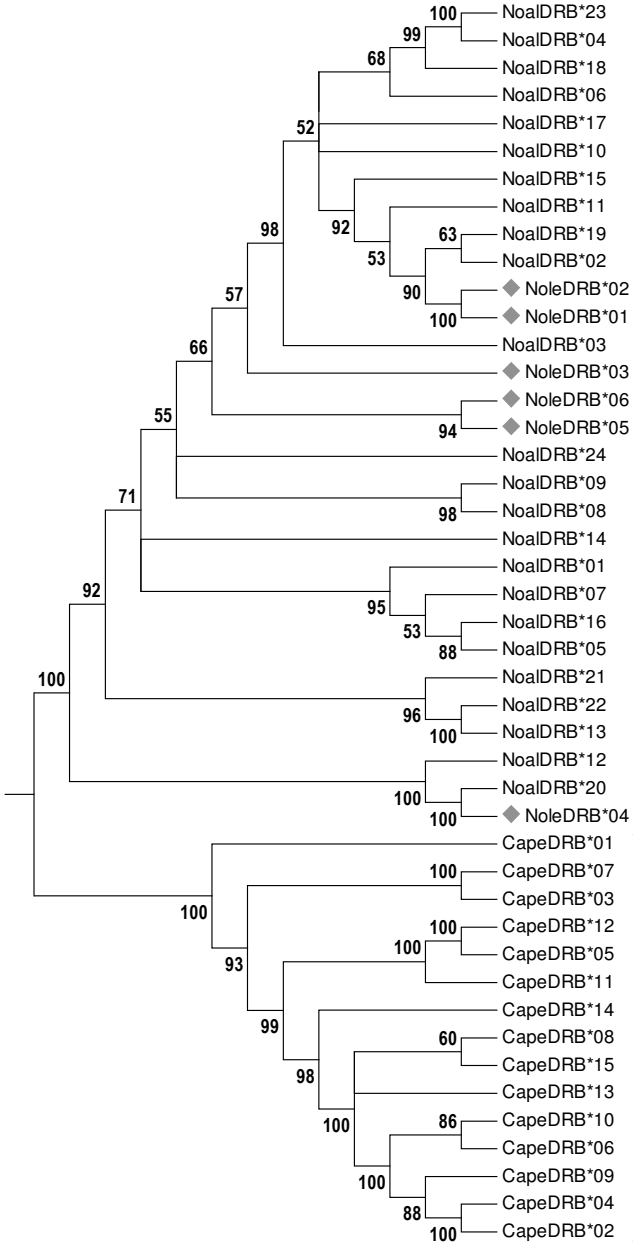

Figure 3 continued.

our knowledge such a high number has never been reported before for any other mammalian species. The highest number reported, are nine *DRB* loci within humans, of which four are functional (Klein et al. 2007), but haplotypes exhibit between one to four *DRB* loci only (Doxiadis et al. 2010). Recent evidence for a comparable polymorphism of MHC class II B genes comes from birds. In the common yellow throat, *Geothlypis trichas*, a minimum of eight expressed loci with possibly a total number of 20 MHC II B loci was detected (Bollmer et al. 2009). What could be the function of high levels of copy variation in bats? Bats have been suggested as natural reservoir hosts for many newly emerging and re-emerging disease pathogens (Dobson 2005, Calisher et al. 2006; Wibbelt et al. 2010). Pathogens which cause severe zoonoses in humans and other wildlife are known to infect and persist in apparently healthy bats. Scientists have argued that bats may have important qualitative and quantitative differences in their innate and acquired immune response compared to other mammals. Thus, pathogens may have coevolved with bats and circulated for thousands and even million of years in bat populations (Calisher et al 2006, Dobson 2005, Wibbelt et al 2010). Duplication of MHC loci plays an important role in the adaptive evolution of immune response by increasing the number of MHC alleles present in individuals, thereby allowing for the detection of a larger number of pathogens (Hughes 1994).

Sequence variation, recombination and selective pressure

Sequence variation between alleles of the same taxa was high and concentrated primarily in the β1 region encoded by exon 2, the region responsible for antigen binding. In all bat species we found compelling evidence that past selection has been acting on MHC diversity. First, we found more non-synonymous than synonymous substitutions especially in the antigen binding β1-domain; even though, this difference was not significant in *S. bilineata*. We used a more conservative approach and calculated substitutions over the whole sequence and not only in nucleotides identified as antigen binding sites in humans (Brown et al. 1993), which might explain why results were not significant for *S. bilineata* (see also Srithayakumar et al. 2011). Likely nucleotides involved in antigen binding were analysed in a species-specific approach. In this approach, maximum likelihood models allowing for selection fitted the data significantly better than models that considered only neutral sites. This was true for each analysed bat species. Bayesian inference revealed evidence for positive selection on specific codons. Positively selected codon sites coincided with putative antigen binding sites of human *DR*1 β-chain (Brown et al. 1993) or were located in close proximity. These facts suggest that sequence diversity in the *DRB* of all investigated bat species may to a large extent be generated by positive selection on codons directly involved in antigen binding.

Recombination and gene conversion are presumed to be additional processes responsible for the high polymorphism typically seen in MHC genes (Otha 1999; Hughes 2000). Evidence for both intra- and interlocus gene conversion at the MHC has been found in a number of taxa (e.g. Richman et al. 2003; Reusch and Langefors 2005; Schaschl et al. 2006; Bollmer et al. 2010), but are assumed to be less important in mammals (Yeager and Hughes 1999). We found evidence that recombination or gene conversion contributed to the *DRB* diversity within the investigated bat species. Interestingly, we detected no evidence for recombination or gene conversion between different bat species, even not between the single *DRB*-locus of the closely related *Noctilio spp*. Gene conversion events between MHC sequences of different species have been demonstrated for a number of taxa signifying that balancing selection maintain ancient alleles across speciation (e.g. Schaschl et al. 2005; Suarez et al. 2006; Burri et al. 2008). Instead, we detected recombination or gene conversion only among *DRB*-alleles within the same bat species, a probable sign of an independent evolution of the *DRB* region in the recent past. We also found support for interlocus gene conversion events especially in *S. bilineata*. Even though, the program Genconv was evaluated as having one of the highest probabilities of correctly inferring gene

conversion events (Posada 2002), the power of this program might be reduced when the rate of interlocus gene conversion is too high. The length of the gene conversion tract is strongly correlated with the gene conversion rates, rather then reflecting true tract length. Thus, long tracts actually confer to a high gene conversion rate, because repeated gene conversions can create long regions with very few mismatches (Manai and Innan 2010). This might be true for the detected long tracts situated in the third exon of *S. bilineata*. Alternatively, these alleles are the product of recent gene duplication, and homology is retained in the conserved exon 3, whereas exon 2 has undergone the typical processes of diversifying selection, as implied by the high number of *DRB* loci. Regardless which of the two scenarios evoked the found pattern it might signify the MHC *DRB* region of this species to be highly recombinant in general.

Phylogenetic relationships

We are the first to examine phylogenetic relationships of MHC genes in bats. All phylogenetic trees indicated that the expressed MHC class II *DRB* sequences from three bat species belonging to three different families are members of monophyletic groups, and are clearly distinct from each other. When analyses were restricted to different functional parts of the *DRB* molecule, those are the highly variable antigen binding region (exon 2) and in contrast the more conserved parts of the molecule (exon 1, 3, 4), results always revealed the same monophyletic clustering, with best posterior probabilities when all exons were combined. Different clustering between exon 2 and other parts of the gene (introns and exons) has been found in several taxa, indicating different evolutionary pathways for different parts of the molecule, depending on function and selection pressure (Kriener et al. 1999, 2000). In our analyses, all exons of the expressed *DRB* sequences clustered species/family specific. Therefore we presume that this relationship is preserved even when considering the highly variable exon 2. Our results imply that the studied loci are paralogues and are the products of recent gene duplication after speciation, demonstrating most probably an independent mode of evolution for each bat species. However, we can not completely rule out the alternative scenario that the locus duplications are old, but ongoing frequent gene conversion events homogenized the loci such that similarities predominate, as we found evidence for recombination/gene conversion among *DRB*-sequences of the same but not between bat species.

Although we analyzed cDNA of single individuals, there is good reason to believe that additional individuals would not substantially change our results. In our holistic phylogenetic tree search, which also included all published chiropteran MHC *DRB*

sequences, we had to restrict our analyses to parts of exon 2. We included six bat species of four families, Emballonuridae, Phyllostomidae, Noctilionidae and Vespertilionidae. We observed a family specific clustering, but the relatively short fragment length (207 bp) together with very high sequence variation obviously affected robustness of trees as evidenced by low posterior probabilities. The intermingled clustering of *DRB* sequences of species from the same genus, i.e. *Noctilio* and *Myotis*, might signify trans-species polymorphism, suggesting that some allelic lineages have been retained by balancing selection beyond the speciation event. Trans-species polymorphism is a common phenomenon seen in MHC genes and has been documented in numerous mammalian groups, primarily between species of the same genus, but also between groups of higher taxonomic levels (e.g. felids: Wei et al. 2010; lagomorphs: Goüy de Bellocq et al. 2009; rodents: Musolf et al. 2004; Babik and Radwan 2007). Shared parasites and thus similar pathogenic selection pressure over long periods of time is thought to be the driving force of trans-species polymorphism (Klein et al. 2007; Goüy de Bellocq et al. 2009). But trans-species-like patterns in *DRB* exon 2 sequences can arise also by convergence, with the consequence that trans-species persistence of allelic lineages may be wrongly inferred where none occurs (Kriener et al. 2000, 2001). Thus, final conclusions about potential trans-species polymorphism in the genera *Noctilio* and *Myotis* have to be treated with caution.

In order to fully understand the time scale at which diversification of the MHC genes in bats occurred, the effects of ongoing recombination, gene conversion on the variability pattern as well as the potential role of copy number variation in disease resistance needs to be studied in more detail. Therefore, large scale genomic studies incorporating a high number of different bat genera and species as well as information on pathogen loads are required.

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Research Institute – Institutional Animal Care and Use Committee (STRI-IUCAC). Permits were issued from the Panamanian authority Autoridad National del Ambiente (ANAM, SE/A 98-08, SEX/A78-08, SEX/A-138-08) and from the Ministerio del Ambiente y Energia, Costa Rica (MINAE, Permit Number: No 183-2008-SINAC). This work was supported by the Leibniz Institute for Zoo and Wildlife Research (IZW), Germany.

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MHC class II *DRB* diversity, selection pattern and population structure in a neotropical bat species, *Noctilio albiventris*

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Abstract

Genes of the major histocompatibility complex (MHC) play a crucial role in the immune response of vertebrates, alter the individual odour and are involved in shaping mating preferences. Pathogen-mediated selection, sexual selection and maternal-fetal interactions have been proposed as the main drivers of frequently observed high levels of polymorphism in functionally important parts of the MHC. Bats constitute the second largest mammalian order and have recently emerged as important vectors of infectious diseases. In addition, Chiroptera are interesting study subjects in evolutionary ecology in the context of olfactory communication, mate choice and associated fitness benefits. Thus, it is surprising that they belong to the least studied mammalian taxa in terms of their MHC diversity. In this study, we investigated the variability in the functionally important MHC class II gene *DRB*, evidence for selection and population structure in the group-living lesser bulldog bat, *Noctilio albiventris* in Panama. We found a single expressed, polymorphic *Noal-DRB* gene. The substitution pattern of the nucleotide sequences of the 18 detected alleles provided evidence for positive selection acting over the evolutionary history of the species in shaping MHC diversity. Roosting colonies were not genetically differentiated, but females showed lower levels of heterozygosity than males, which might be a sign that the sexes differ in the selection pressures acting on the MHC. This study provides the pre-requisites for further investigations of the role of the individual MHC constitution in parasite resistance, olfactory communication and mate choice in *N. albiventris* and other bats.

[1] *Authors' contributions: The study was conceived by all authors. I carried out all laboratory procedures, statistical analyses, data interpretation, and drafted the manuscript. S. Sommer initiated the collaboration, supervised the research, and revised the manuscript. D. Dechmann collected the samples and together with C. Voigt provided comments and suggestions on the manuscript.*

Introduction

Genes of the major histocompatibility complex (MHC) play an essential role in the adaptive immune response of vertebrates and are crucial for the understanding of the influence of natural selection on genetic diversity in wild populations (Bernatchez and Landry 2003). MHC-encoded cell surface glycoproteins present antigens of intracellular (MHC class I genes) and extracellular (MHC class II genes) origin to T-cells which trigger the appropriate immune response (Klein 1986; Janeway and Travers 2002; Kummanovicas et al. 2003). Class II-derived molecules are heterodimers of two associated polypeptide chains, an α- and a β-chain. The β-chain consists of an antigen-binding β1-domain, an immunoglobulin-like β2-domain and a transmembrane domain. Certain amino acid positions of the β1- region, the so called antigen-binding sites (ABS, encoded by exon 2) show high levels of genetic variation (Brown et al. 1993) with a higher rate of nonsynonymous (d_N, amino-acid altering) over synonymous (d_S, silent) nucleotide substitutions (Hughes and Nei 1988, 1989). Under neutrality theory (Kimura 1977), the rate of synonymous nucleotide substitution is predicted to be larger than the rate of nonsynonymous substitutions because a change of the amino acid composition is more likely to be deleterious. The pattern of an elevated rate of nonsynonymous substitutions at ABS is considered as clear evidence for 'positive Darwinian selection' (Hughes and Nei 1988, 1989) shaping genetic variation (reviewed in Hughes 2007). Others used the term 'historical positive selection' (Hedrick 1999) or 'positive selection over evolutionary time scale' (Bernatchez and Landry 2003) to describe this observation. This polymorphism enables the immune system to recognize an extensive range of pathogens and is therefore crucial for the immunological fitness of individuals and, thus, animal populations (Bernatchez and Landry 2003; Edwards and Potts 1996). The high levels of polymorphism at MHC class II loci found in most vertebrate species are thought to be maintained by different forms of balancing selection including heterozygosity advantage (Doherty and Zinkernagel 1975) and rare allele advantage (also called negative-frequency-dependent selection; Slade and McCallum 1992; Takahata and Nei 1990) as well as selection that varies in space and time (Hill 1991; Hedrick 2002). Distinguishing between the different forms of balancing selection in natural populations is difficult as they are not mutually exclusive and may operate together with other selective and neutral forces (Sommer 2005; Piertney and Olivier 2006; Spurgin and Richardson 2010).

Genes of the MHC are known to alter the individual body odour (e.g. Penn and Potts 1998; Milinski 2006; Kwak et al. 2009) and may therefore be involved in mate choice decisions (e.g. Wedekind et al. 1995; Chaix et al. 2008; Ilmonen et al. 2009), thus promoting genetic structure within and among social groups (Cutrera and Lacy 2006; Chesser 1990; Matocq and Lacy 2004; Kundu and Faulkes 2004). Sexual selection processes may not only be supported in direct mate choice situations, but also through post-copulatory mechanisms including maternal-fetal interactions (e.g. Wedekind et al. 1996; Ober and Van der Ven 1997). Several studies have already highlighted that reproduction among MHC-dissimilar mates is favoured (e.g. primates: Schwensow et al. 2008; humans: Chaix et al. 2008; Ilmonen et al. 2009); mostly because a genetically diverse offspring genotype is advantageous in the defence against pathogens (Penn and Potts 1999; Parham and Ohta 1996). But still the debate is controversial. In some populations MHC-based mate choice was not affirmed (humans: Hedrick and Black 1997; Chaix et al. 2008; ruminants: Paterson and Pemberton 1997). And some studies have suggested that an intermediate, rather than the highest level of MHC diversity is optimal (Wegner et al. 2003; Woelfig et al. 2009).

Bats are not only the second largest mammalian order, but also the most gregarious of all mammals. Some bat colonies harbour several million individuals. Thus, social communication among bats can be complex, involving not only visual and acoustical but also olfactory signals (McCracken and Wilkinson 2000; Altringham and Fenton 2003). Many bat species produce distinct odours from a variety of glands (e.g. Quay 1970; Dapson et al. 1977; Voigt and von Helversen 1999; Scully et al. 2000; Caspers et al. 2009). Pilot studies have shown that bats use odours for kin and individual recognition (Bouchard 2001; Gustin and McCracken 1987; Safi and Kerth 2003) and during male-female interactions (Bouchard 2001; Voigt and von Helversen 1999). Recently, it has been discovered that bats are reservoir hosts and potentially important vectors of many infectious diseases (Calisher et al. 2006; Wong et al. 2007; Field 2009; Wibbelt et al. 2010). Given the combination of these facts, it becomes apparent that the study of bat immunogenetics is of fundamental relevance, not only for understanding the mechanisms underlying the zoonotic potential of bats but also to shed light on the link between MHC, pathogen resistance and olfaction-based mate choice in mammals.

In the present study, we examined the MHC class II *DRB* region of a neotropical bat species, the lesser bulldog bat *Noctilio albiventris*, in Panama. *Noctilio albiventris* lives in social groups of so far unknown relatedness. Besides acoustical signals, group members also communicate among each other via volatile compounds from so-called oily spots in the subaxillary region. In addition, males possess inguinal pockets next to

the scrotum which provide a distinct odour, particularly during the reproductive period (Brook and Decker 1996; Studier and Lavoi 1984). In summary, *N. albiventris* harbours morphologically distinct scent-producing organs, suggesting that volatiles are of key importance not only for individual communication, but also for mate choice.

Our specific aims were: (1) to design reliable primers to characterize MHC class II *DRB* exon 2 diversity in *N. albiventris* using a genome-walking-approach that enables the amplification of specific DNA fragments in situations where the sequence of only one primer is known (modified from Ko et al. 2003), (2) to examine evidence for selection acting on MHC in *N. albiventris*; i.e. a higher rate of non-synonymous over synonymous substitutions in ABS would indicate positive selection acting over the evolutionary history of the species, and (3) to investigate whether subpopulations, roosting colonies and the sexes differ in their genetic population structure. Differences in the MHC constitution of subpopulations and roosting colonies could indicate local adaptation as a result of recent selection events, whereas differences between sexes could indicate MHC related sexual selection mechanisms.

Material and Methods

Study site and sampling

Our main study site was the village Gamboa (09.07° N, 079.41° W) in Panama. Bats were caught with mist nets (see Dechmann et al. 2009) as they emerged at dusk from their roost during 2006-2008. Individuals caught from the same daytime roost were assumed to be from the same colony. A second study site was Barro Colorado Island (BCI, 09.10° N, 079.51° W), situated 15 km away from Gamboa, where animals foraging above water were caught in mist nets set up along the boat docks of a marina (Fig. 1). Bats from the two sites were considered separate subpopulations as their foraging areas did not overlap (Dechmann et al. 2009). Bats were sexed, aged and body measurements taken. From all 215 bats, we collected a 4-mm skin sample from the wing membrane using a sterile biopsy punch (Worthington-Willmer and Barrett 1996). Skin samples were stored in 96% ethanol until DNA isolation. For optimal primer design and to prove expression of MHC-alleles a liver sample was collected from an euthanized male *N. albiventris.* The liver sample was preserved in RNAlater (Sigma-Aldrich, Steinheim, Germany) and stored at -20° C until subsequent analysis.

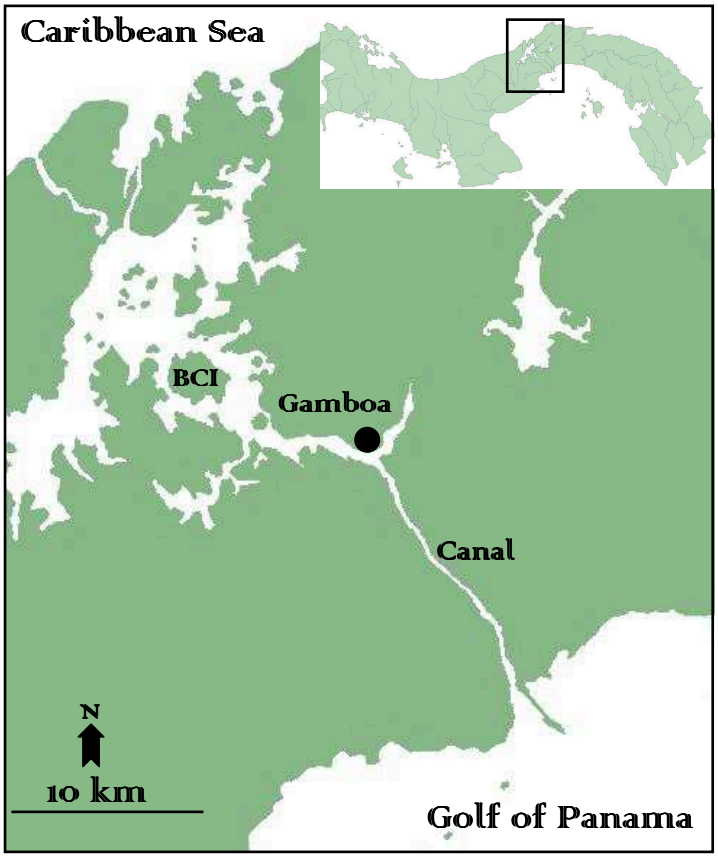
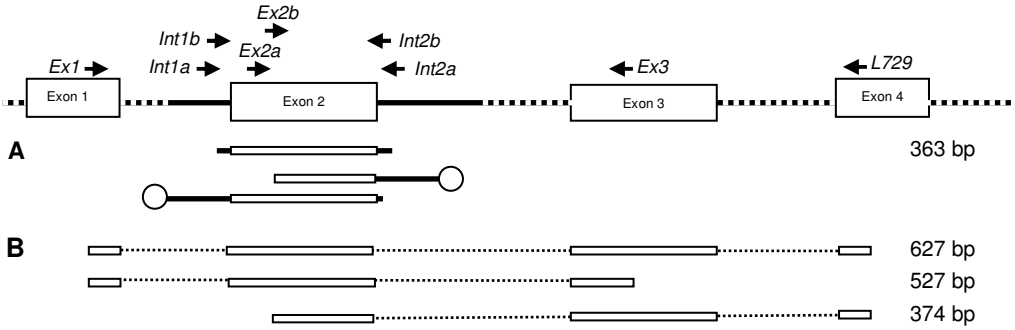


Figure 1. Map of the study sites Gamboa and Barro Colorado Island (BCI) in Panama (modified from the Smithonian Tropical Research Institute mapserver).

gDNA/RNA isolation and cDNA construction

Genomic DNA (gDNA) was extracted using the DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's manual. RNA was isolated from the liver using the RNeasy Kit (Qiagen, Hilden, Germany) which includes a DNase treatment according to the manufacturer's instructions. The quantity was determined by measuring the absorbance at 260 nm and purity (i.e. presence of contaminants) was assessed by the ratio at 260/280 nm which should be ~1.8 for DNA and ~2.0 for RNA (Nanotrop, Peqlab, Erlangen, Germany). Up to 5 µg of total RNA were used to construct a first-strand complementary DNA library (cDNA). For reverse transcription we included 5 µg Oligo(dT)12-18 Primer (Invitrogen, Karlsruhe, Germany) and 200 U of Revert Aid^{TM} H-Minus M-MuIV reverse transcriptase (Fermentas, St. Leon-Rot, Germany) together with 20 U of a ribunuclease inhibitor (RiboLock^{TM}, Fermentas, St. Leon-Rot, Germany) as instructed in the manufacturer's protocol. cDNA was checked spectrophotometrically (Nanotrop, Peqlab, Erlangen, Germany) and by electrophoresis on a 1.5% agarose gel. Reproducibility was ensured by two independent repeats of the whole procedure.



Primer		Sequence 5' to 3'
JSex1-DRB	*Ex1*	GCTCCYGGATGRCAGCTCTGA
JSex3-DRB	*Ex3*	AGAGCAGACCAGGAGGTTGTG
DRB-L729	*L729*	ACTCAMCATCTTGCTCTG
JSN2-DRB	*Ex2a*	GAGTGTCATTTCTMCAAYGGGAC
JSN2R-DRB	*Ex2b*	GTGCGCTTCGACAGCRACGT
JSi2N-DRB	*Int2a*	CACACGCACGTACACAAGTACACA
JSi2A-DRB	*Int2b*	CCGCCCCGCCGCGCTCAC
JSi1N-DRB	*Int1a*	GGCGCCCGGCCTGGCCGACGTC
JSi1N2-DRB	*Int1b*	CAGCTGCRTSACGGTGGTTCCTG

Figure 2. Positions and sequences of PCR primers to amplify the indicated fragments of the MHC class II *DRB* gene in *N. albiventris* based on gDNA (A) and cDNA (B). Boxes symbolize exons, dark lines mark introns and dotted lines not sequenced parts of the introns. Open circles indicate positions of ligated vectorettes (see methods for details). The primer *DRB*-L729 is taken from Bowen et al. (2004).

Primer design using cDNA and vectorette PCR

An MHC class II *DRB* cDNA alignment of different mammalian species was constructed by a BLAST search (www.ncbi.nlm.nih.gov) of the GenBank database. Primers complementary to conserved parts of exon1 (*Ex1*) and exon3 (*Ex3*) were designed (Fig. 2). They were used together with primer *L729* situated in exon 4 (Bowen et al. 2004, Fig. 2) in different combinations to amplify *DRB* sequences from the cDNA of *N. albiventris*. PCR amplification was carried out in a total reaction volume of 20 µl including 20 ng cDNA, 1 U of proofreading polymerase (Hotstar Hifidelity polymerase, Qiagen, Hilden, Germany), 1x Hotstar Hifidelity PCR buffer, 0.3 mM dNTPs and 0.4 µM of each primer (Sigma-Aldrich, Steinheim, Germany). Thermocycling started with an initial denaturation step at 96°C for 10 min, followed by 35 cycles of denaturation at 96°C for 45 s, annealing at 58°C for 45 s and elongation at 72°C for 2 min. An additional 15 min elongation step followed the last cycle. PCR products were purified (Cycle pure Kit, Peqlab, Erlangen, Germany) and cloned using the pCR®4-TOPO® TA cloning Kit (Invitrogen, Karlsruhe, Germany). At least 23 recombinant clones per primer combination were amplified using the vector-primers *T7*for and *M13*rev, sequenced

using BigDye Terminator v.3.1 Cycle Sequencing Kit (ABI) and analyzed on ABI PRISM 310 Automated Genetic Analyzer (Applied Biosystems, Foster City, Ca USA). All primer combinations amplified the same two MHC class II *DRB* alleles in *N. albiventris*. These *NoaI*-sequences were used to design the species-specific primers *Ex2a* and *Ex2b* (Fig. 2) binding to conserved sites of exon 2 (inferred from the established cDNA alignment and available sequences of the bat *Saccopteryx bilineata* (GenBank accession numbers: EF533888-EF533900, Mayer and Brunner 2007) which were used for the subsequent first vectorette PCR essay.

Vectorette PCR is a method to amplify DNA fragments of interest where the sequence information is only available on one side and is described in detail by Ko and co-workers (2003). We constructed vectorette libraries with gDNA of five individuals. Up to 5 µg of gDNA due to available template of each animal were digested with restriction enzymes EcoRI, XapI, Fsp and CspI (Fast Digest™, Fermentas, St. Leon-Rot, Germany). 1 to 5 µg gDNA was digested by 1 to 5 U restriction enzyme in a total volume of 50 or 100 µl depending on the amount of gDNA. Double-stranded vectorettes (1 µM) consisting of vect53 and vect57TTAA (EcoRI, XapI) or vect53 and vect57AT (Fsp and CspI) (Ko et al. 2003) were ligated to the sticky ends of the digested gDNA using 2 U of T4DNA ligase per 1 µg digested gDNA (Rapid DNA Ligation Kit, Fermentas, St. Leon-Rot, Germany).

Vectorette PCR essays consisted of two PCRs. The first PCR was followed by a second nested PCR to avoid false positives. A step-down scheme was always applied using Hotstar *Taq* Master Mix (Qiagen, Hilden, Germany) according to the user's manual in a total reaction volume of 25 µl with following conditions: initial activation of Hotstar *Taq* and denaturation at 95°C for 14 min, followed by 5 cycles of denaturation at 95°C for 60 s, annealing at 67°C for 60 s and elongation at 72°C for 2 min, five cycles with annealing at 63°C, followed by 15 cycles denaturation at 95°C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 120 s, 15 cycles with annealing at 57°C and a final extension at 72°C for 10 min. Amplification products were checked on a 1.5% agarose gel and purified with cycle Pure Kit (Peqlab, Erlangen, Germany).

The first vectorette PCR essay was performed with the primer *Ex2a* and the vectorette primer *C20* (Ko et al. 2003) and the nested vectorette PCR included the nested primer *Ex2b* in combination with the nested vectorette primer *B21* (Ko et al. 2003). The resulting intron 2 sequences allowed the design of *Noctilio*-specific intron 2 primers (*Int2a* and *Int2b*) (Fig. 2). These were used to amplify intron 1 sequences in another vectorette-PCR essay. Finally, according to the intron 1 sequences the primers *Int1a* and *Int1b* were developed. *Int1a* and *Int2a* were used to amplify the whole 270 bp MHC

DRB class II exon 2 and partial introns (45 bp in intron1, 48 bp in intron2, Fig. 2) and were applied to genotype all 215 *N. albiventris* individuals.

Genotyping and allele identification

Amplification was performed in 25 µl volumes containing 0.4 µM of each primer (Sigma-Aldrich, Steinheim, Germany), 1x reaction buffer (10 mM TrisHCl, 50 mM KCl, 0.1% Triton x100, 0.2 mg/ml BSA), 0.2 mM of each dNTP and 1 U *Taq* polymerase (MPBiomedicals, Heidelberg, Germany). Step down PCR was performed as follows: initial incubation at 95°C for 5 min followed by 5 cycles of denaturation at 95°C for 10 s, annealing at 67°C for 10 s and elongation at 72°C for 30 s, five cycles with annealing at 64°C and 25 cycles denaturation at 95°C for 10 s, annealing at 60°C for 10 s and elongation at 72°C for 30 s, final extension was at 72°C for 3 min. Amplicons were genotyped by single strand confirmation polymorphism (SSCP) on a polyacrylamide gel as described in Schad et al. (2004). Allele identification was done by excising the single strands from the gel matrix and diluting them in dH_2O. Then a re-amplification of diluted single strands with primers *Int1a* and *Int2a* was done prior to sequence analyses as described above. An autonomous amplicon with primer *Int1b* and *Int2b* of each individual was directly sequenced to confirm the individual SSCP pattern.

Statistical analyses

We edited and aligned nucleotide sequences manually using MEGA 4.0 (Tamura et al. 2007). We also used this program for calculating the p-distance of amino acid sequences as a measurement of functional MHC class II *DRB* divergence (Nei and Kumar 2000) and for analyzing the relative rates of non-synonymous (d_N) and synonymous (d_S) base pair substitutions according to Nei and Gojobori (1986) applying the Jukes-Cantor correction for multiple hits (Jukes and Cantor 1969). Calculations were applied for all sites and separately for putative antigen binding sites (ABS) and non-ABS assuming functional congruence to human ABS of the *HLA-DR1* (*DRA*/*DRB1**0101) molecule (Brown et al. 1993). The d_N/d_S ratios of all sites, as well as for ABS and non-ABS separately, were compared with an implemented Z-test (Nei and Kumar 2000) to test for positive selection.

We identified species-specific positively selected sites (PSS) with maximum-likelihood analysis using CODEML (included in PAML version 3.15 software package; Yang 1997, 2007) and compared these PSS with the human ABS. First, we fitted models with different assumptions of selection patterns to the sequence data. We used the models M7 (beta) and M8 (beta and ω) as described in Yang et al. (2007). M7 served as a null model where the ω ratio varies according to the beta distribution and does not allow

positive selected sites ($0<\omega<1$). M8 adds a class of sites to account for the possible occurrence of positively selected sites ($\omega>1$). The models were compared using a likelihood ratio test (LRT) by calculating the likelihood difference $2\Delta l = 2(l_1-l_0)$ and then compared to a X^2-distribution with the degrees of freedom equal to the difference in the number of estimated parameters (Yang and Bielawski 2000). In the next step, after LRT provided evidence for positive selection, the Bayes empirical Bayes method (BEB; Yang et al. 2005) integrated in CODEML was used to identify the sites under positive selection with the cut-off posterior probability set at $P_b = 95\%$.

Allele frequencies, observed and expected heterozygosity, and deviation from Hardy-Weinberg expectations were estimated using the software ARLEQUIN version 3.0 (Excoffier and Schneider 2005). Pairwise F_{ST} based on haplotype frequencies were calculated to infer population subdivision (10,000 permutations, Wright 1951, 1965). Allelic richness (*R*) as a measure of the number of alleles independent of sample size was estimated using the rarefaction method as implemented in FSTAT version 2.9.3 (Goudet 2001). Chi-square tests were used to compare the number of heterozygote individuals between the sexes. Differences in the mean individual amino acid distance of males and females were investigated by ANOVA. Calculations always were two-tailed with significance level at $\alpha = 0.05$ and performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Bonferroni corrected significant levels were applied for multiple comparisons (Rice 1989).

Results

Capture and sampling

In total we caught 215 individuals (91 males, 123 females), 185 in Gamboa (59 males, 108 females) and 29 on BCI (22 males, 7 females). All of them were genotyped for the MHC class II *DRB* gene. In Gamboa, we caught bats from seven roosts. We included only colonies with more than ten individuals in statistical analyses (Colony A: N = 52, 22 males, 30 females; Colony B: N = 74, 20 males, 54 females; Colony C: N = 27, 13 males, 14 females; Colony D: N = 14, 4 males, 10 females).

MHC class II *DRB* diversity

Using the cDNA of one male *N. albiventris,* all primer combinations amplified the same two MHC class II *DRB* alleles differing only in fragment length (628 bp, 527 bp and 376 bp respectively, Fig. 2). We designed species-specific intron primers (*Int1a* and *Int2a*)

Table 1. Allele frequencies of MHC class II *DRB* alleles in the whole population and in subpopulations, roosting colonies and sexes. Sample size (N).

Level	All	*Subpopulation*		*Roosting Colonies*				*Sexes*	
Category		BCI	Gamboa	A	B	C	D	Females	Males
N	215	29	185	52	74	27	14	123	91
***Noal-DRB**01**	0.040	0.069	0.035	0.048	0.027	0.056	0.036	0.024	0.060
***Noal-DRB**02**	0.175	0.172	0.176	0.154	0.182	0.296	0.072	0.175	0.176
***Noal-DRB**03**	0.009	-	0.012	0.010	0.014	-	-	0.012	0.006
***Noal-DRB**04**	0.117	0.138	0.113	0.135	0.095	0.056	0.143	0.106	0.124
***Noal-DRB**05**	0.044	0.017	0.049	0.087	0.054	0.019	-	0.045	0.044
***Noal-DRB**06**	0.040	0.069	0.035	0.039	0.020	0.074	0.072	0.049	0.026
***Noal-DRB**07**	0.005	-	0.005	0.010	0.007	-	-	0.004	0.006
***Noal-DRB**08**	0.042	0.034	0.043	0.087	0.027	0.019	0.036	0.033	0.055
***Noal-DRB**09**	0.054	0.034	0.057	0.029	0.081	0.037	0.036	0.057	0.050
***Noal-DRB**10**	0.255	0.190	0.264	0.183	0.297	0.259	0.464	0.293	0.209
***Noal-DRB**11**	0.028	0.034	0.027	0.039	0.027	-	-	0.012	0.050
***Noal-DRB**12**	0.088	0.172	0.076	0.087	0.061	0.074	0.071	0.081	0.100
***Noal-DRB**13**	0.028	-	0.032	0.019	0.014	0.037	0.071	0.029	0.022
***Noal-DRB**14**	0.005	0.017	0.003	-	0.007	-	-	0.004	0.006
***Noal-DRB**15**	0.005	-	0.008	-	0.007	0.019	-	0.008	-
***Noal-DRB**16**	0.007	-	0.007	0.010	0.014	0.019	-	0.008	0.011
***Noal-DRB**17**	0.044	0.017	0.049	0.058	0.054	0.037	-	0.053	0.033
***Noal-DRB**18**	0.014	0.034	0.012	0.010	0.013	-	-	0.008	0.022

BCI: Barro Colorado Island

for amplification of the whole MHC class II *DRB* exon 2 (270 bp) of *N. albiventris* flanked by short intron sequences with a total length of 363 bp (Fig. 2) by using the vectorette PCR approach based on gDNA. No indels or stop codons were found and never more than two alleles per individual were amplified suggesting that a single *DRB* locus was expressed. We named this locus *MhcNoal-DRB* according to the established MHC nomenclature (Klein et al. 1990; Ellis et al. 2006). The nucleotide and deduced amino acid sequence showed high homology with human (84%), canine (83%), equine (83%) and other mammalian class II *DRB* genes.

We detected 18 different alleles of *Noal-DRB* in the 215 individuals of *N. albiventris* (GenBank accession numbers: HM347941-HM347958). In the nucleotide sequences, we observed 71 (26.3%) variable positions and the alleles differed by two to 40 (average 26.3 ± 2.9) nucleotide positions. All alleles had a unique amino acid sequence, whereas 38 (43%) out of 89 amino acids were polymorphic. They differed by one to 26 (average 17.6 ± 2.5) amino acid positions. The most common allele *Noal-DRB**10 occurred at a frequency of 0.255, followed by *Noal-DRB**02 (0.175) and *Noal-*

Table 2. MHC class II *DRB* exon 2 variability in *N. albiventris* in the whole population and in subpopulations, roosting colonies and sexes. Sample size (N), number of alleles (#), allelic richness (*R*) adjusted to the smallest sample size per level, observed (H_{obs}) and expected (H_{exp}) heterozygosity and the mean individual amino acid distance between alleles (Ind Dist ± standard error) are shown.

Level	Category	N	# Alleles	*R*	H_{obs}	H_{exp}	Ind Dist
All		215	18		0.902	0.871	0.177 ± 0.005
Subpopulation	**BCI**	29	13	13	0.931	0.886	0.189 ± 0.013
	Gamboa	185	18	13.6	0.817	0.868	0.175 ± 0.006
Roosting Colonies	**A**	52	16	11.1	0.981	0.901	0.190 ± 0.009
	B	74	18	10.4	0.838	0.856	0.164 ± 0.010
	C	27	13	10.1	0.889	0.839	0.166 ± 0.016
	D	14	9	9.0	0.857	0.767	0.171 ± 0.022
Sexes	**Females**	123	18	17.2	0.854	0.856	0.164 ± 0.008
	Males	93	17	17.0	0.967	0.888	0.195 ± 0.006

BCI: Barro Colorado Island

*DRB**04 (0.117). The remaining alleles occurred in two to 36 individuals with a frequency ranging between 0.005 and 0.089 (Table1). Observed heterozygosity (0.902) was higher than expected (0.871) and did not deviate from Hardy-Weinberg expectations (Table 2). The individual *Noal-DRB* exon 2 distance of an individual based on amino acid sequence ranged from zero (homozygote) to 0.287 with an average of 0.177 ± 0.078 (Table 2).

Evidence for historical selection

Two approaches were used to test for historical positive selection acting on the examined exon 2 of *Noal-DRB* locus. First, the averaged rates of non-synonymous (d_N) and synonymous (d_S) base pair substitutions of all sequences were calculated for all sites and separately for putative ABS and non-ABS assuming functional congruence to human ABS of the *HLA-DR1* molecule (Brown et al. 1993). Non-synonymous substitutions occurred at a significantly higher rate than synonymous ones (d_N/d_S =

Table 3. Non-synonymous (d_N) and synonymous (d_S) substitutions (± standard error) as well as their ratio in antigen binding (ABS) and non-antigen binding sites (non-ABS) assuming concordance with the human *HLA-DR1* molecule (Brown et al. 1993). N is the number of codons in each category. P is the probability ($\alpha \leq 0.05$) that d_N and d_S are different using a Z-test; ns, not significant.

Region	N	d_N	d_S	d_N/d_S	P
ABS	25	0.353 ± 0.077	0.112 ± 0.044	3.139	0.002
Non-ABS	64	0.053 ± 0.013	0.034 ± 0.018	0.942	ns
All	89	0.124 ± 0.021	0.055 ± 0.018	2.904	0.004

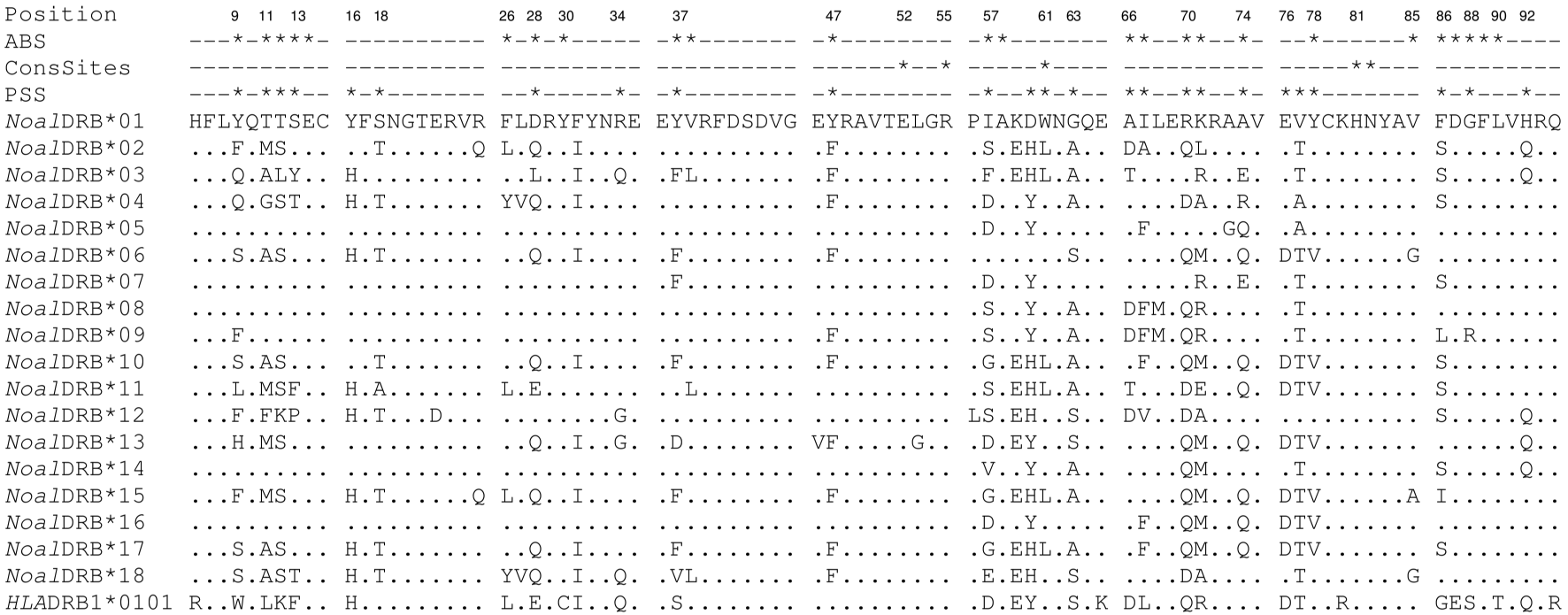


Figure 3. Amino acid sequence variation of 18 MHC class II *DRB* exon 2 alleles of *N. albiventris and the human HLA-DRB1*0101 sequence*. Dots mark identity with the top sequence. Numbers indicate the amino acid positions of the β1-domain, * signify the amino acid positions of antigen binding sites (ABS) and conserved sites (ConsSites) of the human *HLA-DR1* β-chain (Brown et al. 1993, Stern et al. 1994). PSS indicate species-specific positive selected sites identified by CODEML (Yang et al. 2005).

Table 4. Identification of species-specific positively selection sites (PSS) by likelihood analysis in MHC class II *DRB* exon 2 amino acid sequences of *N. albiventris*. Numbers indicate the amino acid positions in the β1-domain. Mean ω indicates the ratio of non-synonymous and synonymous substitutions at PSS and its probability (P_b (ω >1)) using a cut-off posterior probability of 95%. Distance (amino acids) to nearest human ABS of the *HLA-DR1* β-chain (Brown et al. 1993) is shown. ** α ≤ 0.01, * α ≤ 0.05.

PSS	mean ω	P_b (ω >1)	Dist. to ABS
9	4.561 ± 0.496	1.000**	**0**
11	4.561 ± 0.496	1.000**	**0**
12	4.490 ± 0.693	0.982*	**0**
13	4.556 ± 0.514	0.999**	**0**
16	3.908 ± 1.434	0.836	2
18	4.353 ± 0.950	0.948	4
28	4.464 ± 0.740	0.976*	**0**
34	3.633 ± 1.604	0.768	4
37	4.552 ± 0.526	0.998**	**0**
47	3.460 ± 1.677	0.724	0
57	4.561 ± 0.496	1.000**	**0**
60	3.733 ± 1.530	0.793	1
61	4.512 ± 0.643	0.988*	**1**
63	4.462 ± 0.747	0.975*	**0**
66	4.334 ± 0.970	0.944	0
67	4.524 ± 0.604	0.991**	**0**
70	4.560 ±0.493	1.000**	**0**
71	4.561 ± 0.496	1.000**	**0**
74	4.561 ± 0.496	1.000**	**0**
76	3.581 ± 1.650	0.755	2
77	4.529 ± 0.592	0.992**	**1**
78	4.559 ± 0.503	1.000**	**0**
86	4.561 ± 0.496	1.000**	**0**
88	3.646 ± 1.731	0.778	0
92	4.545 ± 0.549	0.996**	**2**

2.90, Z-test, p = 0.004) especially in the regions that code for ABS (d_N/d_S = 3.14, Z-test, p = 0.002; Table 3).

Second, we observed a significantly higher log likelihood estimate (2Δl = 59.67, df = 2, p < 0.0001) for model M8 (positive selection) than for its corresponding null model M7 (no positive selection). The Bayes empirical Bayes approach under model M8 inferred 17 significant sites to be under positive selection (PSS) with the cut-off posterior probability set at 95%. Fourteen of these sites were congruent with predicted ABS of the human *HLA-DR1* β-chain (Brown et al. 1993). The other three sites were located in close proximity, within one to two amino acid positions to the human ABS (Table 4, Fig.

3). In the *Noal-DRB* sequences all but one (61 W) of certain amino acid positions, which are conserved in human *HLA-DR1* molecules (Brown et al. 1993; Stern et al. 1994), were also conserved presenting identical amino acids. At position 61, tryptophan was replaced by leucine in six *Noal*-alleles (33.3%) and identified as PSS (Table 4, Fig. 3).

Population structure and MHC

We observed no genetic differentiation between the two subpopulations of Gamboa and BCI (F_{ST} = 0.003, p = 0.23). Allele frequencies of the two subpopulations are shown in Table 1. Numbers of alleles were similar after correction for differences in sample size. Observed heterozygosity was high in both subpopulations without a significant deviation from Hardy-Weinberg expectations (Table 2).

All four roosting colonies with more than ten individuals captured in the village Gamboa showed high levels of heterozygosity and allelic richness (*R*) ranging between 9 and 11.1. Observed heterozygosity exceeded the expected value in all but one colony (colony B) and did not deviate significantly from Hardy-Weinberg equilibrium (Table 2). Pairwise F_{ST} statistics showed a slight differentiation between colonies A, C and D, but this significance was lost after Bonferroni correction (α' ≤ 0.008; Table 5).

Table 5. Pairwise differentiation between roosting colonies using conventional F-statistic based on haplotype frequencies (Wright, 1965). F_{ST}-values are provided below the diagonal and corresponding p-values above diagonal. Not significant (ns), Bonferroni corrected significance level α'≤ 0.008.

Colony	A	B	C	D
A		ns	ns	0.010
B	0.006		ns	ns
C	0.012	0.002		0.031
D	0.036	0.013	0.036	

We found no significant differentiation within males and females when comparing colonies (males: range of F_{ST}: 0.001 - 0.026, p-values not significant; females: F_{ST}: < 0.001 - 0.039, p-values not significant; Bonferroni corrected significance level α'≤ 0.008). We found no significant differentiation between males and females within colonies either (F_{ST}A = 0.009, p = 0.87; F_{ST}B = 0.001, p = 0.44; F_{ST}C = 0.007, p = 0.54; F_{ST}D = 0.030, p = 0.82; F_{ST} All = 0.002, p= 0.87).

However, males and females differed significantly in their individual amino acid distance (Anova: F = 8.48, p = 0.004, df = 1, Fig. 4, Table 2). Furthermore, the

observed heterozygosity of males was significantly higher than in females (overall: χ^2 = 7.73, df = 1, p = 0.005). The analyses were not significant when colonies were analysed separately (A: χ^2 = 0.81, p = 0.36; B: χ^2 = 2.54, p = 0.11; C: χ^2 = 3.13, p = 0.07; D: χ^2 = 0.93, p = 0.33). In males, the observed heterozygosity exceeded the expected value in the overall sample as well as in all colonies analysed separately but did not deviate significantly from Hardy-Weinberg equilibrium (overall: H_{obs} = 0.97, H_{exp} = 0.89; A: H_{obs} = 1.00, H_{exp} = 0.91; B: H_{obs} = 0.96, H_{exp} = 0.89; C: H_{obs} = 1.00, H_{exp} = 0.88; D: H_{obs} = 1.00, H_{exp} = 0.89; BCI: H_{obs} = 1.00, H_{exp} = 0.89). In females the observed heterozygosity was always lower than in males but close to the expected value in the overall sample as well as in two out of four colonies (overall: H_{obs} = 0.85, H_{exp} = 0.86; A: H_{obs} = 0.96, H_{exp} = 0.90; B: H_{obs} = 0.83, H_{exp} = 0.86; C: H_{obs} = 0.78, H_{exp} = 0.79; D: H_{obs} = 0.75, H_{exp} = 0.75; BCI: H_{obs} = 1.00, H_{exp} = 0.92; Table 2).

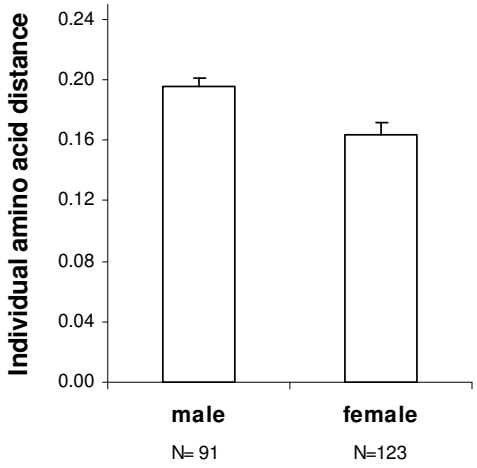


Figure 4. Mean (±standard error) amino acid distance between individual MHC class II *DRB* exon 2 alleles of *N. albiventris* in males and females.

Discussion

Up to date not much is known about the genetic structure and polymorphism of the MHC in bats (Mayer and Brunner 2007) mainly due to a lack of sequence data for primer design. This is astonishing given the size of the order, the importance of olfactory signals for social communication in bats and their zoonotic relevance as reservoir hosts for many pathogens. Therefore, our primary aim was to determine MHC class II *DRB* exon 2 diversity in the lesser bulldog bat, *N. albiventris*. Further, we tested for evidence of positive selection acting over the evolutionary history of the species.

And third, we were interested whether local adaptation and sexual selection may shape the contemporary genetic structure of *N. albiventris* in a Central American population.

MHC class II *DRB* diversity

Well-designed primers are essential for population genetic analyses. In this study, cDNA analyses followed by a vectorette PCR approach offered the possibility of intron-mapping. Species-specific primers binding to conserved segments of the flanking introns of *N. albiventris* were designed to amplify the whole *DRB* exon 2. The reliability of the resulting species-specific sequence patterns of the locus of interest is very high, because the incidence of non-amplifying alleles can be neglected. This might turn into a problem when exon primers are used to amplify parts of polymorphic genes. The occurrence of non-amplifying alleles would preclude the use of data for many population genetic purposes, because they can seriously bias population genetic analyses (Cummings et al. 2010; Dakin and Avise 2004).

We found evidence for a single expressed MHC class II *DRB* locus in *N. albiventris* with moderate allelic-variability of 18 alleles detected in 215 individuals. This allelic-variability is within the range of MHC class II *DRB* polymorphism of other mammalian species (e.g. Sommer 2005). The only other *DRB* gene studied in a bat, *Saccopteryx bilineata*, revealed a rather low allelic variability (11 alleles in 85 individuals; Mayer and Brunner 2007). However, this might be an underestimate of the actual variability, because preliminary studies on RNA and DNA with species-specific developed primers revealed evidence for at least 5 *DRB* loci in *S bilineata*. (Schad et al. unpublished data). In general, the occurrence of different MHC class II genes (*DP*, *DO*, *DM*, *DQ* and *DR*) is conserved in mammals. But the number of functional alpha and beta genes is highly variable due to species-specific local duplication events. It can even vary between individuals of the same species (see Kumanovics et al. 2003). The *DRB* is the most widely studied and usually the most diverse class II gene, not only with respect to high allelic richness but also in terms of gene duplications (e.g. Doxiades et al. 2000; Bowen et al. 2004; Babik et al. 2005; Schwensow et al. 2007). We cannot rule out that other MHC class II genes may provide a higher variability in bats as it is described for instance in the cetacean MHC class II *DQB* gene (Baker et al. 2006). As a future task it will be necessary to investigate other loci to evaluate MHC diversity in bats more comprehensively.

Evidence for historical selection

We found a higher rate of non-synonymous versus synonymous nucleotide substitutions over the entire sequence and especially in the putative ABS but not in non-ABS (Brown et al. 1993). Such elevated d_N/d_S-ratios are a widely accepted sign for historical positive selection in polymorphic MHC genes (Hughes and Nei 1988, 1989; Nei and Kummar 2000; Nielsen 2001; Hughes 2007). They indicate positive selection on ABS acting over the evolutionary history of the species (Hedrick 1999; Bernatchez and Landry 2003; Sommer 2005). We subsequently confirmed these results in a species-specific analysis where different codon evolution models were compared by maximum likelihood analyses (Yang 2007; Yang and Bielawski 2000). This method has been suggested as a powerful tool with a high likelihood of detecting effects of positive selection (Wong et al. 2004). Our data fitted best with the model incorporating positive selection and 17 species-specific positively selected sites (PSS) were identified by the Bayes empirical Bayes analysis. Fourteen were congruent with human ABS of the *HLA-DR1* β-chain. Three PSS were situated outside the human ABS and some of the human ABS were not identified as PSS in *Noal-DRB*. Comparable results have been reported from other species (Kundu and Faulkes 2004; Schwensow et al. 2007; Meyer-Lucht et al. 2008; Babik et al. 2008). In general, high congruence of positive selected sites with human ABS is assumed to demonstrate homologous functionality of the molecule. Contrarily, human ABS which are not identified as PSS might be not involved in the antigen recognition and binding in the respective species. Furthermore, in most species investigated so far, additional PSS have been reported. These findings suggest species-specific selection pressure acting on MHC genes due to a different pathogen exposure. In addition, certain amino acid residuals of *HLA-DR1* molecules are highly conserved and involved in universal hydrogen bond of antigens (61 W, 81 H, 82 N) or are responsible for the stability of *DR1* heterodimers in building salt bridges between the dimers (52 E, 55 R) (Brown et al. 1993; Stern et al. 1994). Also in the *Noal-DRB* sequences all of these positions, except position 61, were conserved indicating similar conserved functionality of the molecules.

Population structure and MHC

Mating behaviour as a correlate and driver of social structure has been suggested in addition to pathogens as another main subject of selection on MHC loci in natural populations (Hambuch and Lacey 2002; Kundu and Faulkes 2004; Cutrera and Lacey 2006). Animals form social groups and colonies in response to cooperative interactions as well as mating tactics. Furthermore, patterns of genetic subdivision are also shaped

by the extent and nature of philopatric behaviour (Travis et al. 1995, Sommer et al. 2002; Solomon 2003; Cutrera and Lacey 2006). We examined the genetic structure of three different levels of social formations or units (subpopulation, roosting colonies, sexes) in our population of *N. albiventris*. We found no genetic differentiation between the two subpopulations (Gamboa, BCI, separated by 15 km), indicating the presence of gene flow at a larger spatial scale. Both subpopulations showed similar levels of polymorphism at the *Noal-DRB* locus. Observed heterozygosity was high in the two subpopulations, and the allele frequencies and the number of alleles were similar after correcting for differences in sample sizes. This implies an equivalent selection pressure maintaining diversity at the *Noal-DRB* locus in both subpopulations in the recent past.

To investigate social structure based on roosting habits, we compared four colonies in the village Gamboa all located in the range of 1.5 km^2. All colonies showed similar levels of genetic variation (heterozygosity, allelic richness and allele frequencies). F-Statistics revealed only limited effects of subdivisions. The colony D showed a slight differentiation compared to the colonies A and C (not significant after Bonferroni correction) which might be rather the result of missing rare alleles due to small sample size than an effect of population structure based on roosting habits. Dechmann and co-workers (2009) distinguished social groups of females (2-5 individuals) by the fact that they emerged simultaneously from a roost. We could not find genetic differences between male and female members of different colonies. The composition of colonies might vary in time indicating a fission-fusion society rather than stable associations. Neutral markers would offer the possibility to gain insights into population dynamic processes like kin-relationships of social groups of females and roosting colonies. Thus, further ecological studies on demographic structure as well as genetic analyses adding neutral markers will help to fully understand the social system of this species.

We tested for gender-specific differences in the MHC constitution to assess the occurrence of sexual selection. We found no population differentiation between males and females. However, in the overall sample males showed a significantly higher heterozygosity rate and also a higher individual amino acid distance than females. In males the observed heterozygosity exceeded the expected value in the overall sample as well as in all within-colony comparisons. In females the observed heterozygosities were always lower than in males but were almost identical with the expected ones in the overall sample and in two out of four colonies. As in both sexes observed and expected heterozygosities did not significantly deviate from Hardy-Weinberg expectations it remains unclear whether the difference between sexes is due to a higher heterozygosity rate in males or a lower heterozygosity rate in females. An

increased heterozygosity rate in males could indicate balancing selection in form of a heterozygote advantage and a deficit in heterozygote females could suggest a reduced selection pressure to maintain diversity in females. It might be that the selection intensity in the investigated *Noctilio* population is not strong enough to detect significant deviations from Hardy Weinberg expectations. It is well known that levels of allelic diversity in relation to sample size have an effect on the statistical power to detect significant deviations from Hardy Weinberg expectations (Seddon and Ellgren 2004). We did not find small scale population structure that would offer a likely explanation for this gender specific difference. Male based long-distance dispersal is unlikely as it would promote the occurrence of new alleles in the male population raising the overall polymorphism at the MHC locus which we did not observe. A methodological error due to DNA quality differences between the sexes seems also to be unlikely because collection and treatment of samples have been the same throughout the study. While direct female choice for heterozygote males would lead to heterozygote offspring in general including daughters it cannot explain the sex-specific bias in heterozygosity. Currently, the differences in heterozygosity in males and females is most likely due to MHC mediated post-copulatory mechanisms (e.g. caused by myotic drive, gametic selection and maternal fetal interactions) or by sex-specific survival differences which result in an increased rate of MHC heterozygote males or a deficit in heterozygote females.

The possibility of the existence of post-copulatory mechanisms resulting in sex-specific differences in MHC heterozygosity was reported only in a few studies so far. Dorak and co-workers (2002) found an increased heterozygosity for MHC class II *DRB* lineages in newborn male babies and suggested that negative selection of homozygotes might be restricted to male offspring only. A deficit in MHC homozygosity in newborn males was also observed in mice (Hamilton and Hellstrom 1978) and rats (Palm 1969, 1970, 1974). Some studies in humans investigated the compatibility at different HLA loci between mothers and infants and observed differences in the sex ratio assuming a different fetal loss in males and females. The results are heterogeneous and differ between *HLA* loci (Ober et al. 1987; Astolfi et al. 1990, 1996). In all of these studies the underlying mechanisms have not been investigated. The ongoing discussion has been reviewed by Fernandez et al. (1999) and Ziegler et al. (2005). The overall conclusion is that the MHC is critical for numerous aspects of mammalian reproduction concerning spermatogenesis (Ziegler et al. 2002, 2005), a sperm selective egg-cumulus complex (Wedekind et al.1996; Rülike et al. 1998; Eisenach and Giojalas 2006) and viability and development of the foetus (Gill 1992; Ober et al. 1987; Astolfi et al. 1990; Wedekind et

al. 1996; Ziegler et al. 2005). However, in all these processes the selective forces might act due to MHC-linked genes, e.g. olfactory receptor genes, transcription factors and others, rather than by the MHC itself (Ho et al. 1990; Gill 1992; Ziegler et al. 2002, 2005; Eisenach and Giojalas 2006). To the best of our knowledge sex-specific differences in offspring survival based on MHC heterozygosity have not been reported yet. But they have been investigated using a microsatellite-based measure of outbreeding (mean d^2) in a few species. In the bat *Rhinolophus ferrumequinum* outbreeding was positively associated with significantly increased survival in male offspring only (Rossiter et al. 2001). The authors postulate that outbreeding at the microsatellite markers reflects immunocompetence, which in turn influences mortality. They also suggest that characterization of MHC loci may provide a suitable test for their hypothesis. Even so, in the red deer (Cervus elaphus) a contrary pattern was found as male offspring survival was negatively associated with outbreeding at microsatellite markers (Coulson et al. 1999). In mice a reduced survivorship was reported for inbreed adult males but not for females most likely as a consequence of males aggressive interactions in the defence of territories (Meagher et al. 2000). Ongoing studies might reveal the mechanisms which have contributed to the sex biased diversity pattern in the investigated population of *N. albiventris.* In addition, neutral markers would provide more detailed information about social structure, dispersal behaviour and gene flow of both sexes.

In this first study on the MHC class II variability of the lesser bulldog bat we detected high genetic variation and evidence for historical positive selection acting on a single expressed *Noal-DRB* locus. The polymorphism at the antigen binding region of the molecule is considered as the pre-condition to cope with a variety of pathogens. No population differentiation between subpopulations, roosting colonies and sexes was observed, but males revealed a significantly higher heterozygosity rate and genetic variability in terms of the genetic distance between the individual MHC alleles than females. We are aware that at this state of the investigation no conclusions on the underlying mechanisms can be made, but our data will lay the basis for further research on the role of the MHC constitution in host-pathogen interactions, individual body odours and sexual selection in a highly interesting bat species.

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Evidence for the 'good genes' model: association of MHC class II *DRB* alleles with ectoparasitism and reproductive state in the neotropical lesser bulldog bat, *Noctilio albiventris*

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Abstract

The adaptive immune system has a major impact on parasite resistance and life history strategies. Immunological defence is costly both in terms of immediate activation and long-term maintenance. The 'good genes' model predicts that males with genotypes that promote a good disease resistance have the ability to allocate more resources to reproductive effort which favours the transmission of good alleles into future generations. Our study shows a correlation between immune gene constitution (Major Histocompatibility Complex, MHC class II *DRB*), ectoparasite loads (ticks and bat flies) and the reproductive state in a neotropical bat, *Noctilio albiventris*. Infestation rates with ectoparasites were linked to specific *Noal-DRB* alleles, differed among roosts, increased with body size and co-varied with reproductive state particularly in males. Non-reproductive adult males were more infested with ectoparasites than reproductively active males, and they had more often an allele (*Noal-DRB**02) associated with a higher tick infestation than reproductively active males or subadults. We conclude that the individual immune gene constitution affects ectoparasite susceptibility, and contributes to fitness relevant trade-offs in male *N. albiventris* as suggested by the 'good genes' model.

[1] *Authors' contributions: The study was conceived by S. Sommer and me. I conducted laboratory procedures, statistical analyses, data interpretation, and drafted the manuscript. S. Sommer initiated the collaboration, supervised the research, and revised the manuscript. D. Dechmann collected the samples and together with C. Voigt contributed to discussion of the results and provided comments and suggestions on the manuscript.*

Introduction

The possible involvement of immune function in trade-offs with life-history related traits is increasingly being recognized as an important aspect of life-history evolution. In particular, it is expected that competitive allocation of resources occurs between reproductive effort and immunocompetence (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002). In vertebrates, trade-offs between costs (reproductive investment or immunocompetence) and benefits (current or future reproductive success) have to be mediated in both sexes. Investment in reproductive effort may lead to suppressed immune function with the consequence of an increased susceptibility to parasites (Folstad and Karter 1992; Knowles et al. 2009). But evidence has also been reported for a reverse interaction with immune activation restraining reproductive investment especially in males as an effect of reduced testosterone levels (Mc Callum and Trauth 2007; Boonekamp et al. 2008; Greiner et al. 2010). According to the for males developed 'good genes' model, the immune response of individuals with a well adapted immune system to parasites should be less costly leaving more resources to other fitness enhancing traits. Consequently, males with 'good genes' for parasite resistance may tolerate the high costs of reproduction better, leading ultimately to an increased fitness and to a spread of these immune genes into subsequent generations (Scheldon and Verhulst 1996; Folstad and Karter 1992; Hamilton and Zuk 1982).

The most important immune genes in the context of parasite resistance and reproduction are those found in the major histocompatibility complex (MHC; reviewed e.g. Piertney and Oliver 2006). Genes within the MHC are involved in the adaptive immune response and are among the most variable genes in vertebrates (Klein 1986). This polymorphism enables the immune system to recognize an extensive range of extra- (e.g. bacteria, helminths, arthropods via MHC class II genes) and intracellular (e.g. viruses, cancer cells via MHC class I genes) pathogens and is crucial for the immunological fitness within an individual and across animal populations (Bernatchez and Landry 2003; Sommer 2005). Haematophageous ectoparasites induce host immune regulatory and effector pathways, which involve antibodies, complement and cytokines of the innate immune system, as well as antigen-presenting cells and T-lymphocytes of the adaptive immune pathway (Wikel 1996; Andrade et al. 2005). Antigens derived from anticoagulants, antiplatelets, vasodilators and immune-modulaters, which are present in the saliva of ectoparasite arthropods to evade host haemostatic defences, are processed and presented to antigen-specific T-lymphocytes at ectoparasite attachment sites by specific host MHC class II molecules.

Subsequently, T-lymphocytes provide immunoregulatory signals for the production of cell-mediated antibody responses which impair the ability of a constant blood flow throughout the blood meal by inactivating saliva mediated proteins. Acquired resistance to ectoparasite infestation may lead to a reduced feeding time, affects number and viability of ova, and may even cause death of ticks during feeding (Wikel 1996; Milleron et al. 2004). On the other side, for both rapidly feeding insects and slowly feeding ticks the reduction of host immunity to their salivary components enhances the likelihood that a host will be a suitable source of future blood meals driving a co-evolutionary arms race (reviewed Andrade et al. 2005; Wikel 1999; Schoeler and Wikel 2001; Francischetti et al. 2009). Furthermore, in the host immunological mediators contribute to an itch sensation, which stimulates self-grooming (Alexander 1986; Giorgi et al.2001), an important factor in reducing ectoparasite burden (Marshall 1982). Thus, immunologically acquired host resistance to ectoparasite feeding may decrease ectoparasite infestation intensity (Wikel 1996; Andrade et al. 2005; Francischetti et al. 2009). However, immunological defence to haematophagous ectoparasites is costly both in terms of activation and maintenance and is therefore subject to trade-offs among an organism's competing energy requirements (Giorgi et al. 2001; Marshall 1982; Møller 1993).

In addition to the immunological and behavioural defences, ectoparasite abundance is also influenced by environmental factors (temperature and humidity) and host characteristics such as home range, social system, sex, reproductive state, age and body size (Møller 1993; Christe et al. 2000; Altizer et al. 2003; Krasnov et al. 2005). The relevance of these factors in determining ectoparasite abundance is likely to be specific for each host-parasite system (Presley and Willig 2008). Bat ectoparasites spend their entire lives either on the body or in the roosts of their hosts. Thus, for most bat ectoparasites, contact between host individuals is required for host transfer or is restricted to host individuals that inhabit the same roost (Presley and Willig 2008; Patterson et al. 2007). Whereas some ectoparasites may infest different bat species, some show high host specificity, indicating co-evolutionary adaptation processes (Giorgi et al. 2004; Dick and Patterson 2007). Bats provide a favourable opportunity to study effects and adaptive processes between ectoparasites and host immune defence, especially with regard to host's MHC genes.

In a previous study we investigated MHC class II polymorphism in a natural population of the lesser bulldog bat, *Noctilio albiventris*, in Panama. The single expressed highly variable MHC class II *Noal-DRB* locus showed clear signs of selection shaping the diversity pattern (Schad et al. 2011). The population is infested by two main

haematophaegeous ectoparasites, the tick *Ornithodoros hasei* (Argasidae), which is known to infest also other bat species and the host-specific bat fly *Paradyschiria parvuloides* (Streblidae) (Dechmann, personal observation, Hood and Pitocchelli 1983). *Noctilio albiventris* lives in social groups year-round, and these social groups consist commonly of several females and non-reproductive as well as reproductive males (Dechmann, personal observation). Reproductive and non-reproductive adult males are observed throughout the year, which suggests that not all adult males in a population are reproductively active at the same time (Krutzsch 2000). However, the underlying ecological and physiological causes and mechanisms have not been investigated so far. Together these make *N. albiventris* an ideal candidate to investigate the interaction between immune genes, ectoparasite susceptibility and reproductive state.

In this study, we recorded the ectoparasite loads (ticks and bat flies), the reproductive state and MHC class II *DRB* gene variability in several roosts of free-ranging *N. albiventris* and tested predictions of the 'good genes' model. According to the 'good genes' model we expected males with good genes (i.e. the *Noal-DRB* alleles) to have lower parasite loads, allowing them to invest more resources in reproduction. Thus, *Noal-DRB* alleles with a protective effect on ectoparasite burden should be more frequent in reproductive individuals, whereas *Noal-DRB* alleles that associate with high ectoparasite burden should accumulate in non-reproductive individuals.

Methods

Ethics Statement

All capture and handling of animals as well as collection and export of samples was done in concordance with Panamanian laws. Permits were issued from the Panamanian authority Autoridad National del Ambiente (ANAM, SE/A 98-08, SEX/A78-08, SEX/A -138-08) and field work and animal handling was carried out according to the protocol by the Smithsonian Tropical Research Institute – Institutional Animal Care and Use Committee (STRI-IUCAC).

Study site and sampling

From February to June, and September to November of the years 2006-2008, we captured 214 individuals of the lesser bulldog bat, *N. albiventris*, in the village Gamboa (09.07° N, 079.41° W) and on Barro Colorado Island (BCI, 09.10° N, 079.51° W). Both sites are located at the Panama Canal. Bats were captured with mist nets when they emerged at dusk from one of six investigated roosts in Gamboa or when foraging over

the Panama Canal along boat docks on BCI (see Schad et al. 2011). Age class (adult or subadult) was distinguished by illuminating the surface of the extended wing and examining the epiphysal-diaphyseal fusion of the fourth metacarpal-phalangeal joint which is a highly reliable method to qualitatively distinguish between these age categories. Those with open joints were classified as 'subadults' and those with fused joints as 'adults' (Brunet-Rossini and Wilkinson 2009). Bats were sexed and reproductive condition of females was determined by abdominal palpation and by examination of teats as advised by Racey (Racey 2009). They were categorized as 'pregnant' when a foetus was detectable (this condition was probably only recognized when the gestation period was about half over), as 'lactating' when milk could be expressed from the nipples and as 'non-reproductive' when neither was observed (Racey 2009). Reproductive status in males is usually evaluated by externally visible changes in testicular and epididymal size, which are thought to signal reproductive readiness (Krutzsch 2000; Racey 2009). In *N. albiventris* testes are temporarily enlarged and thought to indicate reproductive readiness. Simultaneously an important secondary sexual trait was considered. Glandular cells in inguinal pockets of the scrotum, visible only when testes are enlarged, produce a male specific odour, which is used most probably for sexual displays (Hood and Pitocchelli 1983; Studier and Lavoie 1984). Accordingly, males were considered as 'reproductively' active when testes were distended and inguinal pockets visible. All other adult males were categorized as 'non-reproductive' (Racey 2009).

Body mass of bats was measured by using a handheld balance (accuracy ± 0.5 g). Body mass does not correlate linearly with body surface area, which is the measure of interest when analyzing ectoparasite abundance. Body surface area of small mammal species can be estimated by scaling the body mass to the power of 2/3 (Heusner 1985; Glazier 2005). Hence, we used $bodymass^{2/3}$ as a proxy of body surface area to quantify linear relationships between ectoparasite abundance and host body size in our analyses (see Presley 2007; Presley and Willig 2008). From all bats, we collected a 4-mm skin sample from the wing membrane using a sterile biopsy punch (Worthington-Wilmer and Baratt 1996). Skin samples were stored in 96% ethanol until DNA isolation.

Parasite Screening

Direct counts of large ectoparasites (bat flies, ticks) were conducted for each captured *N. albiventris*. Bat flies were counted visually by removal from the bat. In order to minimize handling time, we could not verify minute diagnostic characters on every ectoparasite specimen counted. Thus, we recorded numbers of bat flies per bat. Ticks

almost exclusively occurred on the naked surfaces of the wing and tail membrane and reached extremely high numbers. Again to minimize handling time counts were restricted to a representative area, the upper surface of the dorsal uropatagium. Voucher specimens of ectoparasites were collected opportunistically and stored in 70% ethanol. Ectoparasites were identified using dichotomous keys (Wenzel and Tipton 1966; Wenzel 1976; Dick and Miller 2010), and voucher samples were verified by L. Durden (ticks) and by C.W. Dick (bat flies).

Molecular techniques

The molecular techniques to investigate MHC class II *DRB* variability have been described in detail elsewhere (Schad et al. 2011). Briefly, we extracted DNA from tissue sample using DNeasy Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. We used primers *JSi1N-DRB* and *JSi2N-DRB* which amplify the whole 270 bp MHC *DRB* class II exon 2 and partial introns. Amplicons were genotyped by single strand confirmation polymorphism (SSCP) on a polyacrylamid gel. For allele identification SSCP bands were subsequently cut out of the gel and re-amplified prior to cycle sequencing analyses. Cycle sequencing was performed with an Applied Biosystems automated sequencer model 3130, using a dye terminator sequencing kit (Applied Biosystems, Forster City, CA). No more than two alleles per individual were detected which was proven by RNA analyses confirming the presence of a single MHC class II *DRB* locus in *N. albiventris*. To affirm the individual SSCP pattern each individual was screened with a second primer pair (*JSi1N2-DRB* and *JSi2A-DRB*) and alleles were verified by direct sequencing. All nucleotide sequences have been submitted to GenBank (Accession numbers: HM347941-HM347958).

Statistical analyses

ARLEQUIN 3.0 (Excoffier et al. 2005) was used to calculate allele frequencies and pairwise F_{ST} based on haplotype frequencies (10,000 permutations) to infer population subdivision. Given that F_{ST} values can underestimate the differentiation between populations with highly polymorphic loci we also estimated the degree of differentiation using both Hedricks G'_{ST} (Hedrick 2005) and Jost's D_{est} (Joost 2008) with the program SMOGD 2.6 (Crawford 2010). Chi-square tests were used to compare the number of alleles between groups. Alleles of homozygote individuals were counted only once. All calculations were two-tailed with a significance level at $\alpha = 0.05$ and performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A post-hoc power analysis (1-ß err prob) was run to adjust for sample sizes with the program G*POWER 2.0 (Faul and Erdfelder

1992). Also post-hoc tests were two tailed with α = 0.05 and the effect size index was set to w = 0.5 (according to Cohen's effect size conventions between groups, Cohen 1988).

To test the influence of different host characteristics on the ectoparasite infestation, we applied different modelling approaches. Models offer the possibility to obtain a more complete perspective on the relevance of single factors in explaining the variation in a dependant variable, when confounding effects of other variables are included. In all models, we took infestation intensity of ticks and bat flies (number of parasites per bat examined) as response variables. The error structure of both response variables ('ticks', 'bat flies') was not normally distributed, so we used generalized linear models. Using 'raw' count data rather than transforming them has been strongly advised by O'Hara and Kotze (O'Hara and Kotze 2010). The models were fitted to a quasi-Poisson error structure with a log-link function. We tested the effect of the immune gene on parasite load using MHC *Noal-DRB* alleles as presence-absence-covariates and considered also the status of heterozygosity. Further, we tested the influence of ecological host characteristics on ectoparasite infestation by including specific 'roosts' (nominal, 6 categories) and covariates associated with life history traits, 'reproductive state' (nominal, 5 categories), 'sex' (nominal, 2 categories), age (nominal, 2 categories) and 'body size' (continuous) into the models. 'Month' (nominal, 5 categories) as well as 'year' (nominal, 2 (for ticks, not counted in 2006) or 3 (for bat flies) categories) were included to test for a seasonal component.

Prior to model analyses we carried out data exploration to identify outliers and to ascertain collinearity among explanatory variables (Zuur et al. 2010). Consequently, the collinear covariates 'sex' and 'reproductive state' were analysed in independent models. Also the influence of *Noal-DRB* alleles and heterozygosity were tested independently. In addition to Spearman rank correlations, we used variance inflation factors (VIF) to assess the extent of any remaining collinearity in nominal covariates (Zuur et al. 2009, 2010) using a stringent cut-off value of ≤ 1.5 for the VIFs (Booth et al. 1994). Some categories of the nominal covariate 'month' as well as 'year' had a VIF > 5, which might be an effect of a biased sampling. Therefore we included a random effect of 'month' and 'year' in our generalized linear mixed models (GLMM, Pinheiro and Bates 2000) and used Laplace approximation. Laplace approximation approximates the true GLMM likelihood rather than a quasi-likelihood, allowing the use of likelihood-based-inference (for details see Bolker et al. 2009). We applied quasi-AIC (QAIC, ΔQAIC) for random effect and fixed effect model selection. QAIC is similar to AIC, except that the log likelihood is divided by the estimated overdispersion scale

parameter of the full model (Bolker et al. 2009). QAIC and ΔQAIC offer the possibility of multiple model comparisons. Influential *Noal-DRB* alleles were first revealed by separate GLMM models in order to reduce the number of explanatory covariates. *Noal-DRB* alleles with a reasonable impact (ΔQAIC < 2) on the ectoparasite infestation were subsequently tested together with the ecological host characteristics.

In parallel, we estimated generalized estimation equations (GEE, Fitzmaurice et al. 2004) using 'roost' as grouping factor to overcome the likely auto-correlation of animals using the same roost. GEEs include an additional variance component to accommodate correlated data and to allow for differences among clusters. GEEs are semi-parametric because estimates rely on parametric assumptions regarding the mean and variance/covariance. We used the compound-symmetric correlation structure, assuming no specific order between the observations of the same cluster (i.e. roost), while assuming observations from different clusters to be independent. We did model selection starting with the full model and dropping each variable in turn, applying an ANOVA analysis (Wald-test) as implemented in the "geepack" package of R (Fitzmaurice et al. 2004) and removed the last significant variable.

Model validation was verified by checking for normal distribution of the residuals and by plotting standardized Pearson residuals versus fitted values in GLMM and GEEs. To ensure that any extreme effects did not overtly bias the models, models were refitted with these observations removed. In addition, missing data in the predictor "body size" reduced the number of included cases in models to 122 (ticks) and 141 (bat flies) when ecological host characteristics were tested. Limitations in sample size per category combination precluded the use of interaction terms.

To summarize, we analyzed our data by two independent approaches (GLMM and GEE) with different correlation structures as the reliability of inferences can be ascertained when estimated parameters show the same tendency in different model approximations. In addition, handling overdispersed count data is statistically difficult and only few suitable approaches are available for such data. Thus, using different approximations offers several advantages. In our case, GLMMs provide the possibility of multiple model comparison through the use of QAIC and ΔQAIC. GEEs, on the other hand, have the advantage of offering significance values for estimated parameters of categories in nominal covariates with more than two levels. All statistical analyses were conducted in the software program R 2.11.0 (R Development Core Team 2009).

Results

Capture and sampling success

In total, we captured 214 *N. albiventris* (91 males, 123 females) of which 20 were subadults. All 214 bats were genotyped for MHC (Schad et al. 2011). We collected samples from 29 bats on BCI and 185 in Gamboa. In Gamboa, we captured bats from six roosts: A (N = 52), B (N = 74), C (N = 27), D (N = 20), E (N = 4) and F (N = 7). All bats on BCI were captured in nets during foraging and cannot be assigned to specific roosts (electronic supplemental material (S)[2]; Table S1). Reproductive and non-reproductive adult males were captured throughout the year, whereas females had two pregnancy peaks per year with a main parturition in April/May and a second, smaller one at the end of the year. These peaks were followed by an increase in lactating females (Table S2).

Ectoparasite load

The numbers of bats examined for ticks within different roosts were as follows: A (2), B (61), C (24), D (10), E (4), F (7) and on BCI (26) (Table S1). We identified only larval stages of the tick *O. hasei* on *N. albiventris*. Seventy-four percent of the examined individuals (N = 134) were infested with ticks. The number of ticks on the uropatagium per investigated individual averaged 10.4 ± 1.1 (range: 0-55; median: 5.0).

The numbers of bats examined for bat flies within different roosts were as follows: A (19), B (73), C (27), D (20), E (4), F (7) and on BCI (16) (Table S1). Eighty-two percent of the examined bats (N = 166) were infested with ectoparasitic flies. The number of bat flies per investigated individual averaged 8.2 ± 0.7 (range: 0-39; median: 6.0). In addition to the bat fly *P. parvuloides* which was identified from every individual that had bat flies, and was present in all roosts, we found occasionally a second streblide bat fly. *Noctiliostrebla aitkeni,* co-infesting individuals always together with *P. parvuloides*. It was missing in bats captured from roost D, E and F.

Host characteristics influencing the ectoparasite load

Ticks

Both model approximations (GLMM and GEE) gave similar results and led to the same biological conclusions: The extent of a bat's infestation with ticks was influenced by the bat's specific MHC class II *DRB* alleles as well as by ecological characteristics such as roost membership, reproductive state and body size.

Table 1. MHC class II *DRB* alleles influencing ectoparasite infestation in *N. albiventris*. Estimated regression parameters of specific *Noal-DRB* alleles influencing tick (A, N = 131) and bat flies (B, N = 165) infestation validated by GLMM models with ΔQAIC < 2 (Laplace approximation with month in years as

[2] see Appendix II

random effects). Best model and, for simplicity, averaged parameters of models with ΔQAIC < 2 are shown.

A. Ticks	Factors	Estimates ± SE	t-value
Best Model	Intercept	1.989 ± 3.682	0.54
QAIC = 183.9	*Noal-DRB**02	0.342 ± 0.388	0.88
	*Noal-DRB**11	0.628 ± 0.628	1.00
Models with	Intercept	1.992 ± 3.647	0.55
ΔQAIC < 2.0	*Noal-DRB**01	- 0.266 ± 0.328	- 0.31
	*Noal-DRB**02	0.336 ± 0.387	0.87
	*Noal-DRB**04	0.237 ± 0.439	0.54
	*Noal-DRB**10	- 0.132 ± 0.393	- 0.34
	*Noal-DRB**11	0.634 ± 0.621	1.02

B. Bat flies	Factors	Estimates ± SE	t-value
Best Model	Intercept	1.983 ± 1.015	1.95
QAIC = 180.5	*Noal-DRB**05	- 0.415 ± 0.604	- 0.69
	*Noal-DRB**09	0.309 ± 0.377	0.82
	*Noal-DRB**11	0.595 ± 0.511	1.17
Models with	Intercept	1.661 ± 1.737	0.96
ΔQAIC < 2.0	*Noal-DRB**01	- 0.362 ± 0.716	- 0.50
	*Noal-DRB**04	- 0.235 ± 0.340	0.69
	*Noal-DRB**05	- 0.362 ± 0.716	- 0.50
	*Noal-DRB**09	0.262 ± 0.374	0.70
	*Noal-DRB**10	- 0.228 ± 0.286	- 0.80
	*Noal-DRB**11	0.609 ± 0.501	1.22

GLMM: generalized linear mixed model; QAIC: quasi Akaike information criterion where the log likelihood is divided by the estimated overdispersion scale parameter of the full model; t-value: estimated parameter divided by its standard error, indicates the likelihood that the estimated parameter is not zero.

All GLMM models analysing the impact of MHC *Noal-DRB* alleles independent of ecological host characteristics with ΔQAIC < 2 included the alleles *Noal-DRB**02 and *Noal-DRB**11 together with the alleles *Noal-DRB**01, *Noal-DRB**04, *Noal-DRB**10 in different combinations. Alleles *Noal-DRB**02, *Noal-DRB**04 and *Noal-DRB**11 were associated with a higher tick infestation, and alleles *Noal-DRB**01 *and Noal-DRB**10 were associated with a lower tick infestation (Table 1).

Both the GLMM and GEE model approximations that combined these five *Noal-DRB* alleles with ecological host characteristics validated 'roost', 'body size', 'reproductive state' and the five *Noal-DRB* alleles to be influential for individual tick infestation (Table 2). GLMM and GEE models including the five *Noal-DRB* alleles explained significantly more of the variation in the tick infestation than a model without these alleles (GLMM without alleles raised ΔQAIC to 4.3; GEE ANOVA: $\chi^2 = 36.1$, df = 5, $p < 0.001$)

Table 2. Tick infestation in *N. albiventris*. Estimated regression parameters and standard errors of combined ecological host characteristics and specific MHC class II *DRB* alleles on tick infestation obtained by GLMM (Laplace approximation with month and year as random effects) and GEE (with autocorrelation factor of roost: correlation parameter α = 0.03 ± 0.07, overdispersion scale parameter = 7.1). Averaged estimates of GLMM models with ΔQAIC < 2 are shown ($QAIC_{best}$ = 182.9, overdispersion parameter of the full GLMM model = 4.57). N = 122.

GLMM			**GEE**			
Factors	**Estimates ± SE**	**t-value**	**Factors**	**Estimates ± SE**	**Wald-Test**	**p-value**
Intercept	- 5.580 ± 3.560	-1.57	Intercept	- 4.182 ± 1.156	13.10	< 0.001***
Roost B[1]	0.914 ± 0.748	1.22	Roost B[1]	0.723 ± 0.371	3.78	0.052(*)
Roost C[1]	1.188 ± 0.783	1.52	Roost C[1]	0.569 ± 0.440	1.67	0.196
Roost D[1]	2.043 ± 1.253	1.63	Roost D[1]	2.398 ± 0.401	35.73	< 0.001***
Roost E[1]	- 0.208 ± 1.350	-0.15	Roost E[1]	- 0.264 ± 0.598	0.20	0.659
Roost F[1]	3.761 ± 1.600	2.35	Roost F[1]	1.423 ± 0.454	9.79	0.002**
Body size	0.664 ± 0.192	2.89	Body size	0.630 ± 0.098	41.42	< 0.001***
Female lactating[2]	0.163 ± 0.482	0.34	Female lactating[2]	0.168 ± 0.202	0.68	0.408
Female pregnant[2]	- 0.345 ± 0.919	-0.36	Female pregnant[2]	- 0.569 ± 0.483	1.39	0.566
Male non-reproductive[3]	1.300 ± 0.671	1.94	Male non-reproductive[3]	1.286 ± 0.354	13.21	< 0.001***
*Noal-DRB**01	- 0.392 ± 0.733	-0.53	*Noal-DRB**10	- 0.311 ± 0.142	4.80	0.029*
*Noal-DRB**02	0.160 ± 0.312	0.51	*Noal-DRB**11	0.543 ± 0.204	7.07	0.007**
*Noal-DRB**04	0.132 ± 0.349	0.38	Capture year 2008	- 1.257 ± 0.249	25.54	< 0.001***
*Noal-DRB**10	- 0.236 ± 0.302	-0.78				
*Noal-DRB**11	0.516 ± 0.511	1.01				

[1]compared to animals of BCI, [2] compared to non-reproductive adult females, [3] compared to reproductively active males

indicating that these MHC alleles explained a determinant part of the variation in parasite loads. In models where *Noal-DRB* alleles were replaced by the variable 'heterozygosity', heterozygosity was identified to have no influence on the individual tick load in GEEs (χ^2 = 2.05, df = 1, p = 0.15), but heterozygosity was validated to be associated with increased tick load in GLMMs (GLMM without 'heterozygosity': ΔQAIC = 5.01). Of the ecological host characteristics, roost explained a substantial part of the variation in tick infestation (GLMM without 'roost': ΔQAIC = 39.3; GEE: χ^2 = 84.3, df = 5, p < 0.001). Bats from roost D and F showed a significantly higher infestation rate than animals from BCI (Table 2). In addition, body size (GLMM without 'body size': ΔQAIC = 33.4; GEE: χ^2 = 41.4, df =1, p < 0.001) and reproductive state (GLMM without 'reproductive state': ΔQAIC = 18.8; GEE: χ^2 = 28.2, df = 4, p < 0.001) had an effect on the infestation, with non-reproductive adult males showing a significantly higher infestation rate than reproductive males. Non-reproductive adult females did not differ in their infestation rate compared to lactating and pregnant females (Table 2). Bats captured in 2008 were significantly less infected than individuals sampled in 2007 (Table 2). Estimated parameters of the optimal GLMM model revealed the same tendency as in the optimal GEE model, with increased divergences in estimates for different roosts (Table 2).

In models where reproductive state was replaced by the variable 'sex', the results differed in the two model approaches. Whereas sex was validated as not influencing individual tick load in GEEs (χ^2 = 1.18, df = 1, p = 0.28), GLMMs validated males to be less infested compared to females. However a GLMM model excluding the variable 'sex' explained the infestation also reasonable well (GLMM without 'sex': ΔQAIC = 2.94) indicating a minor effect of sex on tick loads. The association of age with individual tick load revealed heterogeneous results, which might be the effect of low sampling of subadults. In GEEs age was identified to be influential with subadults being more infested (χ^2 = 5.35, df = 1, p = 0.02). GLMMs validated the variable 'age' to have a minor effect on infestation, though a model without the variable 'age' explained tick load best, a model including age was validated still to be realistic (GLMM with 'age': ΔQAIC = 1.32). Estimates of all other covariates suggested a similar influence independently whether or not the variable 'sex' or 'age' was included.

Bat flies

Both GLMM and GEE gave similar results and suggested the same biological conclusions: variation in infestation of bats with bat flies was associated with specific MHC class II *DRB* alleles, the inhabited roost, reproductive state and body size.

Table 3. Batfly infestation in *N. albiventris*. Estimated regression parameters and standard errors of combined ecological host characteristics and specific MHC class II *DRB* alleles on bat flies infestation obtained by GLMM (Laplace approximation with month and year as random effects) and GEE (with autocorrelation factor of roost: correlation parameter α = 0.08 ± 0.07, overdispersion scale parameter = 2.8). Averaged estimates of GLMM models with ΔQAIC < 2 are shown ($QAIC_{best}$ = 135.0, overdispersion parameter of the full GLMM model = 3.34). N = 141.

GLMM			**GEE**			
Factors	**Estimates ± SE**	**t-value**	**Factors**	**Estimates ± SE**	**Wald-Test**	**p-value**
Intercept	- 1.947 ± 2.558	-0.76	Intercept	- 0.899 ± 1.039	0.75	0.386
Roost B[1]	0.089 ± 0.631	0.14	Roost B[1]	0.674 ± 0.283	5.65	0.017*
Roost C[1]	- 0.106 ± 0.592	- 0.18	Roost C[1]	0.022 ± 0.294	0.01	0.941
Roost D[1]	0.623 ± 0.737	0.85	Roost D[1]	0.778 ± 0.334	5.41	0.020*
Roost E[1]	- 0.760 ± 0.938	- 0.67	Roost E[1]	0.056 ± 0.389	0.02	0.886
Roost F[1]	3.427 ± 1.105	3.11	Roost F[1]	2.077 ± 0.354	34.5	< 0.001***
Body size	0.283 ± 0.228	1.25	Body size	0.295 ± 0.110	7.08	0.008**
Female lactating[2]	- 0.532 ± 0.361	-1.48	Female lactating[2]	- 0.531 ± 0.188	7.59	0.005**
Female pregnant[2]	- 0.675 ± 0.566	-1.20	Female pregnant[2]	- 0.489 ± 0.215	5.14	0.023*
Male non-reproductive[3]	1.645 ± 0.691	2.39	Male non-reproductive[3]	1.512 ± 0.367	17.00	< 0.001***
*Noal-DRB**04	-0.162 ± 0.249	-0.65	*Noal-DRB**04	- 0.164 ± 0.117	1.97	0.161
*Noal-DRB**09	0.276 ± 0.260	1.06	*Noal-DRB**09	0.207 ± 0.154	1.81	0.178
*Noal-DRB**11	0.364 ± 0.389	0.96	*Noal-DRB**11	0.379 ± 0.166	5.23	0.022*
			Capture year 2007	0.605 ± 0.238	6.15	0.009**
			Capture year 2008	- 0.022 ± 0.283	0.01	0.982

[1] compared to animals of BCI, [2] compared to non-reproductive adult females, [3] compared to reproductively active males

Analysing the impact of MHC *Noal-DRB* alleles independently of the ecological host characteristics, six different models had a ΔQAIC < 2, identifying six different alleles as potentially influencing an individual's likelihood of being infected with bat flies. In all models, *Noal-DRB**01, *Noal-DRB**04, *Noal-DRB**05 and *Noal-DRB**10 were associated with lower and *Noal-DRB**09 *and Noal-DRB**11 with higher bat fly infestation (Table 1).

We combined ecological host characteristics with the allele information in further GLMM and GEE models. Both model approximations confirmed the relevance of the same ecological host characteristics, 'roost', 'body size', 'reproductive state' and the same MHC variables, namely *Noal-DRB**04, *Noal-DRB**09 and *Noal-DRB**11, in explaining infestation of bats with bat flies (Table 3). Models including these alleles explained the variation better than a model neglecting these alleles (GEE: χ^2 = 6.18-7.74., df = 2-3, p < 0.05). Even so, GLMM models including these alleles in different combinations had low ΔQAIC-values (< 0.69), a model without these alleles still explained the variation in the bats' infestation with bat flies reasonably well (ΔQAIC = 1.92). Alleles *Noal-DRB**01, *Noal-DRB**05 and *Noa-lDRB**10 were found to be less important for the bats' infestation with bat flies in both GLMM (ΔQAIC: 2.32 - 4.65) and GEE (p > 0.10). In models where *Noal-DRB* alleles were replaced by the variable 'heterozygosity', heterozygosity was validated not to influence the infestation with bat flies (GLMM with 'heterozygosity' ΔQAIC = 2.4; GEE: χ^2 = 2.47, df = 1, p = 0.12). Of the ecological host characteristics, roost explained a substantial amount of the variation in infestation with bat flies (GLMM without 'roost': ΔQAIC = 59.8; GEE: χ^2 = 73.8, df = 5, p < 0.001). Bats of roost B, D and F showed a significantly higher infestation rate compared to animals from BCI (Table 2). Infestation intensity rose significantly with increasing 'body size' (GLMM without 'body size': ΔQAIC = 3.08; GEE: χ^2 = 7.08, df = 1, p = 0.008). Also, reproductive state had a significant effect on infestation intensity (GLMM without 'reproductive state': ΔQAIC = 18.4; GEE: χ^2 = 28.9, df = 4, p < 0.001) with non-reproductive adult males being more parasitized than reproductive males. Also non-reproductive adult females were more infected than lactating and pregnant females (Table 3). Capture year also influenced the variation in bat fly infestation (GEE: χ^2 = 34.9, df = 2, p < 0.001), with animals sampled in 2007 showing a higher rate of infestation than animals sampled in 2006. Estimated parameters of the optimal GLMM model confirmed the results of the GEE and showed the same tendency (Table 3). 'Sex' had no effect on parasite load in models where sex was used instead of reproductive state (GLMM with 'sex': ΔQAIC = 4.24; GEE: χ^2 = 3.38, df = 1, p = 0.07). The influence of 'age' on bat flies infestation varied: in GEEs age had no effect on infestation (χ^2 = 0.22, df = 1, p = 0.64), but in GLMMs a model including age was best

(ΔQAIC = 0), with subadults showing higher bat fly loads. However, a model without the variable age still was validated to be realistic (ΔQAIC = 2.02) indicating an uncertain effect of age on infestation. Estimates of all other covariates suggested a similar effect on infestation independently whether or not the variable 'sex' or 'age' was included.

Comparison of MHC allele frequencies

We compared MHC-*DRB* allele frequencies of non-reproductive and reproductive adult males and subadults (Table S3) to investigate how alleles were transmitted into the next generation. Neither non-reproductive and reproductive males (F_{ST} = 0.006, p = 0.136 G'_{ST} = 0.055, D_{est} = 0.052; see Table S4 for confidence intervals) nor reproductive males and subadults (F_{ST} = 0.005, p = 0.243, G'_{ST} = 0.036, D_{est} = 0.033; Table S4) showed significant differences in their allele frequencies, but non-reproductive males differed in their allelic composition from subadults (F_{ST} = 0.025, p = 0.022, Bonferroni non-significant (α'≤ 0.016), G'_{ST} = 0.163, D_{est} = 0.152; Table S4).

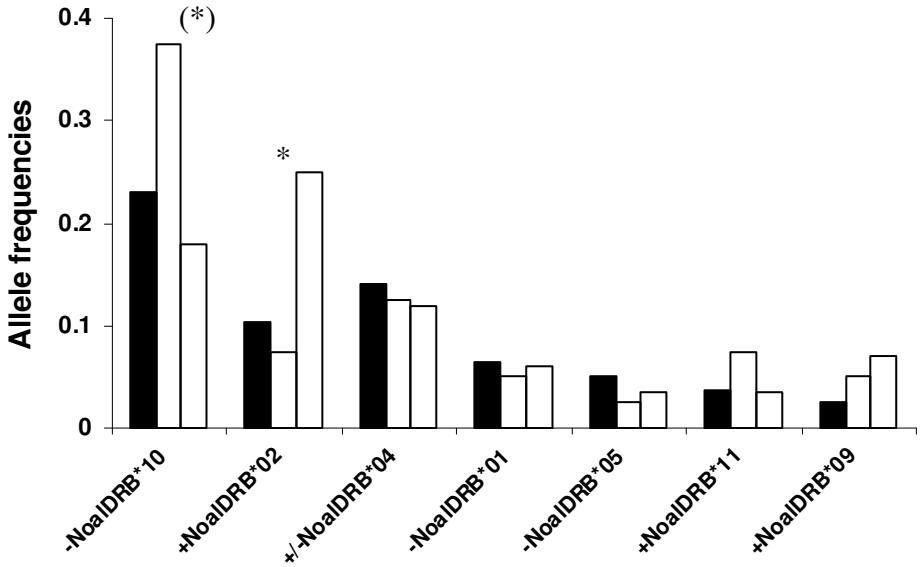

Figure 1. Allele frequencies of MHC class II *DRB* exon 2 influencing the ectoparasite infestation in *N. albiventris.* Distribution of *Noal-DRB* alleles influencing the ticks and bat flies infestation in reproductive males (black bars), subadults (hatched bars) and non-reproductive males (white bars). '+' indicates an association with an increased and '-' with a decreased parasite load. Allele *Noal-DRB**02 is significant accumulated in non-reproductive males and less frequent in subadults (χ^2 = 10.07, p = 0.006, df = 2, Bonferroni significant, power (1-β err prob) 100 %). Contrarily, allele *Noal-DRB**10 is less frequent in non-reproductive males and accumulated in subadults (χ^2 = 3.20, df = 1, p = 0.064, power 98 %).

Further, we analysed differences in the allele distribution with respect to alleles which were identified to influence ectoparasite infestation (*Noal-DRB**01, *Noal-DRB**02, *Noal-DRB**04, *Noal-DRB**05, *Noal-DRB**09, *Noal-DRB**10, *Noal-DRB**11; Table 1). Allele *Noal-DRB**02 which was associated with a higher infestation rate of ticks, was more frequent in non-reproductive males than in reproductive males (χ^2 = 6.95, df = 1, p = 0.008, power: 99 %) and subadults (χ^2 = 6.71, df = 1, p = 0.009, power: 98 %) but no difference was found between reproductive males and subadults (χ^2 = 0.223, df = 1, p = 0.43, power: 97 %; Figure 1). This effect was enhanced when the three groups were tested together (χ^2 = 10.17, df = 2, p = 0.006, Bonferroni significant (α'≤ 0.016), power: 100 %). Interestingly, *Noal-DRB**10, the most frequent allele in the population (Table S3) which was associated with a decreased parasitism in ticks and bat flies showed an opposite pattern. Although not statistically significant, we found that *Noal-DRB**10 tended to be less frequent in non-reproductive males and most frequent in subadults (χ^2 = 3.20, df = 1, p = 0.064, power: 98 %; Figure 1). It occurred at an intermediate frequency in reproductive males. We found no difference, neither between reproductive and non-reproductive adult males (χ^2 = 0.162, df = 1, p = 0.42, power: 99 %) nor between reproductive males and subadults (χ^2 = 1.63, df = 1, p = 0.16, power: 99 %). All other alleles which occurred at minor frequencies in the study population (Table S3, Figure 1) were distributed without any significant differences among groups (χ^2, results not shown).

Discussion

We investigated the relationship between the individual immune gene constitution and ectoparasite loads in a free ranging population of *N. albiventris.* We analysed the impact of host characteristics like age, sex and reproductive state on ectoparasite infestation and we tested predictions of the 'good-genes' model, which postulates that males with well-adapted immune genes to coexisting parasites, have the ability to allocate more resources to reproduction. We found that in the neotropical bat *N. albiventris*, the infestation rate with ticks and bat flies was associated with various ecological traits, the reproductive status especially in males as well as with specific MHC class II *DRB* alleles.

Environmental and ecological host characteristics associated with ectoparasite loads

All collected ectoparasite species have previously been reported for *N. albiventris* in Panama (Wenzel and Tipton 1966). The tick species *O. hasei* is specific to bats and so far the sole tick species found to parasitize *N. albiventris* (Hood and Pitocchelli 1983). *Paradyschira* and *Noctiliostrebla* are bat fly genera that are specific to the bat genus *Noctilio* and may co-parasitize the same individual (Presley 2004). Our data are in line with investigations on ectoparasite assemblages in populations of *N. albiventris* in Paraguay and Venezuela, where a mean of 2.5 ectoparasites are reported to co-parasitize the same individual. Observed ectoparasite assemblages resembled our findings and included the tick *O. hasei*, a very common bat fly species of the genus *Paradyschiria* and a second less abundant fly of the genus *Noctiliostrebla*, which occurred on all bats also infested with the common fly (Presley 2004). During the study period the population has not been found to be faced with other severe diseases. Other ectoparasites like mites were observed only singularly and preliminary studies on intestinal helminth infestation showed that less than 12 % of the population were infested by intestinal parasites. However, selection pressure on MHC class II alleles might also be caused by other external pathogens than the investigated ectoparasites.

Parasite abundance on bat hosts depends on complex interactions between environmental factors such as season and host characteristics such as roost usage, behaviour, body size, age, sex, reproductive state and individual immunocompetence (Marshall 1982; Christe et al. 2000; ter Hofstede and Fenton 2005; Pearce and O'Shea 2007; Patterson et al. 2007; Presley and Willig 2008; Mans 2011). We found seasonal differences in ectoparasite loads on *N. albiventris*, both on a long- (years) and short-term scale (months). This may be caused by changes in environmental conditions as seasonal changes in temperature and humidity may cause fluctuations in the life cycle of ectoparasites (Marshall 1982) and thus may influence the infestation intensity of the host (Krasnov et al. 2005). Since we cannot rule out the possibility that the observed seasonal differences were biased by unbalanced sampling, we controlled for random effects caused by capture year and month by using GLMMs.

Membership to a specific roost was identified to have an important impact on the infestation of bats with ectoparasites by both model approaches. This is not surprising, because roosts have been suggested as a primary source of ectoparasite transmission in bats (Marshall 1982; Patterson et al. 2007; ter Hofstede and Fenton 2005), since ticks and bat flies depend on sheltered cavities for reproduction (Dick and Patterson 2007; Presley 2004). In our *N. albiventris* population, infestation with ectoparasites was

influenced by body size. The individual body size as a measure of a bat's surface area predicted increased prevalence of ticks and bat flies. Body size is thought to influence ectoparasite loads directly by limiting the available resources (Rosenzweig 1995; Christe et al. 2003; Hawlena et al. 2005; Poulin and George-Nascimento 2007). Across many host-parasite systems, male-biased parasitism has been postulated to be related to sexual size dimorphism (reviewed in Moore and Wilson 2002). Studies on bat hosts contrast this finding, with female bats being generally more heavily infested by ectoparasites (Presley and Willig 2008; Christe et al. 2007; Patterson et al. 2008). We did not find any sex-biased differences in the infestation with bat flies in *N. albiventris,* and we found equivocal results concerning ticks. Females were validated only in one of the two model approaches (GLMM) to have higher tick loads than males, indicating also a minor effect of sex on tick burden.

Analysing the effect of individual reproductive state on parasite loads yielded intriguing results albeit sample sizes were quite low. Non-reproductive males were more heavily infested with both ectoparasites than reproductive males, suggesting a link between fitness and ectoparasite resistance. There is evidence that susceptibility to parasites might be related to the reproductive state also in female bats (Christe et al. 2000; Pearce and O'Shea 2007; Sharifi et al. 2008). Females at different reproductive stages did not vary in their infestation rate with ticks. But, similar to that found in males, non-reproductive females showed higher infestation with bat flies than reproductive females (both, lactating and pregnant), indicating that individuals with better parasite resistance are more likely to reproduce. However, we did not detect early pregnancies, which refer to non-reproductive females and might have biased the results concerning ticks and bat flies. Variable results have been reported in other studies investigating infestation by ectoparasites of female bats at different reproductive stages (Christe et al. 2000; Pearce and O'Shea 2007; Sharifi et al. 2008). These contrasting finding preclude general conclusions and indicate that complex processes act in specific bat-parasite systems.

Little is known about the roosting behaviour of *N. albiventris* in the wild and we cannot exclude that ectoparasite infestation might be influenced by roosting behaviour in relation to sex, age or even reproductive state. From captive individuals it is known that females and juveniles usually roost more closely to each other than do males (Dechmann, personal observation), which could explain the tendency of a higher ectoparasite load on females as well as subadults. Also age might influence an individual's susceptibility to parasites with an increased parasite load in juveniles and very old bats, most probably due to an ineffective immune system and self grooming

capability (Marshall 1982). According to our results a higher susceptibility to both ectoparasite taxa according to age was not unequivocally confirmed by the two modelling approaches applied and might be a result of the limited number of subadults investigated or may indicate a minor importance of age on ectoparasite loads. Unfortunately we do not know whether very old individuals were captured during the study, because once bats reach full size no field methods are available that include age determination of very old bats (Brunet-Rossini and Wilkinson 2009). Long-term recapture studies would be necessary to ascertain this point.

Impact of MHC class II DRB alleles on ectoparasite infestation

In addition to the complex ecological host characteristics and as a precondition to investigating the 'good-genes' model, we observed significant relationships between ectoparasite infestation and specific *Noal-DRB* alleles. We identified alleles associated with high (*Noal-DRB**02, *Noal-DRB**04, *Noal-DRB**09, *Noal-DRB**11) and low (*Noal-DRB**01, *Noal-DRB**04, *Noal-DRB**05, *Noal-DRB**10) ectoparasite abundance. Three of them had the same effect on both ectoparasite taxa (*Noal-DRB**01, *Noal-DRB**10, *Noal-DRB**11), whereas others were associated either with tick or bat flies infestation (tick: *Noal-DRB**02, bat flies: *Noal-DRB**05 and *Noal-DRB**09). Allele *Noal-DRB**04 had a dual effect. It was correlated with an increased tick load and associated with a decreased bat fly infestation. It is known that although each MHC molecule has a high peptide binding specificity, it may accommodate several different peptides (Aluvia and Margalit 2004). Moreover, resistance against one parasite can be conferred by multiple different MHC alleles (Goüy de Bellocq et al. 2008). Thus, co-evolutionary processes might not necessarily be entirely species specific (Poulin and Morand 2004).

During host parasite co-evolutionary processes, ectoparasites may also develop immunocompatibility with their hosts by sharing antigenic epitopes (Dick and Patterson 2007; Mans 2011). Salivary proteins of ectoparasites are known to modulate host immunity in inhibiting regulatory as well as effector pathways involved in acquiring and expressing resistance (Schoeler and Wikel 1999, Mans 2011). For example, antigen presenting macrophages and T_H1 lymphocyte functions are suppressed by tick salivary gland extracts (Wikel 1999). Shared antigenic epitopes between host and ectoparasites may explain the positive association of specific MHC molecules with specific ectoparasite taxa. Association of specific MHC class II *DRB* alleles with susceptibility to or protection against pathogens in mammals have been reported in numerous studies, including *DRB* alleles resistant to ectoparasites in cattle (*Bos taurus*, Untalan et al.

2007), white-tailed deer (*Odocoileus virginianus*, Ditchkoff et al. 2005) and water vole (*Arvicola terrestris*, Oliver et al. 2009).

Besides direct responses of MHC mediated susceptibility to ectoparasites, the MHC may also contribute to individual attraction during the location of suitable hosts. Hosts are located by ectoparasites not only via respired carbon dioxide and body heat, but also through specific host odours (Marshall 1982). Experimental analyses have shown that the individual body odour in vertebrates is influenced by immune genes of the MHC (e.g. Penn and Potts 1998; Kwak et al. 2009). This finding is supported by the fact that the MHC is in physical linkage with olfactory receptor genes in most vertebrates assessed so far (reviewed in Santos et al. 2010). Host odours are a particularly important cue for ectoparasites to differentiate among species (Krasnov et al. 2002; Lourenço and Palmeirim 2008), and might also be used to differentiate between sexes and reproductive stages (Christe et al. 2000; Christe et al. 2007). Furthermore, there is evidence that odours produced from skin determine levels of attractiveness of human beings to mosquitoes (Logan 2008; Verhulst et al. 2011). Verhulst and co-workers (2011) found that the microbial community on the skin causes these differences in odorant cues. The authors hypothesize that the MHC may exert this influence of attractiveness by changing the skin microbiota composition and hence the volatiles produced by the bacteria and/or the human host. Our results could indicate that in *N. albiventris* specific *Noal-DRB* alleles might be responsible for attracting ectoparasite species to particular host individuals and may support the hypothesis of Verhulst and co-workers.

There is broad evidence that MHC mediated odours are used in mate selection with the consequence that reproduction among MHC dissimilar mates is favoured (Penn and Potts 1999; Ilmonen et al. 2009) presumably to generate a genetically heterozygote offspring. Thus, there might be a reasonable possibility that beside direct selection through parasites also mating strategies might influences the MHC allele composition in the investigated *N. albiventris* population to some extend. However, an association between heterozygosity and ectoparasite load was only observed regarding tick infestation in one (GLMM) of the two model approaches. Against the predictions of the heterozygote advantage hypothesis (Doherty and Zinkernagel 1975) heterozygote individuals were associated with higher tick loads compared to homozygotes. We think that this equivocal result was caused by high frequency of *Noal-DRB**02 but also *Noal-DRB**11, which predominantly occurred in heterozygote individuals, both associated with increased tick burden.

The variation in the infestation with ectoparasites of a bat was best explained when ecological and genetic host characteristics were combined for analyses. Whereas ecological host characteristics showed a strong influence on the infestation the impact of immune genes were comparatively less powerful but still significant. Obviously ecological predictors which reflect the availability or exposure of ectoparasites, such as roost, season and available source (host body size) will be of overriding importance in the ectoparasite infestation compared to an individual's immune gene constitution or reproductive state with the latter acting both more on fine-scale parameters.

Testing the 'good-genes' model based on MHC class II *DRB* constitution

To test the predictions of the 'good genes' model we focused on immunogenetic differences between non-reproductive and reproductive adult males to better understand the link between individual MHC class II *DRB* constitution, ectoparasite infestation and investment in reproduction. We additionally compared *DRB* variation between reproductive and non-reproductive males with that of subadults to detect indicators for selective reproductive success related to the immune gene constitution. Subadults differed in their overall *Noal-DRB* allele frequencies from non-reproductive, but not from reproductive males, possibly a consequence of the limited inheritance of alleles from non-reproductive males to subsequent generations. But using conventional F-statistics, statistical support disappeared after Bonferroni correction. However, Hedrick's G'_{ST} and Jost's D_{est} which are used to asses subtle genetic structuring in highly polymorphic loci such as MHC showed higher support for an allelic differentiation between these groups. Non-reproductive and reproductive adult males differed at an intermediate level. We are aware that sample size limitations might have biased these results in both directions. However, these population differentiation tests are based on frequencies of all *Noal-DRB* alleles, including those alleles which were not relevant in the association to both ectoparasite taxa and might thus not precisely answer our question. When we analysed the distribution of specific alleles relevant for ectoparasite infestation, we obtained an unambiguous result concerning the two most frequent alleles in the population. Allele *Noal-DRB**02, which was associated with a higher tick infestation, was significantly more frequent in non-reproductive males compared to reproductive males and was even less frequent in subadults. Furthermore, a noticeable, although non-significant accumulation of allele *Noal-DRB**10, which was associated with low tick and bat fly loads, was observed at a high frequency in subadults but was less frequent in non-reproductive males.

Our results suggest that the MHC-*DRB* constitution contributes to the fitness of male bats as less infected individuals might have a higher reproductive success. Alleles which were common in strongly infected adult males were rare in subadults. This supports the assumptions of the 'good-genes' model: genetically well adapted males to prevailing parasites seem to be able to tolerate elevated costs of reproduction, whereas poorly adapted males suffer from increased parasite loads and seem not to be able to invest in the costly process of reproduction. Whether this holds true also for subsequent years requires ongoing investigations. It has been suggested by the immune competence handicap hypothesis (Folstad and Karter 1992) that testosterone might be the physiological mediator regulating the competition between reproductive investment and parasite defence in males. Genetic quality in terms of parasite resistance might modify this trade-off essentially (Folstad and Karter 1992; Sheldon and Verhulst 1996). A potential association between MHC-types and testosterone production has been reported in white-tailed deer (*Odocoileus virginianus*), suggesting that males, carrying a certain MHC-type, bear the cost of elevated testosterone levels (Ditchkoff et al. 2001). The relevance of the MHC for the reproductive effort in male fish has been demonstrated by Milinski and co-workers (2010). In male three-spined sticklebacks (*Gasterosteus aculeatus*) MHC dependent odour signals which are involved in mate choice are produced only when males are in reproductive state. The authors postulate that producing the MHC mediated olfactory signal is costly to the senders. Less healthy or parasitized males might thus stop producing this sexually selected trait or reduce their investment in reproduction when they can no longer afford to produce the costly signal. It remains to be investigated whether the susceptibility to ectoparasites in male *N. albiventris* increases during the mating period, and whether this has an effect on the investment in developed testes and the odour produced in the inguinal pockets for mating.

To conclude, our study indicates that besides the impact of ecological factors (e.g. roost, season), ectoparasite load is also influenced by MHC class II *DRB* allelic composition of an individual, shaping the trade-off between the cost of reproduction and immune defence. Thus, it provides evidence for the 'good-genes' model based on immune gene variation which has rarely been investigated under natural conditions so far. Whether this also holds true for MHC class I in relation to virus infections will be the focus of ongoing studies.

Acknowledgments

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General Discussion

Up until now, not much is known about the structure and polymorphism of MHC genes in bats (Mayer and Brunner 2007; Richmann et al. 2011). This is astonishing given the size of the order, their zoonotic relevance as reservoir hosts for many pathogens and the importance of olfactory signals for the complex social communication mechanisms. Especially since the MHC is assumed to alter the individual body odour and is known to be in physical linkage with olfactory receptor genes (Penn and Potts 1999; Santos et al. 2010). Thus, the intention of my doctoral research was to investigate the evolution of adaptively important MHC genes in bats. I examined the MHC class II *DRB* diversity pattern in four New World bat species belonging to three bat families and provided for the first time phylogenetic analyses of MHC loci in bats by establishing species-specific molecular techniques. In a second part of my thesis I examined the functional importance of *DRB* diversity in a free ranging population of *N. albiventris* and aimed to understand potential causes and consequences of the observed diversity pattern at population level taking population structure, ectoparasite burden and life history traits into account.

Patterns of MHC diversity and evolution in bats

Among the investigated bat species I found a heterogeneous pattern of MHC variability which mainly concerned the number of duplicated MHC class II *DRB* loci. I found a single *DRB* locus in the genus *Noctilio*, three *DRB* loci in *C. perspicillata* and a minimum of ten functional *DRB* loci in *S. bilineata*. The number of identified functional *DRB* loci in *C. perspicillata* and *Noctilio* lies within the normal range observed in numerous mammalian species (one to four; Castro-Pietro et al. 2011) and is in line with the two loci described in the bat genus *Myotis* (Richmann et al. 2011). However, such a high number of functional *DRB* loci as in *S. bilineata* has never been described for other mammals. In a previous study of this species, which used general primers instead of species-specific only two to three *DRB* loci were detected (Mayer and Brunner 2007). The best-studied mammalian species to date is the *Homo sapiens* which was so far thought to exhibit the highest number of nine *DRB* loci. However, among those loci only four are functional (Klein et al. 2007; Doxiadis et al. 2010). So far, the only other example of such a high copy number has been described in a bird,

the common yellow throat (*Geothlypis trichas,* Bollmer et al. 2010) where up to eight expressed MHC class II B loci were reported. The mode of MHC diversification through locus duplication is a well known mode of evolution in the MHC gene family. Duplication provides a way of retaining one duplicate with a useful molecule function, whilst the twin is liberated to mutate and possibly acquires novel helpful protein functions in the arms-race between host and parasite (Charbonnel et al. 2006).

In all investigated bat species I confirmed evolutionary mechanisms for generating and maintaining MHC diversity at the nucleotide level which underline the functional relevance of the MHC class II in bats. Levels of nucleotide diversity among *DRB* sequences were similar to each other and to those described for functional MHC *DRB* loci in other vertebrates (Sommer 2005), indicating that balancing selection mechanisms maintain sequence polymorphism. Evidence for positive Darwinian selection which maintains polymorphism over an evolutionary timescale was signified by the fact that non-synonymous substitutions were more frequent than synonymous ones in the section coding for the antigen binding region (exon 2/ß1-domain, but see *S. bilineata*). Thereby positive selection acts particularly on specific codons which might be directly involved in antigen adhesion. As a sign for homologous functionality of the molecule the amino acid positions of the codons in question were in most cases analogue to those of the other bat species and to those described for humans (Brown et al. 1993, Stern et al. 1994). But also bat-specific and species-specific exclusive positions were found, indicating different selection pressures due to a bat- and species-specific pathogen exposure. These are well known phenomena described also for other taxa (e.g. Schwensow et al. 2007; Babik et al. 2008; Meyer-Lucht et al. 2008).

The observed sequence diversity between alleles appeared not only to be generated by point mutations but also through recombination and gene conversion. These mechanisms can generate new sequence variants by converting smaller DNA tracts, thereby creating chimaeric sequences. But gene conversion can also decrease variation by homogenizing formerly diverse loci, especially when gene conversion tracts are large or occur at very high frequencies (known as ‘concerted mode of evolution’, Otha 1980, 1999; Hess and Edwards 2002). Therefore, the role of inter- and intragenic recombination in MHC gene diversification is a controversially discussed theme (Hess and Edwards 2002; Reusch and Langefors 2005; Klein et al. 2007).

In mammals recombination is considered to be of minor importance and it is suggested that MHC genes evolve independently so that duplication history (i.e. orthology) usually can be traced back within lineages, which implies a trans-species mode of evolution as the driving force (Klein et al. 2007). In contrast, inter- and intragenic recombination is

assumed to play a central role in the evolution of avian MHC class II *B* genes. This is underpinned by the fact that avian MHC genes usually cluster by species indicating either concerted evolution of paralogue loci or orthology by recent duplication events (Hess and Edwards 2002, but see Burri et al. 2010). The phylogenetic relationship which I revealed in the chiropteran MHC-*DRB* challenge the patterns of MHC evolution stated in mammals so far. In bats *DRB* loci clustered in a species-specific manner, regardless which region of the gene was analysed and support was even strongest when the whole sequence was taken into account. Thus, it seems that the mode of evolution in bats resembles more the pattern mainly found in birds (but see Burri et al. 2006, 2010) and my results question the generality of the conclusion about the mode of MHC evolution in mammals.

Yet, I found considerable differences among the investigated bats concerning not only the number of *DRB* loci detected, but also in the pattern of recombination and gene conversion. Whereas in *C. perspicillata* and in *Noctilio*, few inter- or intragenic recombination events occur – resulting mainly in a shuffling of short sequence motives especially within exon 2 and thus contribute to diversification in the antigen binding region – *DRB* genes in *S. bilineata* have apparently experienced more and longer gene conversion events. Long gene conversion tracts were situated in exon 3 indicating higher rates of concerted evolution or more likely frequent gene duplication in the recent past leading to homogenization rather than diversification. This is underlined by the fact that I did not find an excess of non-synonymous substitutions over synonymous ones in the antigen binding ß1-domain in this species. Further work on the genomic structure of the investigated loci as well as information from other bat species within the investigated families will have to show to what extent recent gene duplication, gene loss and/or concerted evolution contribute to the found pattern and probably will shed light on the timescale at which diversification and/or homogenization in bats MHC evolution took place.

Possible mechanisms causing the differences in MHC diversity among the investigated bat species

I found astonishing variability in the MHC-*DRB* polymorphism between bat species. Whereas the polymorphism in all investigated species but *S. bilineata* are in line with those of other vertebrates and mammals, the loci polymorphism in *S. bilineata* is a remarkable exception. It is known, that duplication of MHC loci plays an important role in the adaptive evolution by increasing the number of alleles present in individuals,

thereby allowing for the detection of a larger number of pathogens (Hughes 1994). *Saccopteryx bilineata* seems to be only occasionally and rarely infested with parasites, whereas *C. perspicillata* as well as *N. albiventris* seem to be faced with a large diversity of endo- or at least ectoparasites (Hood and Pitocchelli 1983; Cloutier and Thomas 1992; Yancey et al. 1998). It needs to be investigated whether the high MHC diversity in *S. bilineata* causes the generally low infestation status in this species. Although some studies have suggested that within an individual an intermediate rather than a maximal MHC diversity is optimal for resistance to multiple parasites (Wegner et al. 2003; Woelfig et al. 2009).

Also sexual selection has been proposed to be a major selective force in triggering MHC diversity, because disassortative mating is a promising strategy to generate an immune-competent offspring (Piertney and Olivier 2006). Thereby an individual's MHC constitution might be recognized by olfaction (e.g. Penn and Potts 1998; Kwak et al. 2009). This is underlined by the fact that olfactory receptor genes are known to be in physical linkage to MHC genes (Santos et al. 2010). Olfactory signals are used to a great extent for social communication in the Emballonuridae, of which various species possess sac-shaped pouches in the outer wings. Known from *S. bilineata*, these sacs are filled by drops of urine and glandular secretions; in combination with bacterial activity the produced pheromones are used by males to attract females (Voigt et al. 2008). It remains to be investigated whether the extensive *DRB* polymorphism in *S. bilineata* is linked to certain olfactory cues exerted for mating or whether loci duplication is a side effect of the generation of multiple olfactory receptor genes in this species. However, in this context, it remains unclear why the MHC diversity in the genus *Noctilio* is rather limited since the use of chemical signals for social communication including mating seems to be of comparable significance in the two species of this genus (Studier and Lavoi 1984).

MHC constitution and selective forces in a natural population of *N. albiventris* in Panama

To gain insights in the evolutionary relevance of the adaptive MHC constitution in relation to an individuals fitness related traits, I focused my studies on a natural bat population. I choose the lesser bulldog bat because with its single expressed *DRB* locus it seemed to be an excellent study species to investigate associations of MHC diversity and its possible underlying selective forces (Spurgin and Richardson 2010).

Population-wide genetic variation and population structure

With 18 MHC-*DRB* alleles detected in 215 individuals in a natural population from Panama, the population-wide allelic diversity at one locus as well as sequence diversity between alleles was in range with levels of MHC class II *DRB* diversity described in numerous wild mammalian populations (summarized in Sommer 2005; Castro-Pietro et al. 2011). The observed heterozygosity was high and exceeded the expected one, but did not differ significantly from Hardy-Weinberg equilibrium. Balancing selection acting in contemporary populations is expected to have a detectable effect on genotypic frequencies. If the intensity of selection is sufficient, there can be detectable deviations from Hardy-Weinberg proportions with an over-representation of heterozygote genotypes (Hedrick et al. 2000). But evidence for such a statistical support is rather limited (reviewed in Piertney and Oliver 2006), probably because deviations can not be detected in a single generation or demographic processes acting on small and fluctuating populations as well as sample size limitations mask any observable bias (Seddon and Ellgren 2004). I found evidence that selection favoring heterozygotes in the investigated *N. albiventris* population is not solely due to a simple overdominance mechanism, but seemed to be influenced also by sexual selection. This was evident by a significant difference in the level of heterozygosity between males and females, which I will discuss later on.

I did not find any detectable demographic structuring of the population reflected in the MHC. Within the investigated subpopulations the social structure based on roosting habits did not influence the MHC composition. This is in accordance with observations on mark/re-capture data, where bat individuals occasionally were caught from different roosts indicating a fission-fusion society rather than stable roost associations (Dechmann personal observation). I did not find any genetically differentiations in the MHC between the two subpopulations either, as a sign for the presence of gene flow at a larger spatial scale and/or an equivalent selection pressure maintaining diversity at the *DRB* locus in both subpopulations in the recent past. Interestingly four additionally investigated *N. albiventris* individuals from Ecuador provided a different *DRB* allele set with a high number of alleles compared to the population in Panama. These geographically distant populations might have experienced different local adaptation events due to a different pathogen exposure, but it could also support the suggestion that more than one species might be present within *N. albiventris* (Vilamiu et al. 2010; Lewis-Oritt et al. 2001). To date no data on neutral markers are available, which would offer insights into the molecular divergence of the species or would help to understand more about demographic and social processes in populations and roosting colonies.

MHC constitution and signs for pathogen-mediated selection – evidence for past and contemporary selection pressures

Pathogen-mediated selection maintaining diversity during the evolutionary history of populations is expected to both increase the apparent rate of non-synoymous nucleotide substitutions relative to neutral expectations and also to retain mutations longer than would be expected under a neutral model (Hughes and Nei 1988; Slade and McCallum 1992). At the nucleotide level, I found clear evidence for such pathogen-driven positive selection patterns. This was evident by an excess of non-synonymous substitutions over synonymous ones when considering the entire sequence, specific codons (ABS) which are known to be responsible for antigen binding in humans (Brown et al. 1993, Stern et al. 1994) and by species-specific codons (PSS) that have undergone positive selection rather than neutral evolution. Those analyses on substitution patterns are useful tools for detecting selection operating over macro-evolutionary timescales, but they provide little insight to the timing or cause of selection (Piertney and Oliver 2006; Spurgin and Richardson 2010).

In search of an explanation for contemporary pathogen-mediated selection mechanisms, I investigated associations of an individual's MHC constitution (heterozygosity, specific alleles) and its infestation intensity with ectoparasites. Whereas the effect of heterozygosity on ectoparasite load was not convincing, I identified associations between specific MHC alleles and ectoparasite loads by ticks and bat flies. The presence of MHC alleles associated with parasite infection is a classical indicator of contemporary pathogen-driven selection (Spurgin and Richardson 2010) and was detected in several mammals (e.g. Schad et al. 2005; Untalan et al. 2007; Oliver et al. 2009). Both beneficial and disadvantageous MHC alleles were found which reduced or increased parasite burden. Whereas some alleles had the same effect on both ectoparasite taxa, others were associated either with tick or bat flies infestation and one MHC allele had even a dual effect. Although each MHC molecule has high peptide binding specificity, a molecule may as well accommodate several different peptides (Altuvia and Margalit 2004) and resistance against one parasite can be conferred by several different MHC alleles (Goüy de Bellocq et al. 2008), so that co-evolutionary processes might not necessarily be entirely species-specific (Poulin and Morand 2004). Ectoparasites may develop immunocompatibility with their hosts by sharing antigenic epitopes as a result of host parasite co-evolutionary processes (Dick and Patterson 2007; Mans 2011). An important component of these interactions is arthropod modulation of the host's innate and acquired, specific immune defence by their salivary proteins. (Mans 2011, Schoeler and Wikel 2001). The positive association

of specific MHC molecules with specific ectoparasite taxa might be an effect of shared antigenic epitopes between host and ectoparasites. An interesting aspect remains the ability of ectoparasites to locate their host through specific host odours (Marshal 1982). Thereby ectoparasites are able to differentiate among species (Krasnov et al. 2001, Lourenco and Palmeirim 2008) and possibly also between the sex and reproductive stages (Christe et al. 2007). Moreover, only recently it has been found that the individual microbial community of human skin is responsible for specific odours, which determine levels of attractiveness to mosquitoes (Verhulst et al. 2011). Thus, it is conceivable that the MHC contributes to this localisation, furthermore because there is clear evidence that the individual body odour in vertebrates is influenced by immune genes of the MHC (e.g. Penn and Potts 1998, Kwak et al. 2009).
Associations between MHC constitution and parasite resistance emphasize the relevance of the MHC for an individual's ability to manage an infection. Usually parasite infections are rarely lethal and thus MHC dependent fitness benefits are mediated also by other mechanisms in an individual's life-history evolution e.g. through MHC dependent sexual selection.

MHC constitution and signs for sexual selection – allele frequencies and reproduction in males

Disease resistance has been suggested to play an important role in mediating life history trade-offs. In particular, it is expected that competitive allocation of resources occurs between reproductive effort and immunocompetence especially in males due to costly sexual selected traits (Folstad and Carter 1992; Sheldon and Verhulst 1996; Boonkamp et al. 2008). Thereby, males with well-adapted immune genes to coexisting parasites might allocate more resources to reproduction and hence 'good' alleles are transmitted into future generations ('good-genes model' Hamilton and Zuk 1982; Folstad and Carter 1992). My data support these expectations. Males which carried an MHC allele associated with increased tick burden seemed to be less reproductively active, because this allele was significantly accumulated in non-reproductive males compared to reproductively active males and non-reproductive males had to deal with higher ectoparasite loads. I could even show that these males might have a reduced reproductive success because they differed significantly in their allele composition compared to that of subadults. This differentiation was mainly due to MHC alleles which were associated with low and high ectoparasite load: whereas non-reproductive males carried more often the MHC allele with a disadvantageous effect on tick load, in subadults the MHC allele with a protective effect on ectoparasite load was more

frequent, which underpins the prediction of a transmission of good alleles in future generations. These findings were also supported by the fact that subadults were genetically not differentiated to reproductively active males.

Despite limitations in our available data set and sample sizes per roost were quite low, the results underline the importance of the MHC also in aspects of life-history evolution. The relevance of the MHC for reproductive effort in males has been demonstrated also in other vertebrates, even though reports are rather few, presumably because the underlying mechanisms are complex and difficult to be sorted out (see Charbonnel et al. 2010). In white-tailed deer (*Odocoileus virginianus*) certain MHC-types were associated with both antler development, a secondary sexual selected trait, and testosterone levels indicating that the MHC may help a male deer to cope with stress related to breeding activity (Ditchkoff et al. 2001). In male three-spined sticklebacks (*Gasterosteus aculeatus*) MHC dependent odour signals which are involved in mate choice are produced only when males are in reproductive state signifying that the MHC might alleviate the investment in a costly sexual selected trait (Milinski et al. 2010). In this context, it remains an interesting open question whether and in which extent the odour produced in the inguinal pockets of the scrotum in reproductively active male *N. albiventris* is associated with the MHC and whether it serves as an honest secondary selected trait of a male's quality.

MHC constitution and signs for sexual selection – gender specific differences in heterozygosity

I found no population differentiation between males and females based on MHC allele frequencies, but males had a significantly higher heterozygosity rate and also a higher individual amino acid distance than females. Whereas in males the observed heterozygosity exceeded the expected value, the observed heterozygosity in females nearly reached the expected one. It remains unclear whether the difference between sexes is due to a higher heterozygosity rate in males or a lower heterozygosity rate in females because in both sexes no significant deviations from Hardy-Weinberg expectations were observed. But the observed gender-specific difference in heterozygosity signifies that the sexes differ in the selection pressures acting on the MHC. The increased heterozygosity rate in males could indicate balancing selection in form of a heterozygote advantage and the lower heterozygosity rate of females could suggest a reduced or neutral selection pressure on MHC genotypes in females. Possible explanations for the observed pattern can be attributed to MHC-mediated sex-

biased mechanisms during reproduction or sex-specific survival differences which result in an increased rate of MHC heterozygote males or a deficit in heterozygote females. Direct female choice for MHC dissimilar males which has been repeatedly reported (e.g. Chaix et al. 2008; Schwensow et al. 2008; Ilmonen et al. 2009) or a higher reproductive success of heterozygote males is not convincing because it would lead to a heterozygote offspring in general.

There is little evidence in the literature for a sex-biased difference in offspring survival based on genetic outbreeding (e.g. microsatellites) and no study tested genes of the MHC. However, there is support that outbred male individuals had a higher survival rate than inbred ones, whereas outbreeding had no selective effect on survival rates in females (bats: Rossiter et al. 2001; mice: Meagher et al. 2000, but see Coulson et al. 1999). In contrast, several studies emphasize MHC dependent, male-specific selection mechanisms during reproduction, because there is evidence for a deficit of MHC homozygote newborn males in humans, rats and mice (Palm 1969, 1970, 1974; Hamilton and Hellstrom 1978; Dorak et al. 2002). This male-specific heterozygosity advantage to be born can be best explained by MHC dependent myotic drive during spermatogenesis (Ziegler et al. 2002, 2005) or by a sperm-selective egg-cumulus complex (Eisenach and Giojalas 2006), but also conceivable is a better viability of heterozygote male foetuses (e.g. Ziegler et al. 2005). Even though the underlying mechanisms how the MHC contributes to these findings are not yet fully understood, there is unanimity that the MHC is critical for numerous aspects of mammalian reproduction (e.g. Fernandez et al. 1999; Ziegler et al. 2005; Eisenach and Giojalas 2006). Although it seems conclusive that the observed gender-specific heterozygosity pattern in *N. albiventris* is due to a higher selection pressure in males, I can not definitely rule out the possibility that also sex-biased differences in social structure, dispersal behaviour and gene flow of both sexes might shape the observed heterozygosity pattern to some extent.

To conclude, in the investigated *N. albiventris* population I found empirical evidence for selection pressures acting on specific MHC alleles as well as on heterozygosity supporting predictions of the ‘frequency-dependent selection hypothesis’ and the ‘heterozygote-advantage hypothesis’. Thereby, parasite-mediated and sexual selection mechanisms are obviously operating together to form diversity of the MHC. It was conspicuous that males apparently are faced with a higher selection pressure by MHC-mediated sexual selection mechanism and life history trade-offs than females. This concerned effects of specific MHC alleles as well as levels of heterozygosity. Even

though there were no hints for local adaptation events or demographic processes shaping the MHC pattern within the population, the lack of neutral markers makes it difficult to unravel possible effects of 'fluctuating selection'. In fact, completely disentangling the interacting different forms of balancing selection is an almost irresolvable task, because all mechanisms produce similar final effects on MHC dynamics (Spurgin and Richardson 2010).

Prospective future work

The results of my doctoral work on MHC evolution in bats contribute to a deeper understanding of the still not well understood co-evolutionary dynamics of MHC-mediated parasite-host-interactions with aspects of life-history evolution and provide essential information for designing future studies on MHC-related topics in evolutionary ecology within the order Chiroptera.

***Saccopteryx bilineata*:** In order to understand the associations of the extensive *DRB* polymorphism in *S. bilineata* and its possible association with olfactory cues inserted for mate choice an already funded major network project entitled "olfactory mate choice – immune system, olfactory receptors and their adaptive value" was initiated by Prof. Sommer and others taking *S. bilineata* as an example.

***Carollia perspicillata*:** Concerning *C. perspicillata* there is evidence that activation of the skin immune system by immune challenge tests led to a decreased plasma testosterone level, indicating that there is a trade-off between immune activation and possible investment in reproduction (Greiner et al. 2010). As a result of my doctoral research the molecular techniques are now available to investigate whether an individual's MHC constitution might alleviate this trade-off. This is one of the next study projects in the research group of Prof. Sommer and lab work has already started.

***Noctilio albiventris*:** In *N. albiventris* efforts have been made to collect odors from the subaxillary region of males and females as well as from the scrotal inguinal pockets of males. Promising future work at our institute aims to disentangle associations of an individuals MHC constitution and compounds of its odor profile analyzed by gas chromatography and mass spectrometer (GCMS).

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Summary

Immune genes of the major histocompatibility complex (MHC) constitute a central component of the adaptive immune system and play an essential role in parasite resistance and associated life-history strategies. In addition to pathogen-mediated selection also sexual selection mechanisms have been identified as the main drivers of the typically-observed high levels of polymorphism in functionally important parts of the MHC. The recognition of the individual MHC constitution is presumed to be mediated through olfactory cues. Indeed, MHC genes are in physical linkage with olfactory receptor genes and alter the individual body odour. Moreover, they are expressed on sperm and trophoplast cells. Thus, MHC-mediated sexual selection processes might not only act in direct mate choice decisions, but also through cryptic processes during reproduction.

Bats (Chiroptera) represent the second largest mammalian order and have been identified as important vectors of newly emerging infectious diseases affecting humans and wildlife. In addition, they are interesting study subjects in evolutionary ecology in the context of olfactory communication, mate choice and associated fitness benefits. Thus, it is surprising that Chiroptera belong to the least studied mammalian taxa in terms of their MHC evolution. In my doctoral thesis I aimed to gain insights in the evolution and diversity pattern of functional MHC genes in some of the major New World bat families by establishing species-specific primers through genome-walking into unknown flanking parts of familiar sites. Further, I took a free-ranging population of the lesser bulldog bat (*Noctilio albiventris*) in Panama as an example to understand the functional importance of the individual MHC constitution in parasite resistance and reproduction as well as the possible underlying selective forces shaping the observed diversity.

My studies indicated that the typical MHC characteristics observed in other mammalian orders, like evidence for balancing and positive selection as well as recombination and gene conversion events, are also present in bats shaping their MHC diversity. I found a wide range of copy number variation of expressed DRB loci in the investigated species. In *Saccopteryx bilineata,* a species with a highly developed olfactory communication system, I found an exceptionally high number of MHC loci duplications generating high levels of variability at the individual level, which has never been described for any other mammalian species so far. My studies included for the first time phylogenetic relationships of MHC genes in bats and I found signs for a family-specific independent

mode of evolution of duplicated genes, regardless whether the highly variable exon 2 (coding for the antigen binding region of the molecule) or more conserved exons (3, 4; encoding protein stabilizing parts) were considered indicating a monophyletic origin of duplicated loci within families. This result questions the general assumed pattern of MHC evolution in mammals where duplicated genes of different families usually cluster together suggesting that duplication occurred before speciation took place, which implies a trans-species mode of evolution. However, I found a trans-species mode of evolution within genera (*Noctilio*, *Myotis*) based on exon 2 signified by an intermingled clustering of *DRB* alleles. The gained knowledge on MHC sequence evolution in major New World bat families will facilitate future MHC investigations in this order.

In the *N. albiventris* study population, the single expressed MHC class II *DRB* gene showed high sequence polymorphism, moderate allelic variability and high levels of population-wide heterozygosity. Whereas demographic processes had minor relevance in shaping the diversity pattern, I found clear evidence for parasite-mediated selection. This was evident by historical positive Darwinian selection maintaining diversity in the functionally important antigen binding sites, and by specific MHC alleles which were associated with low and high ectoparasite burden according to predictions of the 'frequency dependent selection hypothesis'. Parasite resistance has been suggested to play an important role in mediating costly life history trade-offs leading to e.g. MHC-mediated benefits in sexual selection. The 'good genes model' predicts that males with a genetically well-adapted immune system in defending harmful parasites have the ability to allocate more resources to reproductive effort. I found support for this prediction since non-reproductive adult *N. albiventris* males carried more often an allele associated with high parasite loads, which differentiated them genetically from reproductively active males as well as from subadults, indicating a reduced transmission of this allele in subsequent generations. In addition, they suffered from increased ectoparasite burden which presumably reduced resources to invest in reproduction. Another sign for sexual selection was the observation of gender-specific difference in heterozygosity, with females showing lower levels of heterozygosity than males. This signifies that the sexes differ in their selection pressures, presumably through MHC-mediated molecular processes during reproduction resulting in a male specific heterozygosity advantage. My data make clear that parasite-mediated selection and sexual selection are interactive and operate together to form diversity at the MHC. Furthermore, my thesis is one of the rare studies contributing to fill the gap between MHC-mediated effects on co-evolutionary processes in parasite-host-interactions and on aspects of life-history evolution.

Zusammenfassung

Innerhalb des adaptiven Immunsystems spielen die Gene des MHC (Major Histocompatibility Complex) eine zentrale Rolle. Neben ihrer Funktion für die körpereigene Parasitenabwehr haben sie auch einen entscheidenden Einfluss auf damit verbundene ‚life-history' Strategien. Typischerweise sind die funktional für die Pathogenerkennung wichtigen Genabschnitte hoch variabel, was evolutiv nicht nur durch die Vielfalt der Pathogene bedingt ist, sondern im Zuge der sexuellen Selektion durch entsprechende Partnerwahl gefördert wird. Dabei wird die individuelle MHC-Konstitution sehr wahrscheinlich über körpereigene Duftstoffe vermittelt, denn MHC Gene bestimmen nicht nur den individuellen Körpergeruch, sondern liegen in chromosomaler Kopplung mit olfaktorischen Rezeptorgenen. Außerdem werden sie auch auf Sperma- und Trophoplastenzellen exprimiert, so dass MHC-bedingte sexuelle Selektionsmechanismen nicht nur über die direkte Partnerwahl, sondern auch durch kryptische Mechanismen während der Fortpflanzung wirken können.

Fledermäuse und Flughunde (Chiroptera) bilden die zweitgrößte Säugetiergruppe und gelten als wichtiges Reservoir und Überträger für den Menschen und andere Wildtiere hoch infektiöser Krankheiten. Innerhalb der evolutionären Ökologie sind sie außerdem auf Grund ihrer z.T. komplexen olfaktorischen Kommunikation während der Partnerwahl und den damit verbundenen fitness relevanten Vorteilen interessante Forschungsobjekte. In Anbetracht dessen ist es erstaunlich, dass bisher so gut wie nichts über den MHC in dieser Säugergruppe bekannt ist. Das Ziel meiner Dissertation war es, zum einen Einblicke in die Evolution und Diversität funktional wichtiger MHC Gene (MHC Klasse II *DRB*) bei Fledermäusen zu erhalten, und zum anderen zu untersuchen, inwieweit die individuelle MHC-Konstitution am Beispiel der kleinen Hasenmaulfledermaus (*Noctilio albiventris*) einen Einfluss auf Parasitenresistenz und Fortpflanzung hat und welche Selektionsmechanismen dabei für das entstandene genetische Diversitätsmuster verantwortlich sind.

Meine Arbeit zeigt, dass Prozesse, die bei anderen Vertebratenordnungen das Diversitätsmuster am MHC hervorrufen, wie balancierende und positive Selektion, Rekombination und Genkonversion ebenfalls für Fledermäuse zutreffen. In der Anzahl exprimierter *DRB* loci unterscheiden sich die untersuchten Fledermausarten allerdings beträchtlich. Bemerkenswert ist die extrem hohe Anzahl *DRB* loci bei *Saccopteryx bilineata*, die in dieser Ausprägung noch bei keiner anderen Säugetierart beschrieben wurde, einer Fledermaus mit einem hoch entwickelten olfaktorischen Kommunikations-

system. Die hier erstmals durchgeführten phylogenetischen Untersuchungen zeigen, dass sich anders als für die meisten anderen Säugetiergruppen beschrieben, die duplizierten *DRB* Loci unabhängig voneinander entwickelt haben. Dieser monophyletische Ursprung duplizierter Loci innerhalb von Fledermausfamilien bestätigte sich für alle Bereiche des Genes: dem hochvariablen Exon 2, das für den funktional entscheidenden Pathogen-bindenden Bereich des Proteins kodiert, sowie für Exon 3 und 4, die für die Molekülstruktur erhaltende Bereiche des Proteins kodieren. Innerhalb der Gattungen (*Noctilio, Myotis*), basierend auf Exon 2, fand ich das für andere Säugergruppen typische Bild eines ‚trans-species polymorphism', bei dem MHC-Allele von verschiedenen Arten sich untereinander ähnlicher sein können als Allele der gleichen Art. Meine Ergebnisse sind ein wichtiger Beitrag zum Verständnis der MHC Evolution in der Gruppe der Fledermäuse und liefern hilfreiche Kenntnisse für zukünftige Studien zum MHC in dieser Säugetierordnung.

Meine Studien an einer frei lebenden Population der kleinen Hasenmaulfledermaus zeigten dass der exprimierte *DRB* Locus typische Anzeichen pathogenbedingter aber auch sexueller Selektionsmechanismen zeigt. Ich fand eine ausgeprägte populationsweite Heterozygotie, positive darwinsche Selektion, die den Polymorphismus in Codons die direkt an der Pathogenerkennung beteiligt sind erhält, sowie spezifische Allele die entweder mit einer erhöhten oder einer geringen Parasitenbelastung einhergehen, entsprechend den Annahmen der ‚Frequenz-abhängigen Selektions-Hypothese'. Die individuelle Parasitenresistenz gilt als ein wichtiger Faktor um ressourceabhängige ‚life-history' Strategien auszuloten. Vor allem Männchen mit einem effektiven Immunsystem, sollten mehr Energien für die Fortpflanzung zur Verfügung haben (‚good-genes model'). Meine Daten bestätigen diese Annahme, Männchen die stärker parasitisiert waren, waren weniger häufig reproduktiv aktiv und trugen häufiger ein *DRB*-Allele das mit erhöhter Parasitenbelastung einherging. Genetisch unterschieden sie sich darin nicht nur von den reproduktiv aktiven Männchen der Population sondern auch von den Jungtieren. Die Jungtiere trugen zudem häufiger ein für die Parasitenabwehr vorteilhaftes Allel. Die Ergebnisse zeigen dass die individuelle MHC-Konstitution einen nicht zu unterschätzenden Einfluss auch auf den Reproduktionserfolg eines Männchens haben kann und vorteilhafte Allele sich bereits in nachfolgenden Generationen durchsetzen. Meine Doktorarbeit gehört damit zu einer der seltenen Studien, die nicht nur zeigen konnte inwieweit der MHC an co-evolutionären Prozessen der Parasit-Wirt-Interaktion beteiligt ist, sondern dass er darüber hinaus auch direkt für die individuelle ‚life-history' Entwicklung von Bedeutung ist.

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Appendix I

Table S1. MHC class II *DRB*- and *DQB*-specific amino acid positions and their coding nucleotide triplets in the β2-domain (encoded by exon 3) in different mammal species. Numbers indicate amino acid positions in the β-chain (Brown et al. 1993). For simplification only one sequence per species is shown and GenBank accession numbers are provided in brackets.

Gene	110	121	128	146	164	169
***DRB*-specific amino acids**	**Q**	**G**	**E**	**G**	**V**	**E**
***DRB*-specific nucleotide tripletts**	**CAG**	**GGN**	**GAR**	**GGC**	**GTY**	**GAR**
*HLA-DRB*1 (HM067861)	CAG	GGT	GAA	GGC	GTT	GAG
HLA-DRB3 (U66825)	CAG	GGT	GAA	GGC	GTT	GAG
*HLA-DRB*4 (NM021983)	CAG	GGT	GAA	GGC	GTT	GAG
*Patr-DRB*5 (FN424217)	CAG	GGT	GAA	GGC	GTT	GAA
Mamu-DRB (AJ601364)	CAG	GGT	GAA	GGC	GTT	GAG
Scab-DRB (M97616)	CAG	GGC	GAA	GGC	GTT	GAG
SLA-DRB (AY962313)	CAG	GGG	GAG	GGC	GTT	GAG
*Ovar-DRB*1 (AM182982)	CAG	GGA	GAA	GGC	GTT	GAG
*BoLA-DRB*1 (D45357)	CAC	GGT	GAA	GGC	GTT	GAG
Tuad-DRB (EF507874)	CAC	GGT	GAA	GGC	GTC	GAG
Eqca-DRB (L33910)	CAC	GGT	GAA	GGC	GTC	GAG
Feca-DRB (EU916192)	CAG	GGT	GAG	GGC	GTT	GAG
*DLA-DRB*1 (NM001014768)	CAG	GGT	GAA	GGC	GTT	GAG
Zaca-DRB (AY491457)	CAG	GGT	GAA	GGC	GTT	GAG
***DQB*-specific amino acids**	**N**	**D**	**K**	**variable***	**T/N**	**D**
***DQB*-specific nucleotide tripletts**	**AAY**	**GAT**	**AAR**	**YMN**	**ACT**	**GAY**
*HLA-DQB*1 (NM002123)	AAC	GAT	AAA	CCC	ACT	GAT
HLA-DQB2 (NR003937)	AAC	GAT	AAA	TCC	ACT	GAC
*Poab-DQB*1 (NM001131755)	AAT	GAT	AAA	CCC	ACT	GAC
*Mamu-DQB*1 (EF3625446)	AAC	GAT	AAA	CCC	ACT	GAC
*SLA-DQB*1 (DQ883220)	AAC	GAT	AAA	CCT	AAT	GAT
*Ovar-DQB*1 (HQ7286751)	AAC	GAT	AAG	CCT	ACC	GAT
*BoLA-DQB*1 (AF037315)	AAC	GAT	AAG	CCT	ACC	GAT
Tuad-DQB (EF017816)	AAC	GAT	AAA	CCT	ACT	GAT
Eqca-DQB (L33910)	AAC	GAT	AAA	CCC	ACT	GAT
Urth-DQB (AB473936)	AAC	GAT	AAA	CCA	ACT	GAT
*DLA-DQB*1 (S53615)	AAC	GAT	AAA	CCA	ACT	GAT
Zaca-DQB (AF503397)	AAC	GAT	AAA	CCA	ACT	GAT

** never Guanine present*; *BoLA*, *Bos taurus*; *DLA*, *Canis lupus*; *Eqca*, *Equus callibus*; *Feca*, *Felis cattus*; *HLA*, *Homo sapiens*; *Mamu*, *Maccaca mulatta*; *Ovar*, *Ovis aries*; *Patr*, *Pan troglytis*; *Poab*, *Pongo abelii*; *SLA*, *Sus scrofa*; *Scab*, *Scurius aberti*; *Tuad*, *Tusiop aduncus*; *Urth*, *Urs thibetanus*; *Zaca*, *Zalophus californianus*

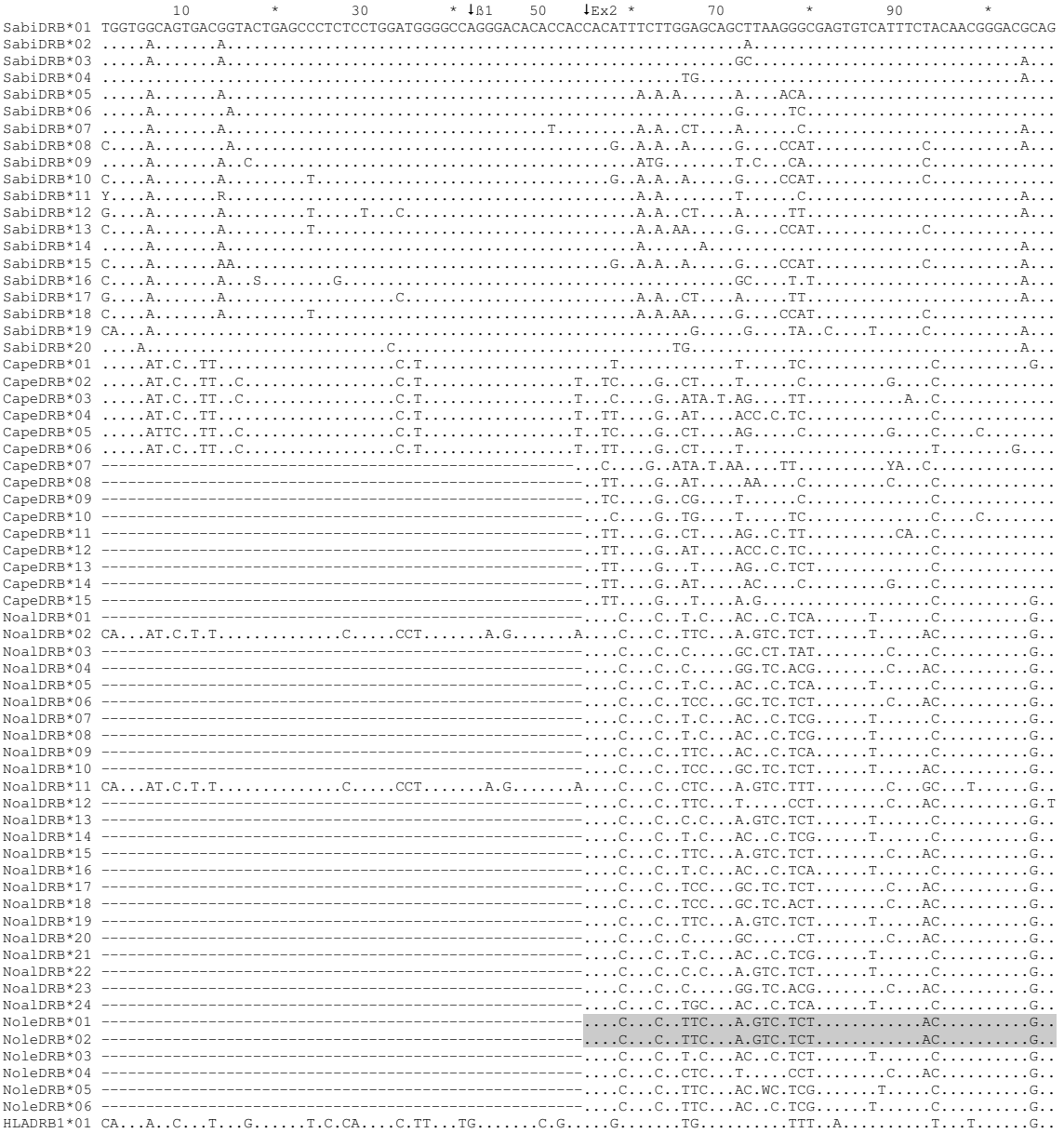

Figure S1. Nucleotide sequence variability of MHC class II *DRB* sequences derived from gDNA (exon 2) and cDNA (exon 1 to 4) of *Saccopteryx bilineata* (*Sabi-DRB*), *Carollia perspicillata* (*Cape-DRB*), *Noctilio albiventris* (*Noal-DRB, Noal-DRB**01-*18 according to Schad et al. 2011) and *Noctilio leporinus* (*Nole-DRB*). The human *HLA-DRB1**0101 sequence (GenBank accession number: HM067843) is included for comparison. Borders of exon 3 to 4 and domains of the β-chain were assigned according to Bowen et al. 2004. Dots mark identity with the top sequence. Dashes indicate not amplified regions. Potential gene conversion events identified by GENECONV (Sawyer 1999) are shaded.

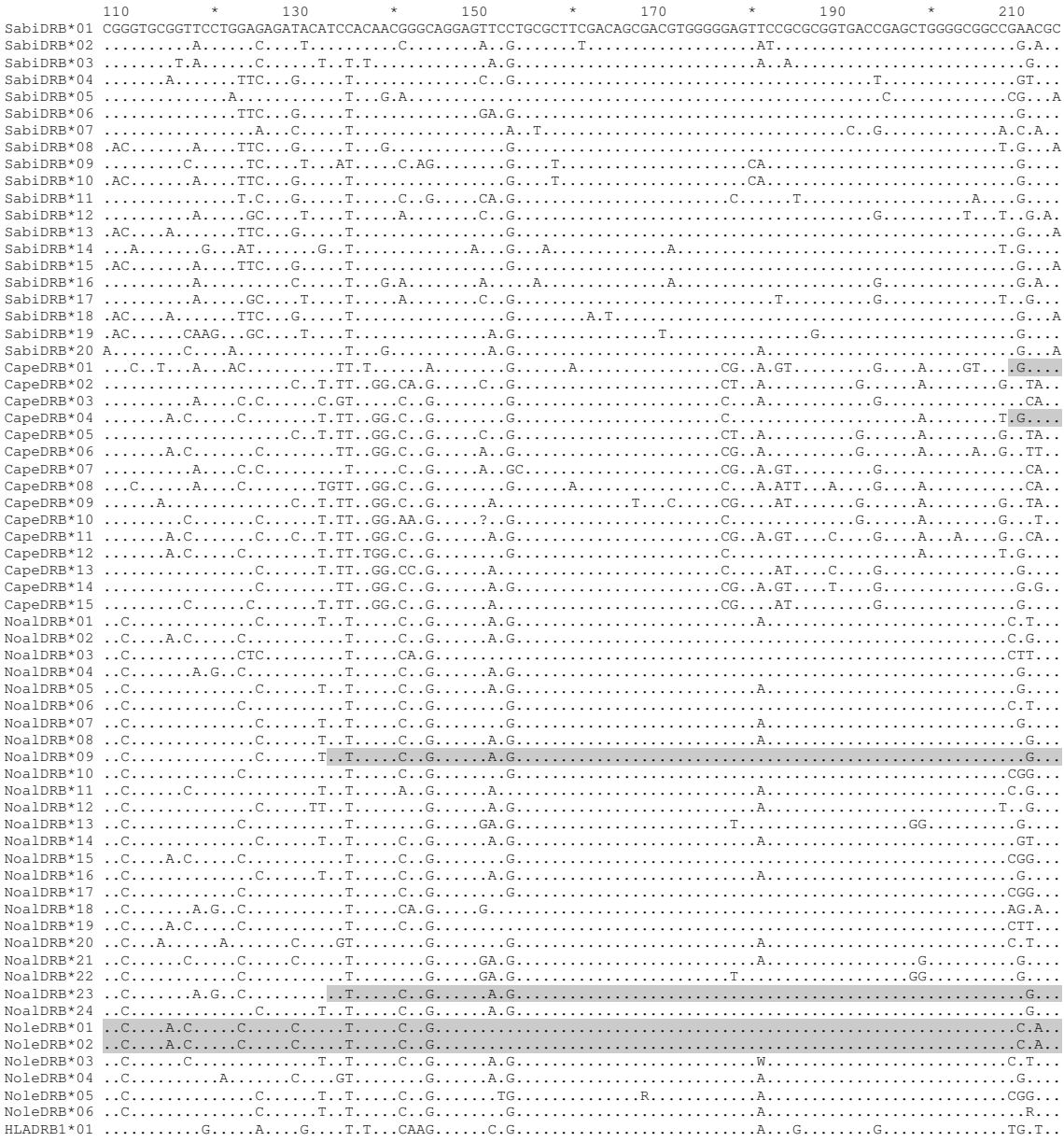

Figure S1 continued

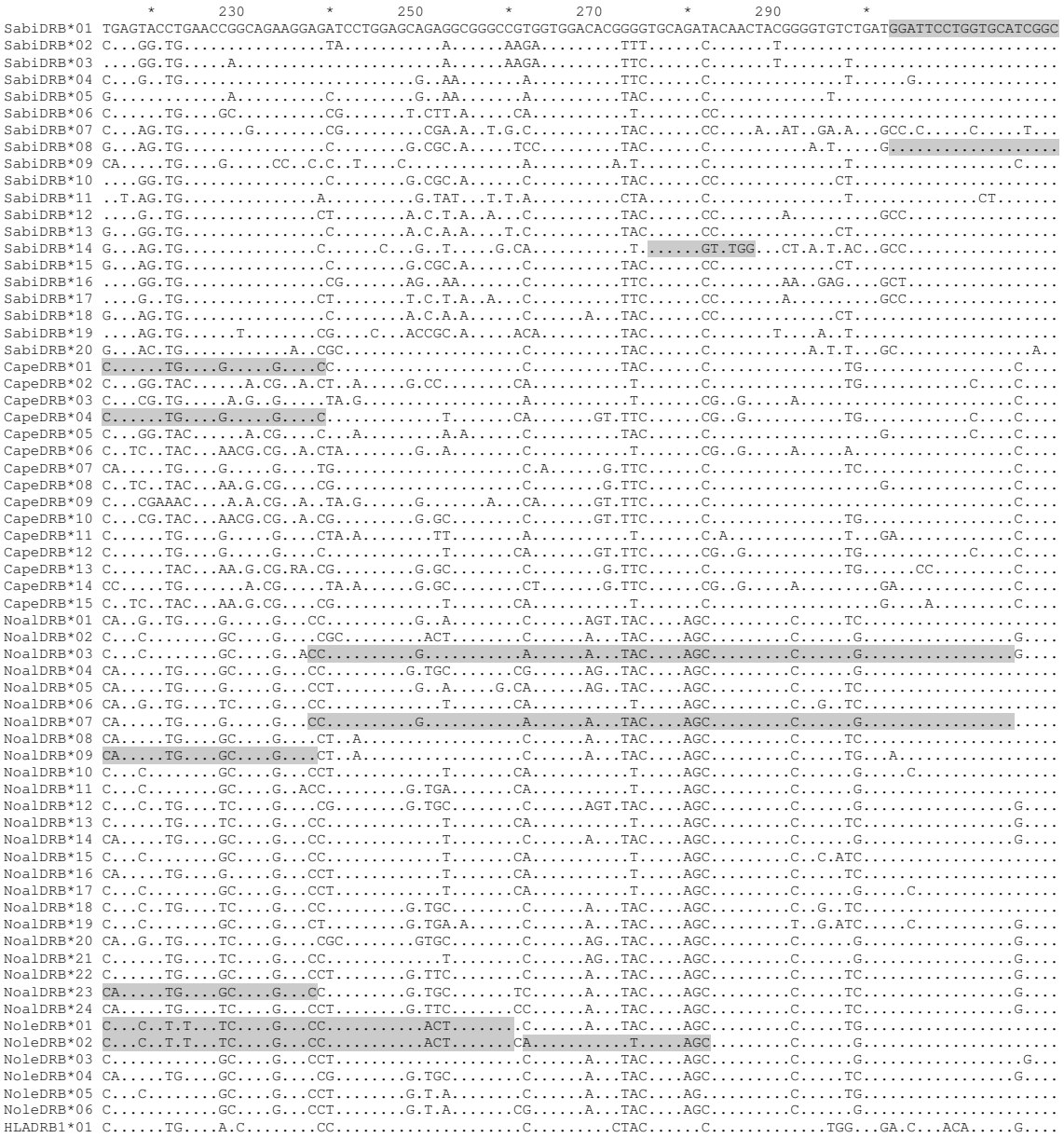

Figure S1 continued

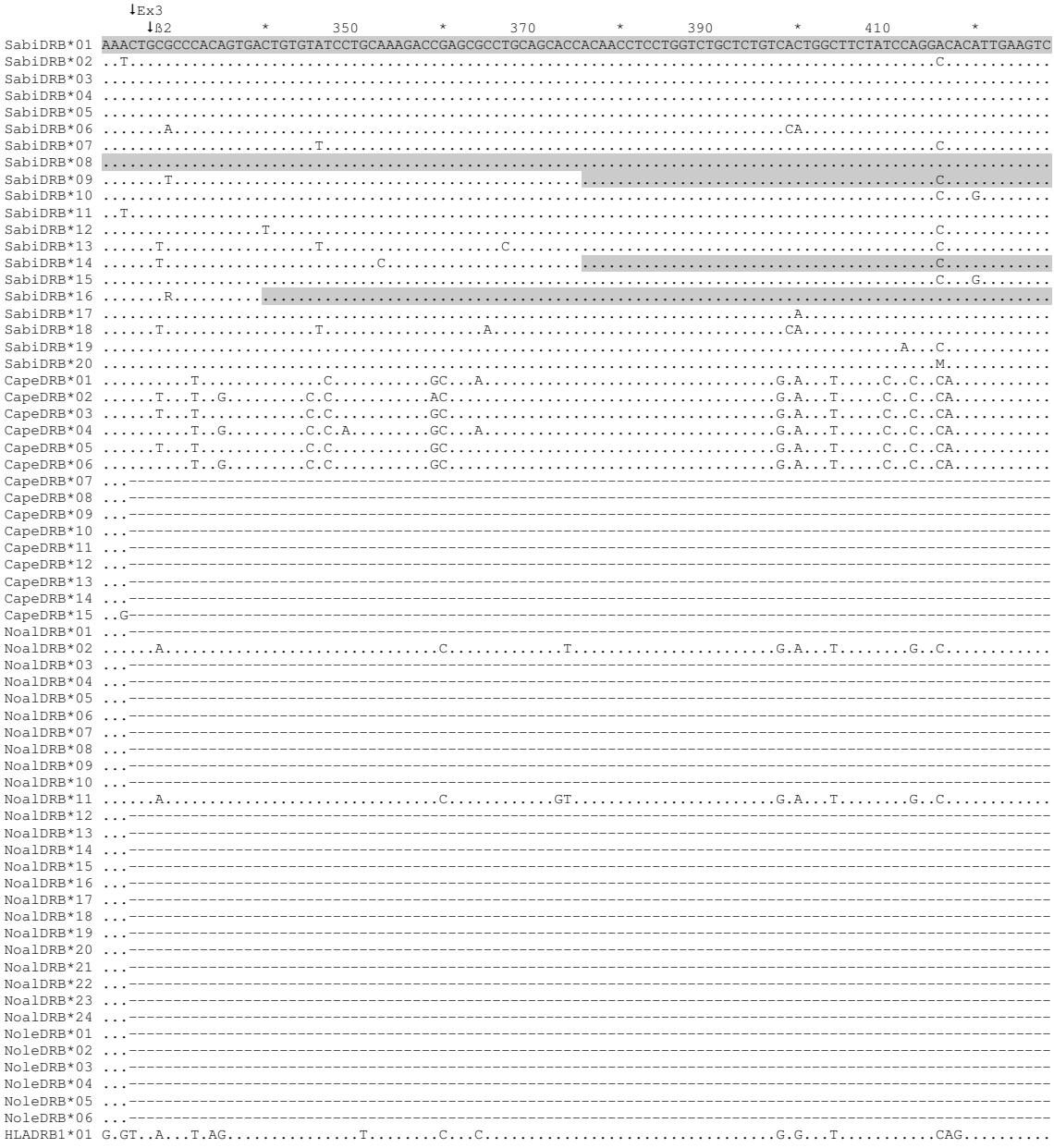

Figure S1 continued

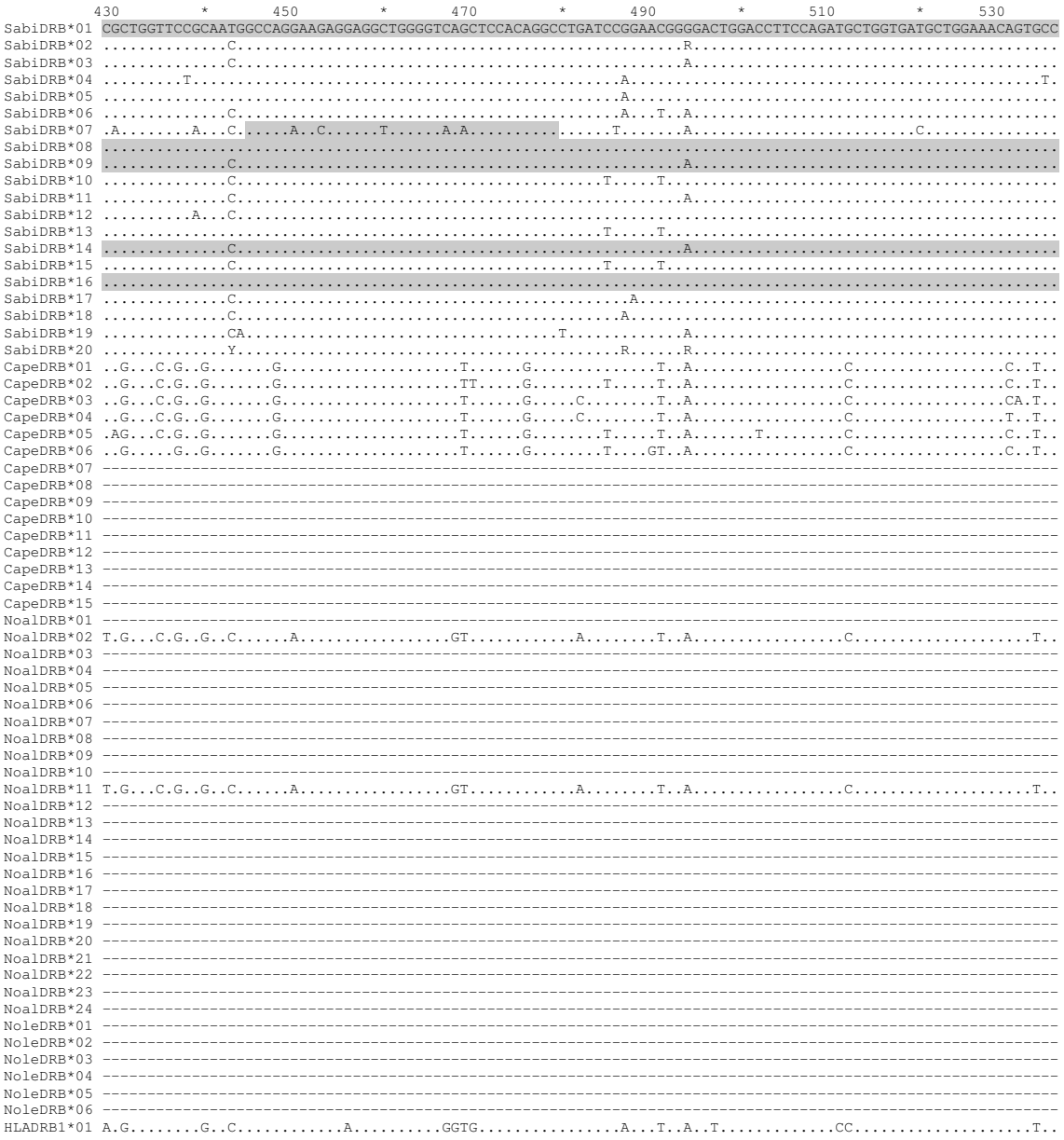

Figure S1 continued

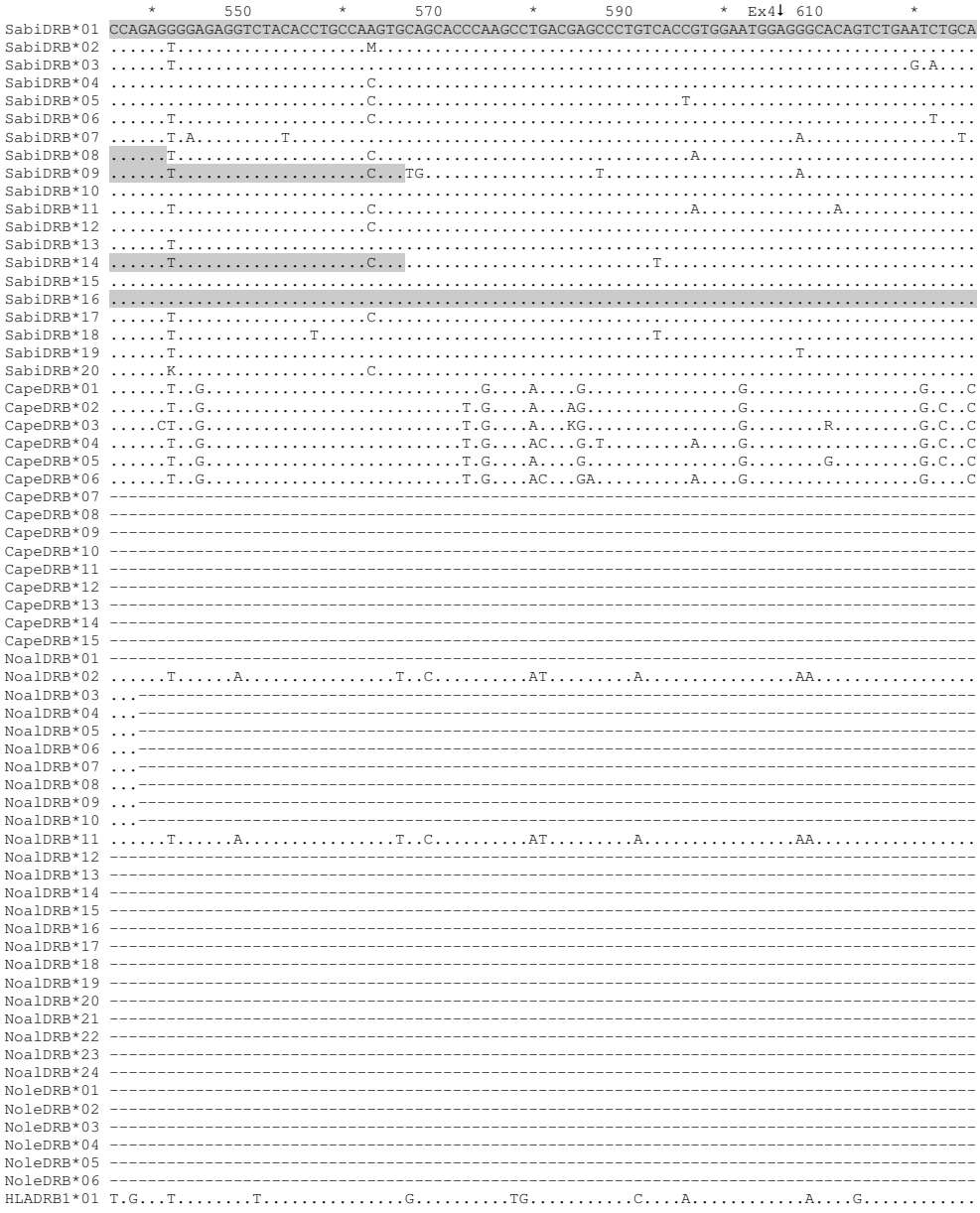

Figure S1 continued

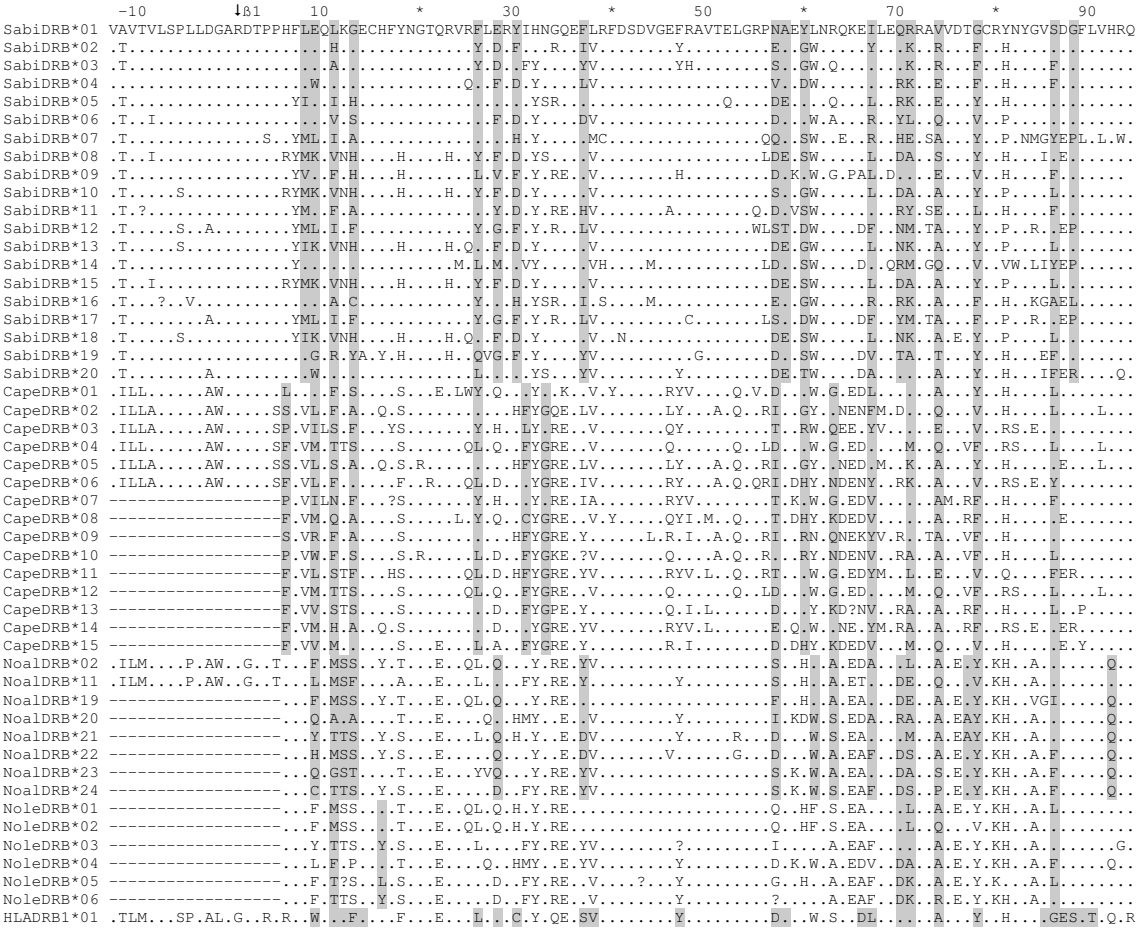

Figure S2. Deduced amino acid alignment of MHC class II *DRB* sequences including the β1 and β2-domain of *Saccopteryx bilineata* (*Sabi-DRB*), *Carollia perspicillata* (*Cape-DRB*), *Noctilio albiventris* (*Noal-DRB*), *N. leporinus* (*Nole-DRB*) and the human *HLA-DRB1**0101 sequence (GenBank accession number: HM067843). Border of the domains are referred from Bowen et al. (2004). Numbers indicate the amino acid positions of the β-chain. Dots mark identity with the top sequence. Question marks indicate unknown amino acids. Positively selected sites identified by CODEML (Yang et al. 2005) are shaded as well as putative antigen binding sites of the human *HLA-DR1* β-chain (Brown et al. 1993). Boxes in the top sequence mark *DRB*-specific amino acids in the β2-domain which differentiate them from *DQB*-molecules.

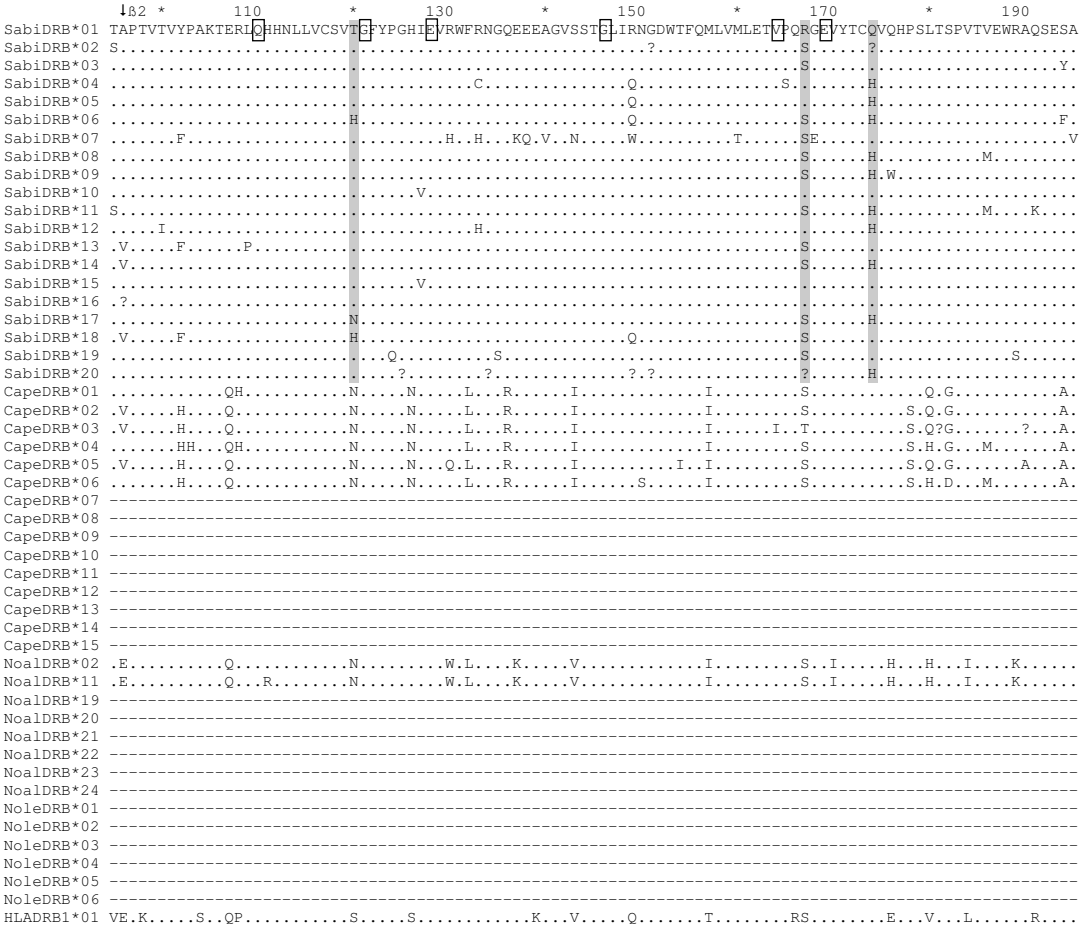

Figure S2 continued

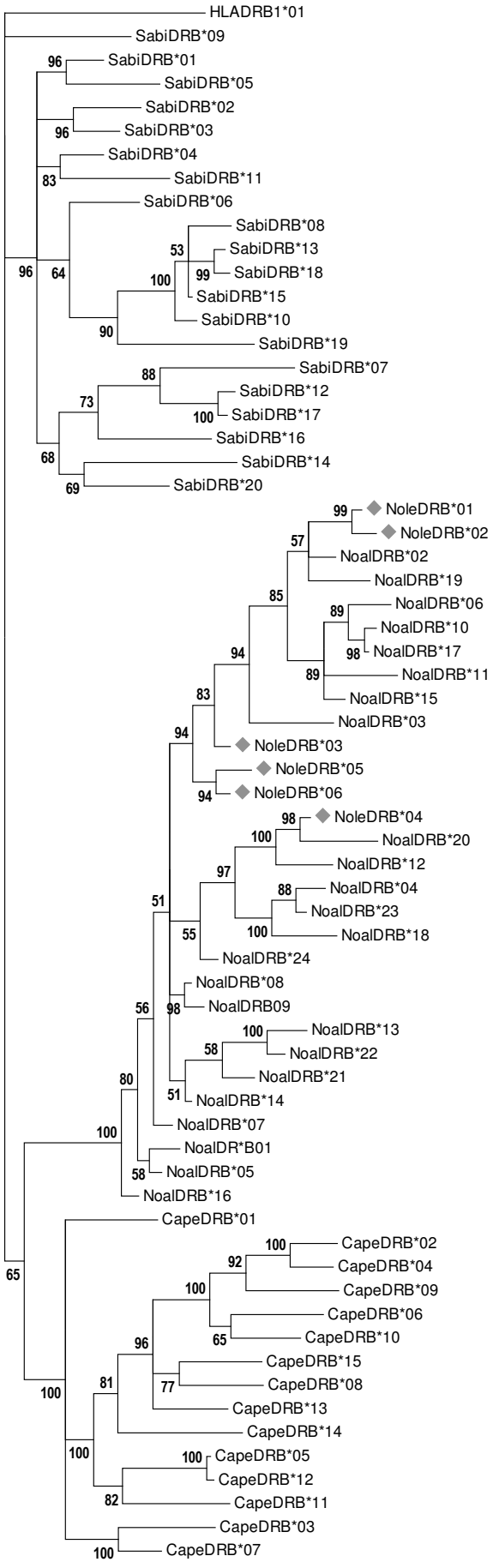

Figure S3. Consensus Bayesian trees (50%-majority-rule) for major histocompatibility complex class II *DRB* sequences based on exon 2 (270 bp; nucleotide position 55-314 according to Fig S1) of the bat species *Saccopteryx bilineata* (*Sabi-DRB**01-20, this study), *Carollia perspicillata* (*Cape-DRB**01-15, this study), *Noctilio albiventris* (*Noal-DRB**01-*24, this study; Schad et al. 2011) as well as *Noctilio leporinus* (◆, *Nole-DRB**01-07, this study). The human *HLA-DRB*1*01 allele (GenBank accession number: HM067843) was used as outgroup to root the trees. Two runs and 4 chains per run were run for 5.11 x 10^6 generations (GTR + Γ + I). Numbers under nodes signify clades supported Bayesian posterior probabilities. Distances are adjusted using best fit models indicated by jMODELTEST (Posada 2008).

Appendix II

Table S1. Data collection of the *N. albiventris* population in Panama in different roosts. Sample sizes according to reproductive state of the whole population (N = 214) are marked in bold, sample sizes of individuals with tick (N = 134) and bat flies (N = 166) data are given in parentheses.

Roost	Females			Males		Subadults
	non-reproductive	lactating	pregnant	reproductive	non-reproductive	non-reproductive
BCI	**4** (4, 1)	**0**	**3** (3, 3)	**13** (11, 8)	**8** (8, 4)	**1** (0, 0)
A	**10** (0, 5)	**16** (0, 5)	**0**	**10** (2, 7)	**8** (0, 2)	**9** (0, 0)
B	**30** (23, 30)	**14** (12, 14)	**8** (7, 7)	**3** (3, 3)	**16** (16, 16)	**3** (0, 3)
C	**8** (8, 8)	**1** (1, 1)	**4** (3, 4)	**3** (3, 3)	**10** (9, 10)	**1** (0, 1)
D	**8** (4, 8)	**2** (0, 2)	**0**	**3** (2, 3)	**1** (0, 1)	**6** (4, 6)
E	**3** (3, 3)	**0**	**0**	**1** (1, 1)	**0**	**0**
F	**0**	**0**	**0**	**7** (7, 7)	**0**	**0**

Table S2. Data collection of the *N. albiventris* population in Panama in different months and years. Sample sizes according to reproductive state of the whole population (N = 214) are marked in bold, sample sizes of individuals with tick (N = 132) and bat flies (N = 166) data are given in parentheses.

Season	Females			Males		Subadults
	non-reproductive	lactating	pregnant	reproductive	non-reproductive	non-reproductive
2006_9	**17** (0, 13)	**17** (0, 6)	**0**	**8** (0, 5)	**8** (0, 2)	**11** (0, 2)
2007_10/11	**3** (3, 3)	**2** (2, 2)	**0**	**9** (9, 8)	**1** (1, 1)	**0**
2007_6	**6** (3, 6)	**3** (0, 3)	**0**	**1** (1, 1)	**1** (0, 1)	**7** (4, 7)
2007_9	**13** (11, 13)	**9** (9, 9)	**0**	**6** (6, 6)	**14** (13,14)	**0**
2008_2/3	**11** (11, 11)	**2** (2, 2)	**11** (9, 10)	**1** (1, 1)	**3** (3, 3)	**0**
2008_4/5	**3** (2, 2)	**0**	**1** (1, 1)	**1**(1,1)	**4** (4, 1)	**0**
2008_9	**0**	**0**	**0**	**2** (2, 2)	**0**	**0**
2008_10/11	**10** (9, 8)	**0**	**3** (3, 3)	**12** (9, 8)	**12** (12, 10)	**2** (1, 1)

Table S3. MHC class II *DRB* exon 2 variability in the whole *N. albiventris* population, and in non-reproductive adult males, in reproductively active males and in subadults. Sample size (N), number of alleles (No) and allele frequencies are shown.

	All	Males non-repro	Males repro	Subadults
N	214	42	40	20
No	18	14	15	12
***Noal-DRB**01**	0.040	0.060	0.064	0.050
***Noal-DRB**02**	0.175	0.250	0.103	0.075
***Noal-DRB**03**	0.009	-	0.013	-
***Noal-DRB**04**	0.117	0.119	0.141	0.125
***Noal-DRB**05**	0.044	0.036	0.051	0.025
***Noal-DRB**06**	0.040	0.036	0.026	0.025
***Noal-DRB**07**	0.005	0.012	-	-
***Noal-DRB**08**	0.042	0.071	0.038	0.100
***Noal-DRB**09**	0.054	0.071	0.026	0.050
***Noal-DRB**10**	0.255	0.179	0.231	0.375
***Noal-DRB**11**	0.028	0.036	0.038	0.075
***Noal-DRB**12**	0.088	0.071	0.128	0.025
***Noal-DRB**13**	0.028	0.024	0.026	0.025
***Noal-DRB**14**	0.005	-	0.013	-
***Noal-DRB**15**	0.005	-	-	-
***Noal-DRB**16**	0.007	-	-	-
***Noal-DRB**17**	0.044	0.024	0.038	0.05
***Noal-DRB**18**	0.014	0.012	0.038	-

Table S4. Test statistics on population differentiation using G'_{ST} (Hedrick 2005) and D_{est} (Jost 2008) between non-reproductive adult males, reproductively active males and subadults.

Groups compared	Df	Diversity Parameter	Pairwise Distance	Bootstrap Estimate	Variance	Std Err	Confidence Interval
Males non-repro	2	G'_{ST}		0.178	0.006	0.002	0.052-0.326
Males repro Subadults		D_{est}		0.162	0.005	0.002	0.048-0.299
Males non-repro	1	G'_{ST}	0.055	0.130	0.007	0.003	0.003-0.287
Males repro		D_{est}	0.052	0.123	0.006	0.003	0.003-0.171
Males non-repro	1	G'_{ST}	0.163	0.234	0.013	0.004	0.042-0.045
Subadults		D_{est}	0.152	0.219	0.012	0.003	0.040-0.423
Males repro	1	G'_{ST}	0.036	0.133	0.010	0.003	-0.017-0.327
Subadults		D_{est}	0.033	1.123	0.008	0.003	-0.015-0.037

Df: degrees of freedom, D_{est}: Jost's diversity index (Jost 2008), G'_{ST}: Hedrick's diversity index (Hedrick 2005), Std Err: Standard error