

ORIGINAL ARTICLE

Non-neutral evolution of the major histocompatibility complex class II gene *DRB1* in the sac-winged bat *Saccopteryx bilineata*

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The immune genes of the major histocompatibility complex (MHC) are classical examples for high levels of genetic diversity and non-neutral evolution. This is particularly true for the regions containing the antigen-binding sites as, for instance, in the exon 2 of the MHC class II gene *DRB*. We surveyed, for the first time in the order Chiroptera, the genetic diversity within this exon in the sac-winged bat *Saccopteryx bilineata*. We detected 11 alleles among 85 bats, of which 79 were sampled in one population. Pairwise comparisons revealed that interallelic sequence differences ranged between 3 and 22%, although nucleotide substitutions were not evenly distributed along the exon

sequence. This was most probably the result of intragenic recombination. High levels of sequence divergence and significantly more nonsynonymous than synonymous substitutions ($d_N/d_S > 1$) suggest long-term balancing selection. Thus, the data are consistent with the hypothesis that recombination gives rise to new alleles at the *DRB* locus of the sac-winged bat, and these are maintained in the population through balancing selection. In this respect, the sac-winged bat closely resembles other mammalian species.

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Introduction

The major histocompatibility complex (MHC) plays a central role in the vertebrate immune system. MHC genes encode two major classes of transmembrane glycoproteins consisting of two chains, α and β . MHC class I genes are expressed in almost all nucleated somatic cells, whereas MHC class II genes are confined to antigen-presenting cells of the immune system (Klein, 1986). Although this general function of the MHC is conserved among vertebrates, the genetic architecture of the MHC can differ within and between vertebrate classes (recently reviewed in Kelley *et al.*, 2005). Gene duplication occurred frequently, and thus the number of classes I and II loci can substantially vary between species (Kelley *et al.*, 2005; Nei and Rooney, 2005). In mammals, the genetic diversity at various MHC loci has been studied in several species of all major mammalian orders except for the second largest order Chiroptera that comprise more than 1000 species worldwide (Simmons, 2006). Bats and flying foxes are of particular immunological interest, because they are hosts of viruses pathogenic to humans including rabies (Fooks *et al.*, 2003; Cliquet and Picard-Meyer, 2004), Ebola (Leroy *et al.*, 2005), SARS-like viruses (Li *et al.*, 2005), and Nipah and Hendra viruses (Wong *et al.*, 2007).

MHC genes are the most variable genes described in vertebrates. Up to 499 alleles were described at the human MHC locus HLA-B (Garrigan and Hedrick, 2003). Highest levels of nucleotide diversity are found in regions containing antigen-binding sites (ABS) and patterns of nucleotide substitutions deviate from neutral evolution expectation in almost all studies. Some form of balancing selection best explains the enormous diversity of certain MHC genes (Hedrick and Thomson, 1983), and two major mutually nonexclusive mechanisms of pathogen-driven selection have been proposed to explain this pattern. First, the frequency-dependent selection hypothesis or the rare allele advantage (Clarke and Kirby, 1966) predicts that rare alleles can have a selective advantage as parasites will adapt to common host alleles. Since an advantageous allele will increase in frequency, parasites resistant to this allele will be favoured. Negative frequency-dependent selection models of host–parasite coevolution showed that a large number of alleles can be retained (Borghans *et al.*, 2004). The spatial and rapid temporal variation in frequency of MHC alleles and resistance provides evidence for frequency-dependent selection (Hill *et al.*, 1994; Westerdahl *et al.*, 2004; Charbonnel and Pemberton, 2005). Second, the overdominance or heterozygote advantage hypothesis (Doherty and Zinkernagel, 1975) assumes that heterozygous individuals have a higher average fitness, because individuals being heterozygous at the antigen-binding region can present a wider array of antigens than homozygous individuals. Therefore, heterozygotes are expected to be more resistant against pathogens than homozygotes. An overdominance effect was shown in

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mice that were coinfecting with *Salmonella enterica* and Theiler's murine encephalomyelitis virus (McClelland *et al.*, 2003). Distinguishing between the frequency-dependent selection and the heterozygote advantage hypothesis is difficult, because both models require a correlation between parasites and particular MHC alleles. Meanwhile, such relationships were found in several species including many non-model organisms (Table 1 in Sommer, 2005). The evolutionary ecology of the MHC was recently summarized in several reviews (Bernatchez and Landry, 2003; Garrigan and Hedrick, 2003; Sommer, 2005; Piertney and Oliver, 2006).

We analysed the antigen-binding region of the MHC class II gene *DRB* of the sac-winged bat *Saccopteryx bilineata* by sequencing cloned PCR products. We selected this neotropical, insectivorous bat species, because completely sampled families consisting of an offspring and both parents were available (Heckel and von Helversen, 2003) that allowed testing Mendelian inheritance of haplotypes. In this study, we describe the genetic diversity within exon 2 of the MHC class II gene *DRB*, and discuss the role of recombination and selection in generating and maintaining high levels of genetic diversity in colonies of the sac-winged bat.

Materials and methods

Sampling

Bats were caught by mist netting at the biological station La Selva in Costa Rica (10°20'N, 84°10'W, $N=79$), on Barro Colorado Island (BCI) in Panama (9°09'N, 79°51'W, $N=3$) and at the Tiputini Biodiversity Station (TBS) in the Amazonian rainforest of Ecuador (~0°38'S, 76°08'W, $N=3$) between 1998 and 2001. Animals were sexed, aged and measured before a circular wing biopsy with a diameter of 4 mm was taken from the proximal plagiopatagium: biopsies were stored in 80% ethanol at room temperature until DNA isolation.

Genetic analysis

DNA was isolated by a salting out procedure (Müllensbach *et al.*, 1989), which yielded about 0.5 µg of total genomic DNA. The exon 2 of the class II MHC gene *DRB* was initially amplified by PCR with the primers GH46 and GH50 (Scharf *et al.*, 1988) but only small amounts of DNA were obtained. The design of two shorter primers ER407B (5'-AACCCCGTAGTTGTGTCTGCA-3') and ER408 (5'-GGATCCTTCGTGTCCCA-3'), which have a similar T_m value and contain only the core sequences of the primers GH46 and GH50, improved the PCR substantially. The reaction was performed in a total volume of 25 µl, which contained about 20 ng total genomic DNA, 0.4% bovine serum albumin, 1 mM of each primer (ER407B and ER408), 0.1 mM of each dNTP, 2 mM MgCl₂, 0.5 U Taq-DNA-polymerase (Promega Corporation, Madison, WI, USA, no. M1665) and 1 × Taq-DNA-polymerase buffer (supplied with the enzyme). A PCR was started with incubation at 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1.5 min and finished by 10 min period at 72°C. Amplified DNA fragments were cleaned with QIAquick PCR Purification columns (QIAGEN, GmbH, Hilden, Germany) and reconstituted in 50 µl Tris-Cl buffer (10 mM, pH 8.5).

The amplified DNA of each individual investigated was cloned using the pGEM-T Easy Kit (Promega) and following the manufacturer's protocol. TAM1-F' Extra competent cells (Active Motif, Rixensart, Belgium) were used in transformation. Positive clones were picked and transferred into a 10 µl PCR mix as described above. If a DNA fragment of the expected length was amplified, the same clone was picked again, transferred into 3 ml LB medium containing 100 µg/ml ampicillin and incubated at 37°C for 14 h. DNA was extracted using the Qiaprep Miniprep Kit (Qiagen) following the recommendations of the manufacturer.

Six to 18 clones with an insert of the *DRB* fragment were sequenced from each individual on a DNA sequencer 4200 (LI-COR) using the ThermoSequenase DYEnamic Direct Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA). The probability of missing one of two gene copies among six clones is $0.5^5 = 0.031$ and decreases to $P < 0.001$ with 11 or more clones. We excluded sequence types from the analysis, if they were found only in one clone to account for nucleotide substitutions or recombination during the PCR or cloning. The inheritance of sequence types was studied in 29 juvenile bats with known mothers and/or fathers from a large colony at the biological station La Selva. They were assigned to their parents by behavioural observations and genotyping of the juveniles and their putative parents at 11 microsatellite loci as described in Heckel and von Helversen (2003).

Data analysis

Allele frequencies were calculated only from adult males and females, which were sampled in Costa Rica. Using these allele frequencies, the heterozygosity at a given amino-acid site was calculated (Hedrick *et al.*, 1991).

Hardy-Weinberg Exact Tests were performed with the program GENEPOP (web version 3.4) (Raymond and Rousset, 1995) using the default values (Markov chain: 100 batches and 1000 iterations per batch). The software package MEGA3 (Kumar *et al.*, 2004) allowed the calculation of the relative rate of nonsynonymous and synonymous substitutions according to Nei and Gojobori (1986) by applying the correction of Jukes and Cantor (1969) for multiple hits. The program Arelequin 2.000 (Schneider *et al.*, 2000) was used to perform the Ewens-Watterson homozygosity test of neutrality. The computer program GENECONV (Sawyer, 1999) was used to test for statistical evidence of gene conversion (Sawyer, 1989). We used the default settings that do not allow mismatches. Global and pairwise comparisons were statistically evaluated with 10 000 permutation runs. P -values were corrected for multiple comparisons. All other statistical tests were performed with SPSS 11.0 for Mac OSX.

Results

Number of loci

We detected 13 sequence types (Figure 1) by sequencing 6–18 clones from each of the 85 individuals studied. Except for two individuals that had three different sequence types, only one or two sequence types were found per individual. The sequence type B1 was detected only in one and B2 in another individual

Variation at the *DRB1* locus; evidence of recombination/gene conversion

Ten alleles were detected within the main sample of 79 individuals from the La Selva population. Allele A1 was most frequent and occurred at a frequency of 80.4% among 69 adult bats (Figure 3). The alleles A5, A8, A10 and A11 were detected in only one individual. Observed heterozygosity (0.30) was lower than expected heterozygosity (0.35). The Hardy-Weinberg Exact Test on heterozygote deficiency (GENEPOP) revealed an almost significant result ($P=0.0575$). Within the Panama sample of three individuals, allele A1 was also most common (four copies) and a single copy of allele A2 and a new allele A11 were found. In contrast, allele A6 was most common in three animals from Ecuador (three copies). The remaining alleles were A1 (two copies) and A7 (one copy).

The DNA sequence consists of 224 base pairs of which 80 (35.7%) were polymorphic. On average, alleles differed by 31.9 nucleotides (14.2%). Alleles A1 and A7 differed by only six nucleotides (2.7%). The most distinct alleles were A9 and A11, which differed by 49 nucleotides (21.9%). Pairwise DNA sequence differences between the alleles are given in Table 1. Allele A11, which was only found in individuals from Panama, was not more distinct to alleles from Costa Rica than alleles from Costa Rica to each other.

Several alleles share different sequence fragments of identical sequence (Figure 1). These shared fragments were significantly similar when the global comparison procedures and permutation test implemented in the

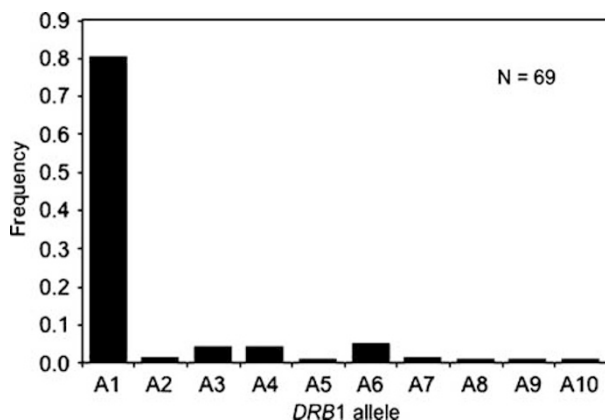


Figure 3 Frequencies of *DRB1* alleles among 69 adult sac-winged bats from the La Selva Biological Station in Costa Rica.

Table 1 Pairwise DNA sequence distances between *DRB1* alleles. Total number of substitutions is given below the diagonal and uncorrected p distance above the diagonal

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11
A1	—	0.08929	0.14732	0.16071	0.05357	0.15625	0.02679	0.08482	0.18304	0.08929	0.17411
A2	20	—	0.15625	0.19196	0.08929	0.13839	0.10714	0.09375	0.16964	0.14732	0.16071
A3	33	35	—	0.15179	0.09375	0.12054	0.16071	0.16964	0.17411	0.20536	0.10714
A4	36	43	34	—	0.19196	0.16518	0.17411	0.17411	0.20536	0.10714	0.16518
A5	12	20	21	43	—	0.17411	0.08036	0.08482	0.18304	0.14286	0.18750
A6	35	31	27	37	39	—	0.17411	0.16964	0.19643	0.17857	0.03571
A7	6	24	36	39	18	39	—	0.07589	0.19643	0.08036	0.18750
A8	19	21	38	39	19	38	17	—	0.12500	0.11607	0.19196
A9	41	38	39	46	41	44	44	28	—	0.21875	0.19643
A10	20	33	46	24	32	40	18	26	49	—	0.19196
A11	39	36	24	37	42	8	42	43	44	43	—

software GENECONV (Sawyer, 1999) was applied. Thus, some alleles are partly characterized by different arrangements of these fragments that is likely due to gene conversion. The two most common alleles (A1 and A6) are distinct at 15.5% of all nucleotide sites and do not share an obvious sequence fragment. The two B sequence types do not share a sequence fragment with each other. Only the sequence type B2 has a short sequence fragment in common with the A4 allele. In contrast, most *DRB1* alleles (that is A sequence types) are linked by shared sequence fragments. This corroborates our view that B sequence types are not alleles of the *DRB1* locus.

Evidence for balancing selection

DRB1 alleles differed in 3 (4%) to 31 (42%) of all 74 amino-acid sites studied. Up to five different amino acids were found at a single codon position and 44 (59.5%) amino-acid sites were variable (Figure 2).

In humans, 18 amino-acid positions, located within the investigated sequence region, are documented to be important in the ABS using X-ray crystallography (Brown *et al.*, 1993). Most variation was concentrated at these ABSs. The proportion of variable positions was significantly higher at ABS than at nonantigen-binding sites (non-ABSs) (15 of 18 (83%) and 29 of 56 (52%), respectively, $\chi^2 = 5.6$, $P < 0.05$). Using the observed allele frequencies in the Costa Rica sample, we calculated the heterozygosity for each amino-acid position (Figure 4).

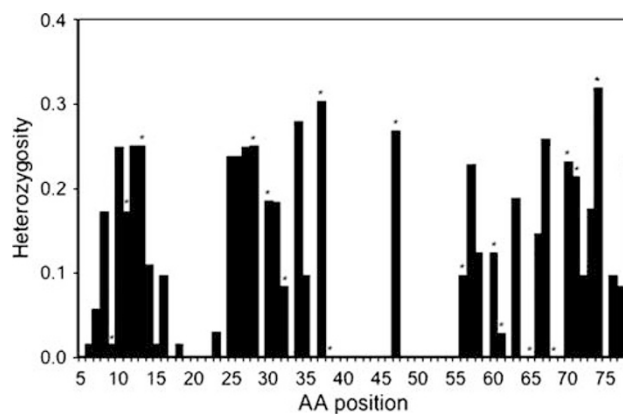


Figure 4 Average heterozygosity for individual amino-acid positions calculated from the allele frequencies in the Costa Rica sample. Asterisks indicate positions, which are assumed to be antigen-binding sites in humans (according to Brown *et al.*, 1993).

The highest heterozygosity was found at position 74 (0.319). An average heterozygosity of 0.153 was observed at ABS, which was significantly higher than the average heterozygosity at non-ABSs (0.066, $U=266$, $P=0.002$). The maximum of five different amino-acid substitutions was only found at two ABS (positions 71 and 74, Figure 2).

Higher divergence among amino-acid sequences than among DNA sequences, and the concentration of amino-acid variation at ABS indicate the existence of balancing selection. Additional evidence comes from the comparison of the relative rate of nonsynonymous (d_N) and synonymous (d_S) substitutions at the 18 antigen (ABS) and the 56 nonantigen-binding amino-acid sites. At ABS, nonsynonymous substitutions ($d_N=0.379\pm 0.180$) were on average 2.4 times more frequent ($Z=-6.33$, $N=55$, $P<0.001$) than synonymous substitutions ($d_S=0.160\pm 0.093$). The corresponding ratio d_N/d_S at non-ABSs was reverse (0.65) and also both substitution rates differed significantly ($d_N=0.111\pm 0.044$, $d_S=0.171\pm 0.078$, $Z=-5.02$, $N=55$, $P<0.001$).

According to the overdominance hypothesis and the negative frequency-dependent selection hypothesis, balancing selection should lead to a more even distribution of allele frequencies than under neutral expectation, which can be tested by the commonly used Ewens–Watterson homozygosity test of neutrality. We did not find a significant deviation from neutral expectation ($P=0.99$) by applying this test to our Costa Rica sample.

Discussion

The first analysis of a MHC gene in a bat species revealed concordant diversity patterns as in species of other mammalian orders.

Number of loci

In the genome of the sac-winged bat *S. bilineata*, we found evidence for two gene copies of the MHC class II gene *DRB*, which is involved in antigen presentation. Cloning and sequencing PCR products amplified by MHC class II β -specific primers produced a maximum of three haplotypes per individual. Three haplotypes were found only in two out of 85 individuals (2%). Only in these two individuals, one haplotype differed from all the other haplotypes detected by a 75 bp insertion and thus likely originated from another (second) locus. The occasional occurrence of three haplotypes can have two reasons. Either duplicated *DRB* loci exist only in some individuals or the primers we used amplify preferably only one locus. At the moment, we cannot distinguish between both hypotheses. Although we used *DRB* exon 2-specific primers, which were also successfully applied in other mammalian orders, including primates and rodents (Gyllensten *et al.*, 1991; Schad *et al.*, 2004; Froeschke and Sommer, 2005), we cannot rule out the amplification failure of a locus due to mutations in a primer-binding site.

In 98% of all bats studied, we amplified one or two haplotypes. We regarded them as alleles from one locus (*DRB1*). This assumption is supported by the Mendelian inheritance of alleles that was tested in 46 parent–offspring pairs and by the observation that genotype frequencies matched Hardy–Weinberg expectations.

Allelic diversity

A total of 10 alleles was found within a small geographic area of the biological station La Selva in Costa Rica. Alleles differed substantially in frequency. Although allele A1 occurred at a frequency of 80%, the frequencies of the remaining alleles were 5% or less. We did not find evidence of an amplification bias among alleles, which could explain such frequency differences. Among 25 heterozygous individuals, clones containing the allele A1 were not more frequent than clones containing another allele (Wilcoxon signed-rank test, $Z=-0.07$, $P=0.94$).

The number of *DRB* alleles varies substantially among mammalian species ranging from one or two in some small and inbred populations (ruminants: Mikko *et al.*, 1999; canids: Aguilar *et al.*, 2004; rodents: Sommer and Tichy, 1999; pinnipeds: Weber *et al.*, 2004) to 321 alleles at the human *HLA-DRB1* locus (Garrigan and Hedrick, 2003). This enormous variation has to be treated with caution because the number of alleles detected also depends on the number of individuals sampled and population coverage. The highest numbers of *DRB* alleles are described from intensively studied species like humans (321 alleles, Garrigan and Hedrick, 2003) or sheep (106 alleles, Konnai *et al.*, 2003). In the sac-winged bat, the number of *DRB1* alleles increased to 11 by analysing additional six individuals from Panama and Ecuador. Therefore, the allele number found in the sac-winged bat is within the range of other mammalian species, although it ranges at the lower end of an outbred population (for example, Mikko *et al.*, 1999).

Recombination

Recombination between alleles (intra-genic recombination) or between loci (inter-genic recombination) can rapidly generate allelic diversity at MHC class II genes (Andersson and Mikko, 1995). Our study provides further evidence for the significance of recombination in generating allelic diversity of MHC class II genes. Several *DRB1* alleles of the sac-winged bat shared different sequence motifs with different alleles (Figure 1). In contrast, the two B sequence types, which are likely encoded by another locus, did not share long-sequence fragments with *DRB1* alleles. This suggests that intra-genic recombination of the *DRB1* locus and less inter-genic recombination plays an important role in generating allelic diversity at the *DRB1* locus of the sac-winged bat. Despite the lack of clear evidence for inter-genic recombination we are not able to rule it out, because we might have missed to amplify paralog *DRB* loci possibly involved in inter-genic recombination. Methodological artefacts, like recombination during PCR, can be ruled out because recombined alleles were independently detected in several individuals. Several recent studies on genetic diversity of MHC class II genes documented intra-genic recombination (human *HLA-DPB1*: Zangenberg *et al.*, 1995; deer mouse *EB*: Richman *et al.*, 2003; chamois *DRB*: Schaschl *et al.*, 2005; stickleback MHC class IIB genes: Reusch and Langefors, 2005). Usually only short-sequence motifs are shared among alleles. In contrast, shared sequence fragments are rather long in the sac-winged bat. A similar pattern of shared sequence fragments among alleles was observed by Zangenberg *et al.* (1995), who studied the rate of evolution within the exon 2 of the human *HLA-DPB1*

locus by screening pools of sperm. The similarity between this sperm analysis study and our study suggests that some recombination events among *DRB1* alleles in the sac-winged bat occurred rather recently. This is further supported by the lack of nucleotide substitutions between some DNA sequence fragments that are shared by two alleles (Figure 1).

Balancing selection

One of the strongest arguments for balancing selection comes from a higher rate of nonsynonymous nucleotide substitutions than synonymous nucleotide substitutions (Garrigan and Hedrick, 2003). The comparison of nonsynonymous to synonymous nucleotide substitutions (d_N/d_S) revealed contrasting results between sites involved in ABS and non-ABS. At ABS, nonsynonymous mutations were 2.4 times more frequent than synonymous mutations, whereas this was not the case for non-ABS. In addition, ABSs were more variable than non-ABSs. The proportion of polymorph ABS was significantly higher than that of non-ABS; and at the protein level, amino-acid sites involved in antigen binding showed higher levels of heterozygosity than nonantigen-binding amino acid sites. The d_N/d_S test is most commonly used in MHC studies to infer a possible role of balancing selection. In 47 of 48 studies, the rate of nonsynonymous substitutions exceeded the rate of synonymous nucleotide substitutions ($d_N/d_S > 1$) (Bernatchez and Landry, 2003), and thus our study on the sac-winged bat is in line with these studies.

Whether balancing selection occurred over the history of the species and whether it still acts in contemporary populations can be tested by different approaches (Garrigan and Hedrick, 2003). The nucleotide sequence of the 11 alleles differed substantially (mean 14.2%). This is in accordance with balancing selection in the past, which can maintain particular haplotype lineages for a long evolutionary period. Therefore, despite the important role of balancing selection for maintaining allelic diversity for a long evolutionary time, its contemporary role remains unclear. Our data do not support ongoing balancing selection at least not within our local population of sac-winged bats from the biological station La Selva. We neither found evidence for heterozygote excess nor did the Ewens–Watterson test reveal a deviation of heterozygosity from a neutral equilibrium distribution. Instead the Hardy–Weinberg–Exact Test on heterozygote deficiency revealed an almost significant result due to an increased proportion of homozygotes of the most common allele. This trend is in contrast to the expectation of ongoing balancing selection due to a heterozygote advantage (Doherty and Zinkernagel, 1975). However, detection of selection from heterozygote excess has low power. Small deme size itself leads to heterozygote excess (Pudovkin *et al.*, 1996; Luikart and Cornuet, 1999; Balloux, 2004) and cryptic population structure could cause homozygote excess. Thus, one would need a comparative study with other markers to see if observed excess was itself in excess of a heterozygote or homozygote excess at other loci.

Positive selection

Despite the diversity of 11 divergent alleles, the observed and expected heterozygosity was very low (0.30 and 0.35,

respectively). Such low levels of heterozygosity are known only from populations of small size or with a bottleneck in the past. They are characterized by a small number of alleles with allele frequencies not exceeding 50% in polymorphic populations (Sena *et al.*, 2003; Aguilar *et al.*, 2004; Amills *et al.*, 2004; Weber *et al.*, 2004). In contrast, in the sac-winged bat, a single-frequent allele (A1) occurred at a frequency of 80% that caused the low heterozygosity. The nine remaining alleles had a frequency of 5% or less. Such a high frequency of a single allele can be the result of genetic drift and is unexpected if heterozygotes (Doherty and Zinkernagel, 1975) or rare alleles (Clarke and Kirby, 1966) have an advantage.

We observed more homozygotes for the common allele than expected, although this result was not significant. An excess of a particular homozygous genotype is expected, if individuals with this genotype have higher fitness, for example, by having a higher chance of survival. One possibility is pathogen-driven selection that was recently shown for a number of wild populations of several species. Particular alleles were significantly increased in frequency in individuals, infected by a particular pathogen compared to uninfected individuals (reviewed in Bernatchez and Landry, 2003; Piertney and Oliver, 2006). For example, the frequency of one allele differed by the factor of more than two between nematode infected and uninfected striped mice *Rhabdomys pumilio* (Froeschke and Sommer, 2005). The high frequency of the A1 allele in the sac-winged bat could be a result of positive selection by an unknown pathogen that invaded our study population. This remains purely speculative at the moment, as we do not have information on the infection of sac-winged bats by parasites. Even when positive selection occurs, allelic diversity can be maintained due to immigrant individuals from other populations, if these populations experience different selection pressures. The analysis of bats from other localities and of neutrally evolving sequence regions close to the *DRB* locus will give some further insights (Garrigan and Hedrick, 2003; Charlesworth, 2006).

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