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PARASITE BURDEN AND CONSTITUTION OF MAJOR HISTOCOMPATIBILITY COMPLEX IN THE MALAGASY MOUSE LEMUR, *MICROCEBUS MURINUS*

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Abstract.—We investigated the importance of the major histocompatibility complex (MHC) constitution on the parasite burden of free-ranging mouse lemurs (*Microcebus murinus*) in four littoral forest fragments in southeastern Madagascar. Fourteen different MHC class II DRB-exon 2 alleles were found in 228 individuals with high levels of sequence divergence between alleles. More nonsynonymous than synonymous substitutions in the functional important antigen recognition and binding sites indicated selection processes maintaining MHC polymorphism. Animals from the four forest fragments differed in their infection status (being infected or not), in the number of different nematode morphotypes per individual (NNI) as well as in the fecal egg counts (FEC) values. Heterozygosity in general was uncorrelated with any of these measures of infection. However, a positive relationship was found between specific alleles and parasite load. Whereas the common allele *Mimu*-DRB*1 was more frequently found in infected individuals and in individuals with high NNI and FEC values (high parasite load), the rare alleles *Mimu*-DRB*6 and 10 were more prevalent in uninfected individuals and in individuals with low NNI and FEC values (low parasite load). These three alleles associated with parasite load had unique amino acid motifs in the antigen binding sites. This distinguished them from the remaining 11 *Mimu*-DRB alleles. Our results support the hypothesis that MHC polymorphism in *M. murinus* is maintained through pathogen-driven selection acting by frequency-dependent selection. This is the first study of the association of MHC variation and parasite burden in a free-ranging primate.

Key words.—Lemur, Madagascar, major histocompatibility complex, *Microcebus murinus*, parasite load.

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The major histocompatibility complex (MHC) plays an important role in the vertebrate immune system. MHC molecules present ''self'' and ''nonself'' peptides to T-cells, and triggers immune reactions in response to foreign peptides (Klein 1986). MHC genes are the most polymorphic loci of all nuclear-encoding genes in vertebrate species, especially the region of the molecule responsible for binding antigens, the so called antigen binding sites (ABS). These show high levels of variation not only in the number of alleles, but also in the extent of sequence variation between alleles (Hughes and Yeager 1998). In particular, these ABS sites display more nonsynonymous than synonymous substitutions, which change the amino acid sequence of the peptide and thus allow binding of a diverse array of antigens (Brown et al. 1988, 1993). Thus, the variability of MHC genes is an indicator for parasite and pathogen resistance, which in turn may influence the long-term survival probability of populations (e.g., Paterson et al. 1998; Hedrick et al. 2001; Langefors et al. 2001). These observations support the hypothesis that selection processes are involved in the maintenance of diversity at MHC loci, even though the processes leading to the extreme polymorphism at MHC loci are strongly debated (reviews in Penn 2002; Bernatchez and Landry 2003). A complementary mechanism to pathogen-driven selection is disassortative mating, acting through olfactory-based mate choice to increase reproductive efficiency and to avoid inbreeding (Potts and Wakeland 1993; Hedrick 1994; Edwards and Potts 1996).

Pathogen or parasite-driven selection and sexual selection can operate by heterozygous advantage (Doherty and Zinkernagel 1975). Heterozygous individuals are assumed to detect and present a wider range of pathogen-driven antigens due to a larger number of different MHC molecules, hence increasing the relative fitness of MHC heterozygotes compared with homozygotes (Hughes and Nei 1989). MHC heterozygosity advantage was indicated in a slower progression to AIDS after HIV infection and in a more effective clearance of hepatitis B viral infections (Thursz et al. 1997; Carrington et al. 1999). MHC-heterozygous mice showed reduced pathogenicity during bacterial infection (streptococcus-induced lesions, Chen et al. 1992; *Salmonella* and *Listeria*, Penn et al. 2002), and they had a faster clearance rate of parasitic worms, *Heligmosomoides polygyrus* (Behnke and Wahid 1991) and *Schistosoma mansoni* (Sher et al. 1984), than the average homozygote.

The second mechanism is negative frequency-dependent selection (Takahata and Nei 1990), in which rare alleles have a selective advantage over common alleles. Host-parasite interactions are considered a dynamic process, with MHC alleles favored at low frequencies and rising in frequency. This induces a corresponding shift in the genetic composition of the parasite population, which reduces the fitness of common host MHC alleles (also described as the Red Queen and moving target hypotheses; reviewed by Penn and Potts 1999; Penn 2002; Bernatchez and Landry 2003). Several studies support the latter mechanism in terms of correlation between certain MHC genotypes or MHC alleles and disease resistance (e.g., malaria, hepatitis B, leprosy, tuberculosis) or other fitness traits (Hill et al. 1991; Thursz et al. 1995; Von Schantz et al. 1996; Jeffery and Bangham 2000; Langefors et al. 2001). In humans, some MHC class II haplotypes were associated with clinical severity of cestode infections (Godot et al. 2000). In feral domestic sheep, MHC variants appeared to play a major role in protection against strongyle nematode invasion, the most prevalent gastrointestinal parasite found (Paterson et al. 1998), and were associated with the level of natural parasitic infection in another study of domestic sheep

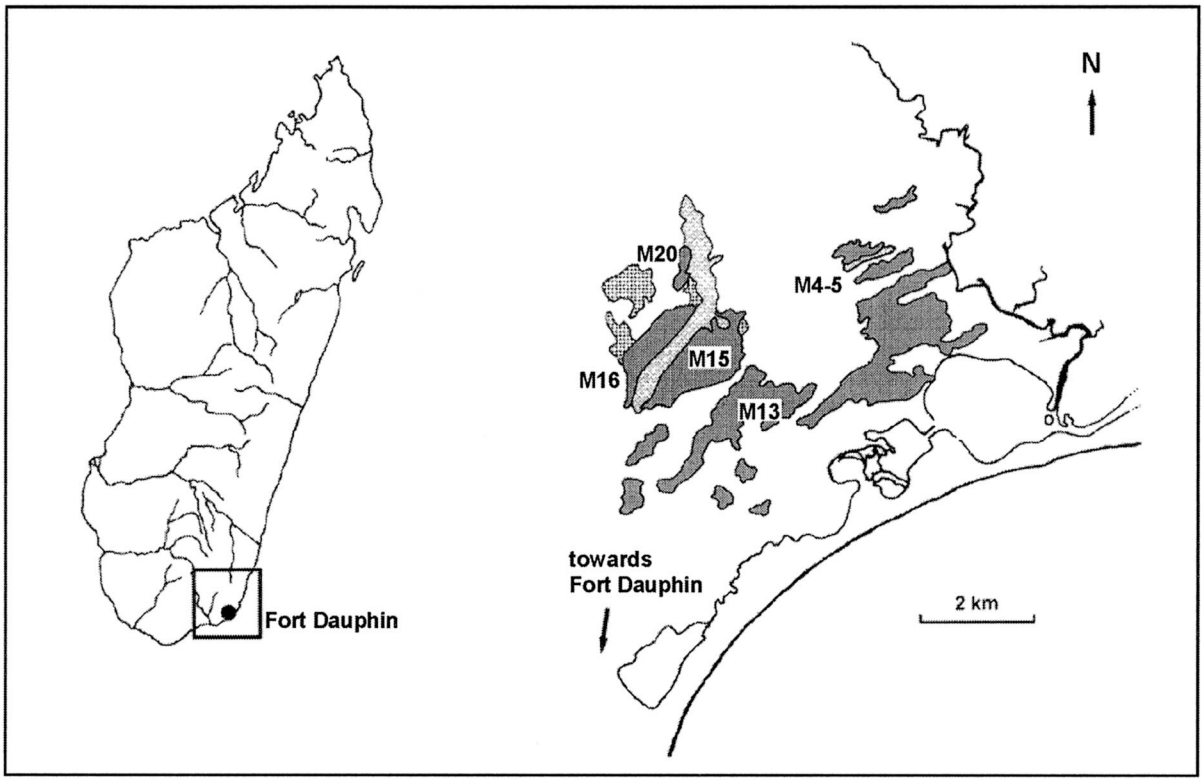

FIG. 1. Location of study areas in southeastern Madagascar (Tolagnaro/Fort Dauphin region). The forest remnants are numbered and shaded. *Eucalyptus* plantations are cross-hatched. Swamps with bordering *Melaleuca* sp. are marked with curved lines (modified from Ramanamanjato and Ganzhorn 2001).

(Buitkamp et al. 1996). These studies indicate functional significance of MHC class II genes in defense against macroparasites. The role of the MHC in the interactions of the intestinal components of the immune system with helminth parasites is not as well understood as for viral or bacterial infections. However, huge progress was made in understanding the cellular and molecular mechanisms in the immune regulation by helminth parasites in recent years (summarized in Maizels and Yazdanbakhsh 2003; Quinnell 2003; Summers et al. 2003). Until now, most of the empirical evidence has been derived from human studies or from studies under laboratory conditions. Studies in free-ranging wild animal populations are still very limited (Bernatchez and Landry 2003).

In this study, we used the endemic mouse lemur (*Microcebus murinus*) in the littoral forests of southeastern Madagascar to investigate the relationship between the MHC constitution and the parasite load under natural conditions. The small (average 60 g) nocturnal, omnivorous lemur is especially suitable for coevolutionary studies due to its high population density and short generation time (about one year). The arboreal species is widely distributed throughout western, southern, and southeastern Madagascar. It is quite adaptable and inhabits not only primary forest but also degraded and secondary vegetation, where its survival is less secure. Negative effects of habitat fragmentation and degradation on population dynamics of *M. murinus* have been indicated (Ganzhorn and Schmid 1998; Rüdel 2004).

To achieve a better understanding of adaptive differences of MHC variation in natural populations we examined MHC polymorphism in a sample of 228 individuals of mouse lemurs and investigated the corresponding parasite load in four different-sized forest fragments in the littoral forest in southeastern Madagascar. The specific aims of the present study on mouse lemurs were (1) to examine levels of parasite burden in the four different fragments, (2) the importance of MHC constitution for resistance to parasites, and, (3) which selective mechanisms may be acting on the MHC in the presence of parasites. Using single-stranded conformation polymorphism (SSCP) and sequencing, we focused on exon 2 of the MHC class II gene DRB, which encodes the major part of the antigen binding sites (Brown et al. 1993).

MATERIALS AND METHODS

Study Areas and Sample Collection

The study was carried out in the littoral forest of Mandena, some 12 km northeast of Tolagnaro/Fort Dauphin in southeastern Madagascar at sites near sea level at an altitude of 0–20 m (24° 56.9′ S; 46° 59.7′ E; Fig. 1). Among the different forest ecosystems in Madagascar, the littoral forests growing on sand along the eastern coastal plain are of particular conservation concern because they belong to the most threatened forest formations of Madagascar with only a few forest islands remaining (Ganzhorn et al. 2001).

For the present study, four littoral forest remnants were chosen, indicated as M4-5 (69 ha), M13 (80 ha), M15-16 (188 ha), and M20 (42 ha) (Fig. 1). The fragments M4-5 and M15-16 were joined because they are connected either by a

replanted corridor (shrubs and trees) or by an introduced Australian tree species *Melaleuca* sp., which can be used by *M. murinus* to move between the fragments. Otherwise, areas between the forest fragments are covered by heath-type vegetation.

For genetic analysis, 228 tissue samples (M4-5: $n = 37$, M13: $n = 29$, M15-16: $n = 136$, M20: $n = 26$) were collected from live-trapped, anaesthetized animals, captured in permanent plots between July 1998 and October 2003. This includes a subsample of 145 individuals from M4-5 and M15-16 that had been analyzed for MHC genotype in a previous study (Schad et al. 2004). A detailed description of site characteristics and trapping procedure is given in Ramanamanjato and Ganzhorn (2001). To investigate parasite infection, 82 individual fecal samples (M4-5: $n = 18$, M13: $n = 19$, M15-16: $n = 30$, M20: $n = 15$) were collected during the tropical winter, a period with stable ecological conditions (between June and October 2001). Genetic and associated parasitic infection data were available for 67 individuals.

Molecular Techniques

We examined variation of a highly polymorphic 171 bp fragment of exon 2 of the MHC class II DRB gene, which includes the functionally important antigen binding and recognition sites. Polymerase chain reaction (PCR) amplification of this region was carried out using primers JS1 and JS2 as described in Schad et al. (2004). To identify allelic diversity, all individuals were subjected to SSCP (Orita et al. 1989). Polymerase chain reaction products were loaded on 15% polyacrylamide gels following the manufacturer's protocol (ETC Elektrophoresetechnik, Kirchentellinsfurt, Germany) and run on a horizontal cooling electrophoresis system (Amersham Pharmacia Biotech, Freiburg, Germany). The configuration of the bands was visualized by silver staining. Samples were rearranged and run again according to assessed similarities. All identified alleles were sequenced bidirectionally. Therefore, at least three examples of each allele were excised from the gel, dissolved in 1× TBE buffer and reamplified under the same PCR conditions mentioned above. Cycle sequencing of the PCR products was performed using a dye terminator sequencing kit (Applied Biosystems, Foster City, CA) and then analyzed by gel electrophoresis with an Applied Biosystems automated sequencer model 377, following the manufacturer's instructions. Details on the molecular techniques are outlined in Sommer and Tichy (1999), Sommer et al. (2002), Sommer (2003) and Schad et al. (2004).

Parasite Screening

To analyze egg shedding, we applied a modification of the McMaster flotation egg counting technique (Sloss et al. 1994), which has been considered a valid method of evaluating worm burdens in several studies (e.g., Gulland et al. 1993; Paterson et al. 1998; Coltman et al. 1999; Cassinello et al. 2001). Feces were screened for nematode eggs per gram of feces (eggs/g) by counting four chambers of McMaster for each sample and by using a flotation-dilution of potassium iodite with a specific weight of 1.5 g/ml (Meyer-Lucht 2003). Nematodes were assigned to morphotypes based on size and morphological characteristics. From all nematodes, photographs were taken and samples stored for later taxonomic classification. We used the number of different nematode morphotypes per individual (NNI) and fecal egg counts (FEC) as a measure of the intensity of parasitism. Both measures are noninvasive and reflect worm burden and fecundity, which are both influenced by the immune state of the host (Stear et al. 1996, 1997).

Statistical Treatment

Expected heterozygosity (gene diversity; Nei 1987) was used as a measure of genetic variation within each population. The extent of population subdivision was examined by pairwise F_{ST} (10,000 permutations; Wright 1965) using the software package Arlequin, version 2.000 (Schneider et al. 2000). Arlequin was also applied to calculate the exact test of sample differentiation based on haplotype frequencies (Raymond and Rousset 1995) by using a Markov chain length of 10,000 steps. MEGA (Kumar et al. 2001) was used to calculate the relative rate of nonsynonymous and synonymous substitutions according to Nei and Gojobori (1986), applying the correction of Jukes and Cantor (1969) for multiple hits.

Nonparametric Kruskal-Wallis and Mann-Whitney *U*-tests were used to analyze differences in the NNI rate between forest fragments. FEC rates (eggs/g) were log-transformed to improve normality and fragments were compared by ANOVA. The influence of heterozygosity on the parasite burden was investigated by χ^2 tests and ANOVA. The χ^2 test was further used to determine whether particular alleles have an effect on parasite resistance and the odds ratio was calculated to estimate the relative risk (Sachs 1992). All tests were two tailed and performed using SPSS version 9.0 (1999; SPSS Inc., Chicago, IL). Bonferroni-corrected significance levels and the Tukey post-hoc tests were used for multiple comparisons and calculations of pairwise differences (Rice 1989; Sachs 1992).

RESULTS

MHC Variability

In the MHC DRB gene-exon 2, the extended dataset from 145 (Schad et al. 2004) to 228 individuals revealed two new alleles (*Mimu*-DRB*12, *Mimu*-DRB*16). No more than two alleles were found in any individual, which suggests that a single copy locus had been amplified. The 14 identified alleles (*Mimu*-DRB*1 to *Mimu*-DRB*10, *Mimu*-DRB*12 to *Mimu*-DRB*14, *Mimu*-DRB*16; GenBank accessions: AJ431266–AJ431270, AJ555835–AJ555841, AJ830740–AJ830741) were based on 71 (41.5%) variable nucleotide positions in a 171 bp sequence. The alleles showed high levels of divergence for an intraspecific comparison, with an average of 23.0 (13.5%) nucleotide differences (minimum: 9 substitutions, maximum: 45 substitutions) between alleles. All alleles had a unique amino acid sequence and the absence of stop codons suggested that all sequences encoded functional proteins. The number of amino acid differences between alleles varied between 5 (8.8 %) and 25 (43.9 %) and reflects the high rate of non-synonymous substitutions.

Of the 17 sites predicted to be involved in antigen recognition (Brown et al. 1988, 1993), 15 sites (88.2%) were

TABLE 1. The estimated rates (± SE) of nonsynonymous (d_N) and synonymous (d_S) substitutions for antigen (ABS) and nonantigen (non-ABS) binding sites and their ratio for DRB-exon2 sequences in *Microcebus murinus*. *n* is the number of codons in each category and *P* is the probability that d_N and d_S are different using a *t*-test. $^*P \leq 0.0001$; ns, not significant.

Positions	*n*	d_N	d_S	d_N/d_S	*P*
ABS	17	0.385 ± 0.073	0.196 ± 0.087	1.96	*
Non-ABS	40	0.076 ± 0.021	0.128 ± 0.037	0.59	*
All	57	0.147 ± 0.027	0.144 ± 0.034	1.02	ns

variable, whereas 16 of 40 non-ABS sites (40.0 %) were polymorph. These variable non-ABS sites were mostly located next to an ABS site. The rate of nonsynonymous (d_N) and synonymous (d_S) substitutions were estimated for both ABS and non-ABS amino acid positions (Table 1). For the antigen-binding sites, d_N (0.385) is significantly greater than d_S (0.196), and the ratio is 1.96 ($t = -8.27$, $P < 0.0001$), whereas in the nonantigen binding sites the ratio between nonsynonymous ($d_N = 0.076$) and synonymous ($d_S = 0.128$) is significantly smaller than unity (d_N/d_S of 0.59, $t = 4.36$, $P < 0.0001$). d_N was 5.07 times higher in the antigen-binding sites than in the nonantigen-binding sites ($t = -18.52$, $P < 0.0001$). This indicates selection processes that maintain polymorphism in the functional important regions of the MHC.

Microcebus murinus populations of all fragments revealed high amounts of genetic variation in the functionally important MHC DRB-exon 2 locus, but the genetic diversity was lower in the fragment M4–5 (0.65) compared to the other fragments (genetic diversity: 0.82–0.84; Table 2). A small but highly significant population differentiation was indicated between the fragment M4-5 and all other fragments (M4-5 vs. M13: $F_{ST} = 0.07$, $P < 0.001$; M4-5 vs. M15-16: $F_{ST} = 0.07$, $P < 0.001$; M4-5 vs. M20: $F_{ST} = 0.07$, $P < 0.001$, all Bonferroni significant). All F_{ST} values are not significant once *Mimu*-DRB*3 is excluded from the analysis, which indicates that the population differentiation is caused by the absence of the common allele *Mimu*-DRB*3 in M4-5 (Table 2).

Parasite Load in Different Fragments

A total of 17 different nematode morphotypes could be distinguished in 82 individual fecal samples (Table 3). One nematode morphotype (N1) was very common and present in 63.4% of the 82 investigated individuals. The remaining 16 morphotypes were found in 1.2 to 11.0% of the sampled individuals. The NNI varied between no infection (23.2%) and one (45.1%), two (24.4%), or three (7.3%) different nematode morphotypes per individual. The morphotype N1 occurred in 82.5% of all infected individuals.

Different numbers of nematode morphotypes were found in the populations of the four fragments: 11 in M4-5, 5 in M13, 10 in M15-16 and 5 in M20. In fragments M4-5, 94.4%; M13, 52.6%; M15-16, 83.3%; and M20, 73.3% of all individuals were infected ($\chi^2 = 10.2$, df = 3, $P < 0.02$). The individuals of the four fragments had significantly different NNI rates ($\chi^2 = 12.47$, df = 3, $P = 0.006$; Fig. 2a). The NNI rate of M4-5 was significantly higher than in all other fragments, and the fragment M13 had the lowest NNI infection rate (M4-5 vs. M13: $Z = -3.17$, $P = 0.002$, Bonferroni significant; M4-5 vs. M15-16: $Z = -2.13$, $P = 0.03$, Bonferroni nonsignificant [ns]; M4-5 vs. M20: $Z = -2.12$, $P = 0.04$, Bonferroni ns; M13 vs. M15-16: $Z = -2.06$, $P = 0.04$, Bonferroni ns).

The four populations also differed significantly in their fecal egg count (FEC) values. Thus, the morphotype N1 accounted for 80.2% of the total FEC rate ($FEC_{\text{all nematodes}}$: $F_{3,78} = 5.17$, $P = 0.003$, Fig. 2b; $FEC_{\text{only N1}}$: $F_{3,78} = 2.96$, $P = 0.037$, Fig. 2c). With regard to the $FEC_{\text{all nematodes}}$, the fragment M4-5 also indicated a significantly higher FEC value than M13 (Tukey post-hoc test: $P = 0.003$), and M13 also had a significantly lower $FEC_{\text{all nematodes}}$ value than M15-16 (Tukey post-hoc test: $P = 0.01$). Considering only the morphotype N1, no significant differences in all pairwise comparisons between the four fragments were found (all Tukey post-hoc tests: ns).

MHC Constitution and Parasite Load

From 67 individuals, both genetic and parasitic infection data were available (Table 3). Within this subsample, the

TABLE 2. Variability and allele frequencies of MHC class II DRB-exon 2 in *Microcebus murinus*. *n* = sample size.

Fragment	M4–5	M13	M15–16	M20	Total
n	37	29	136	26	228
Gene diversity	0.65 ± 0.06	0.82 ± 0.02	0.84 ± 0.01	0.84 ± 0.02	0.82 ± 0.01
Mimu-DRB*1	0.57	0.29	0.29	0.27	0.33
Mimu-DRB*2	0.12	0.19	0.11	0.21	0.12
Mimu-DRB*3	—	0.19	0.16	0.17	0.14
Mimu-DRB*4	0.03	—	0.01	—	0.01
Mimu-DRB*5	0.05	0.14	0.11	0.12	0.10
Mimu-DRB*6	0.10	0.10	0.12	0.10	0.11
Mimu-DRB*7	—	—	0.01	0.02	0.01
Mimu-DRB*8	—	—	0.01	—	0.01
Mimu-DRB*9	0.03	0.03	0.01	—	0.02
Mimu-DRB*10	0.05	0.05	0.05	0.10	0.06
Mimu-DRB*12	—	—	0.14	—	0.08
Mimu-DRB*13	0.03	—	—	—	0.004
Mimu-DRB*14	0.03	—	—	—	0.004
Mimu-DRB*16	—	—	—	0.02	0.002

most common nematode morphotype N1 was present in 59.7% of all and 77.6 % of the infected individuals. N1 accounted for 77.9% of the total fecal egg count rate (FEC). Therefore, the analyses were carried out for all nematodes and for the nematode morphotype N1 separately. Because the genetic and parasitic investigations revealed significant differences between M4-5 and the other fragments (see above), all statistics were calculated for all fragments; for the fragments M13, M15-16, and M20 combined; and for M4-5 which was analyzed separately if possible.

Effects of specific alleles on the infection status

We investigated the frequency-dependent selection hypotheses (Takahata and Nei 1990) by analyzing the effects of specific alleles on the individual parasite load. Analyses of the MHC DRB-exon 2 allele frequencies revealed significant differences between uninfected and infected individuals. The exact tests of sample differentiation based on allele frequencies were significant, irrespective of whether all nematodes or only the most common nematode morphotype N1 were considered, in all fragments (all nematodes: $P = 0.008 \pm 0.004$; only N1: $P = 0.048 \pm 0.008$) or only in the fragments M13, M15-16, and M20 combined (all nematodes: $P = 0.013 \pm 0.006$; only N1: $P = 0.025 \pm 0.004$). Statistical analyses for the fragment M4-5 were not possible due to the high proportion of infected individuals (Table 3).

Considering all fragments, *Mimu*-DRB*1 was significantly associated with infected individuals (all nematodes: $\chi^2 = 4.46$, df = 1, $P = 0.026$; only N1: $\chi^2 = 2.24$, df = 1, $P = 0.096$). The odds ratio for *Mimu*-DRB*1 (all nematodes) was 3.2, and the risk of being infected by a nematode was 1.3 times higher in individuals carrying this allele ($P < 0.05$). In contrast *Mimu*-DRB*6 (all nematodes: $\chi^2 = 13.99$, df = 1, $P < 0.001$; only N1: $\chi^2 = 5.63$, df = 1, $P = 0.022$) and *Mimu*-DRB*10 (all nematodes: $\chi^2 = 4.64$, df = 1, $P = 0.046$; only N1: $\chi^2 = 5.63$, df = 1, $P = 0.022$) were significantly linked with uninfected individuals (Fig. 3a,b). The odds ratio for *Mimu*-DRB*6 was 0.08 (all nematodes) and 0.17 (N1), and the probability of belonging to the group of uninfected individuals was 3.6 and 2.8 times higher in individuals carrying this allele, respectively ($P < 0.05$). Correspondingly, the odds ratio for *Mimu*-DRB*10 was 0.24 (all nematodes) and 0.17 (N1), and the probability of belonging to the group of uninfected individuals was 1.7 and 2.8 times higher in individuals carrying this allele, respectively ($P < 0.05$).

Considering fragments M13, M15-16, and M20 combined, no significant association was found between *Mimu*-DRB*1 and infected individuals (χ^2 tests: ns). However, *Mimu*-DRB*6 (all nematodes: $\chi^2 = 14.66$, df = 1, $P < 0.001$; only N1: $\chi^2 = 7.67$, df = 1, $P = 0.008$) and *Mimu*-DRB*10 (all nematodes: $\chi^2 = 3.49$, df = 1, $P = 0.073$; only N1: $\chi^2 = 5.45$, df = 1, $P = 0.024$) were still linked with uninfected individuals (figures not shown). The odds ratio for *Mimu*-DRB*6 was 0.05 (all nematodes) and 0.09 (N1), and the probability of belonging to the group of uninfected individuals was 6.1 and 5.0 times higher in individuals carrying this allele, respectively ($P < 0.05$). Correspondingly, the odds ratio for *Mimu*-DRB*10 was 0.28 (all nematodes) and 0.17 (N1), and the probability of belonging to the group of uninfected individuals was 1.7 and 2.8 times higher in individuals carrying this allele, respectively ($P < 0.05$).

Effects of specific alleles on number of nematode morphotypes per individual

The number of different nematode morphotype infections differed significantly with respect to specific alleles in all fragments ($\chi^2 = 29.33$, df = 10, $P < 0.001$; Fig. 4a) and in the fragments M13, M15-16, and M20 ($\chi^2 = 22.40$, df = 8, $P = 0.004$; figure not shown). It was not significant in M4-5 ($\chi^2 = 5.73$, df = 4, ns; figure not shown). With regard to all fragments, individuals carrying the alleles *Mimu*-DRB*1 ($Z = -2.98$, $P = 0.003$) had a significantly higher NNI compared to other individuals. *Mimu*-DRB*6 ($Z = -3.37$, $P = 0.001$) and *Mimu*-DRB*10 ($Z = -2.48$, $P = 0.013$) had significantly lower NNI compared to other individuals. No significant differences were found in all other alleles. If the fragments M13, M15-16, and M20 combined were taken into account, *Mimu*-DRB*6 ($Z = -3.39$, $P = 0.001$) and *Mimu*-DRB*10 ($Z = -2.13$, $P = 0.033$) had significantly lower NNI compared to other individuals.

Effect of specific alleles on fecal egg count

Likewise, the FEC value differed significantly with respect to specific alleles in all fragments (ANOVA: all nematodes: $F_{10,123} = 2.46$, $P = 0.01$, Fig. 4b; only N1: $F_{10,123} = 2.09$, $P = 0.03$, Fig. 4c) and in the fragments M13, M15-16, and M20 (ANOVA: all nematodes: $F_{9,102} = 2.41$, $P < 0.02$; only N1: $F_{9,102} = 2.16$, $P < 0.03$), but not in M4-5. If all fragments were considered, individuals carrying the allele *Mimu*-DRB*1 had significantly higher $FEC_{\text{all nematodes}}$ values ($t = -2.23$, $P < 0.03$) compared to individuals with other alleles. Again, individuals with *Mimu*-DRB*6 had significantly lower FEC values in all fragments (all nematodes: $t = 3.74$, $P < 0.0001$; N1: $t = 3.18$, $P = 0.01$) and in fragments M13, M15-16, and M20 combined (all nematodes: $t = 6.45$, $P < 0.0001$; N1: $t = 5.05$, $P < 0.0001$). The association of *Mimu*-DRB*10 and low FEC_{N1} values were significant in all fragments ($t = 2.18$, $P = 0.03$) and in fragments M13, M15-16, and M20 combined ($t = 2.11$, $P < 0.04$).

Effects of heterozygosity on infection status, number of nematode morphotypes per individual, and fecal egg count

The effects of the individual MHC constitution on the parasite load were investigated by analyses of a possible heterozygosity advantage (heterozygosity advantage hypothesis; Doherty and Zinkernagel 1975). All comparisons indicated no effects of homozygosity or heterozygosity on the individual status of being infected or uninfected (all χ^2 tests, ns; figures not shown), on NNI (all χ^2 tests, ns; figures not shown) and on FEC (all ANOVAs, ns; figures not shown).

Discussion

In humans, more diseases have been associated with MHC than with any other area of the genome and thus genes of MHC are placed among the best candidates for molecular adaptation in vertebrates (Hedrick 1994; Jeffery and Bangham 2000; Bernatchez and Landry 2003). Polymorphism is

TABLE 3. Individual MHC constitution and status of nematode infection in the four littoral forest fragments. The individual identification number (Id-Nr), the inhabited forest fragment, the DRB-genotype, the fecal egg count values (FEC, log egg/g + 1) for all nematodes (FEC-all) and for the most common nematode morphotype N1 (FEC-N1), the number of different nematode morphotypes per individual (NNI), and the different morphotypes that constitute the individual parasite load are given. The morphotypes belong to Nematoda (N7, N8, N9, N10, N11, N12, N14, N15), Strongyloidae (N1, N13), Trichosomoides sp. (N2), *Trichuris* sp. (N3, N4), Oxyuridae (N5, N6), and Acanthocephala (N16, N17). A dash indicates no DNA available.

Id-Nr	Fragment	Genotype	FEC-all	FEC-N1	NNI	Morphotypes
0214.46C1	M4–5	—	2.08	2.08	1	N1
01C5.156C	M4–5	—	1.72	1.72	1	N1
01C6.201F	M4–5	—	2.66	2.66	1	N1
01C6.2EF9	M4–5	—	2.34	2.3	3	N1, N7, N8
01C6.E261	M4–5	—	1.93	1.93	1	N1
01C6.E7E7	M4–5	—	1.87	1.48	1	N1
01C6.306C	M4–5	—	1.71	1.25	2	N1, N3
01C5.1CD9	M4–5	*Mimu**1 *Mimu**6	1.92	1.92	1	N1
01C5.2C71	M4–5	*Mimu**1 *Mimu**1	1.61	0	2	N3, N17
01C6.05FD	M4–5	*Mimu**2 *Mimu**2	0	0	0	
01C5.1CDF	M4–5	*Mimu**1 *Mimu**5	1.01	0	1	N5
01C5.1138	M4–5	*Mimu**1 *Mimu**1	1.93	1.84	3	N1, N6, N15
01D0.CBB3	M4–5	*Mimu**1 *Mimu**1	2.28	2.19	2	N1, N3
01C6.E19D	M4–5	*Mimu**1 *Mimu**5	1.84	1.54	2	N1, N3
01C6.35DF	M4–5	*Mimu**1 *Mimu**1	2.68	2.67	2	N1, N18
01C6.2871	M4–5	*Mimu**1 *Mimu**2	2.88	0	2	N6, N9
01C6.250B	M4–5	*Mimu**1 *Mimu**2	2.52	2.5	2	N1, N10
01C6.D1A3	M4–5	*Mimu**14 *Mimu**14	2.28	2.19	2	N1, N6
01C6.E29B	M13	—	0	0	0	
01C5.095C	M13	*Mimu**1 *Mimu**3	0	0	0	
01C5.0C40	M13	*Mimu**2 *Mimu**5	0	0	0	
01C5.11C1	M13	*Mimu**3 *Mimu**3	2.28	2.26	2	N1, N8
01C5.123F	M13	*Mimu**1 *Mimu**2	1.22	1.22	1	N1
01C6.759A	M13	*Mimu**1 *Mimu**3	2.22	2.14	3	N1, N6, N12
01C6.48C3	M13	*Mimu**5 *Mimu**5	2.13	2.13	1	N1
01C5.0365	M13	*Mimu**1 *Mimu**2	1.51	1.51	1	N1
01C5.2C0F	M13	*Mimu**5 *Mimu**10	0	0	0	
01C5.3BB0	M13	*Mimu**3 *Mimu**3	1.59	1.42	2	N1, N11
01C2.02C3	M13	*Mimu**1 *Mimu**1	1.42	1.42	1	N1
01C5.19D6	M13	*Mimu**6 *Mimu**6	0	0	0	
01C5.265D	M13	*Mimu**6 *Mimu**6	0	0	0	
01C5.2BA3	M13	*Mimu**3 *Mimu**5	1.12	0	1	N11
01C6.725C	M13	*Mimu**1 *Mimu**1	1.54	1.54	1	N1
01C6.DDC3	M13	*Mimu**2 *Mimu**3	0	0	0	
01C6.6082	M13	*Mimu**10 *Mimu**10	0	0	0	
01C5.1009	M13	*Mimu**9 *Mimu**9	2.42	2.42	1	N1
01C5.2DC2	M13	*Mimu**6 *Mimu**6	0	0	0	
01C5.03EC	M15–16	—	1.81	1.63	2	N1, N2
01C5.1CD3	M15–16	—	1.43	0	1	N16
016B.B9C9	M15–16	—	2.89	2.52	3	N1, N4, N5
01C5.1990	M15–16	—	2.56	2.4	2	N1, 15
016B.B407	M15–16	—	2.81	2.74	2	N1, N2
01C6.6B11	M15–16	—	1.34	1.34	1	N1
016A.8AAF	M15–16	*Mimu**1 *Mimu**1	2.11	2.11	1	N1
01ED.DFE7	M15–16	*Mimu**1 *Mimu**10	2.71	2.71	1	N1
016B.1CDD	M15–16	*Mimu**3 *Mimu**3	0	0	0	
01EE.4EF9	M15–16	*Mimu**1 *Mimu**3	1.89	1.89	1	N1
01ED.BDB0	M15–16	*Mimu**1 *Mimu**1	2.21	2.21	1	N1
01C5.D1B5	M15–16	*Mimu**5 *Mimu**5	1.96	1.96	1	N1
01F7.EE02	M15–16	*Mimu**3 *Mimu**3	2.32	2.32	1	N1
01C6.459E	M15–16	*Mimu**1 *Mimu**10	0	0	0	
01C5.2B4E	M15–16	*Mimu**12 *Mimu**12	1.77	1.77	1	N1
01C6.C320	M15–16	*Mimu**12 *Mimu**12	0	0	0	
01C6.173D	M15–16	*Mimu**12 *Mimu**12	2	0	1	N14
01C6.1FFD	M15–16	*Mimu**12 *Mimu**12	2.8	2.8	1	N1
01C6.274D	M15–16	*Mimu**12 *Mimu**12	1.22	1.22	1	N1
01C6.C40E	M15–16	*Mimu**12 *Mimu**12	1.71	1.42	2	N1, N5
01C6.1830	M15–16	*Mimu**12 *Mimu**12	1.45	0	1	N6
01C6.E973	M15–16	*Mimu**12 *Mimu**2	2.1	1.71	2	N1, N2
01C6.E65F	M15–16	*Mimu**12 *Mimu**12	2.54	2.54	1	N1
01C6.C3B1	M15–16	*Mimu**12 *Mimu**12	2	1.91	2	N1, N7
01C6.2DA3	M15–16	*Mimu**12 *Mimu**3	1.41	0	1	N5
01C5.05D3	M15–16	*Mimu**12 *Mimu**2	2.85	2	3	N1, N3, N5
01C5.0615	M15–16	*Mimu**12 *Mimu**12	2.8	2.78	2	N1, N3

TABLE 3. Continued.

Id-Nr	Fragment	Genotype	FEC-all	FEC-N1	NNI	Morphotypes
01C5.172A	M15–16	*Mimu**12 *Mimu**12	0	0	0	
01C5.32B9	M15–16	*Mimu**12 *Mimu**12	0	0	0	
01C5.3F5D	M15–16	*Mimu**12 *Mimu**12	1.61	1.61	1	N1
01D0.CD56	M20	—	0	0	0	
01EE.4FB2	M20	*Mimu**5 *Mimu**5	2.24	2.24	1	N1, N6
01C6.D31F	M20	*Mimu**1 *Mimu**2	1.9	1.68	2	N1, N6
01C5.08BD	M20	*Mimu**3 *Mimu**7	1.66	1.57	2	N1, N11
01C5.C5A9	M20	*Mimu**5 *Mimu**10	0	0	0	
01C6.0666	M20	*Mimu**3 *Mimu**3	2.17	2.17	1	N1
01C5.1587	M20	*Mimu**2 *Mimu**16	2.28	0	1	N11
01C5.1722	M20	*Mimu**1 *Mimu**1	2.17	0	3	N3, N4, N6
01C5.172D	M20	*Mimu**6 *Mimu**10	1.39	1.39	1	N1
01C5.28B5	M20	*Mimu**1 *Mimu**1	0	0	0	
01C6.D60B	M20	*Mimu**10 *Mimu**10	2.38	0	1	N6
01C6.DA69	M20	*Mimu**3 *Mimu**5	1.52	1.52	1	N1
01C6.E0BB	M20	*Mimu**5 *Mimu**6	0	0	0	
01C6.E9C5	M20	*Mimu**2 *Mimu**2	0.99	0.99	1	N1
01C5.0FDE	M20	*Mimu**1 *Mimu**2	1.84	1.26	2	N1, N6

thought to be maintained by balancing selection through heterozygosity advantage (Doherty and Zinkernagel 1975) or frequency-dependent selection (Takahata and Nei 1990). To date, most of the empirical evidence to assess mechanisms that maintain MHC diversity is derived from clinical studies in humans or from model organisms such as mice under laboratory conditions. Few studies have attempted to test for an association between MHC polymorphism and disease resistance in wild vertebrates (summarized in Bernatchez and Landry 2003). In this study, we investigated the association between MHC constitution and parasite burden and possible selection mechanisms in a free-ranging lemur population (*M. murinus*) living in four different forest fragments (M4-5, M13, M15-16, and M20) in the littoral forest of southeastern Madagascar. The study included a previously analyzed subsample of 145 individuals from M4-5 and M15-16 (Schad et al. 2004).

MHC Variability

Fourteen different MHC class II DRB-exon 2 alleles (*Mimu*-DRB*1 to *Mimu*-DRB*10, *Mimu*-DRB*12 to *Mimu*-DRB*14, *Mimu*-DRB*16) were found in 228 individuals with high levels of sequence divergence between alleles. In our *M. murinus* study populations no individual had more than two alleles, suggesting that only one DRB locus was amplified. The *Mimu*-DRB alleles showed high levels of sequence divergence for an intraspecific comparison, and all alleles had a unique amino acid sequence. All sequences were structurally functional: they did not show stop codons or deletions and insertions, which considerably changed the reading frame. Polymorphism was highest in the functionally important antigen recognition and binding sites. In these positions, significantly more nonsynonymous than synonymous substitutions were found (ratio: 1.96). This is considered a clear indication for positive selection (Hughes and Nei 1988, 1989) and is characteristic for proteins with antigen-presenting function (Bergstrom and Gyllensten 1995). By contrast, purifying selection has been shown to act on codons in the nonantigen binding sites (Nei and Gojobori 1986) and the ratio between nonsynonymous and synonymous substitutions was significantly smaller than unity (0.59). The results of the MHC variability analyses of the enlarged dataset agree with the previous study (Schad et al. 2004). The *M. murinus* populations of all four fragments revealed high amounts of genetic variation, but genetic diversity was lower in fragment M4-5 (0.65) compared to the other fragments (genetic diversity: 0.82–0.84). A small but highly significant population differentiation was indicated between fragment M4-5 and all other fragments, probably due to the absence of the common allele *Mimu*-DRB*3 in M4-5.

Parasite Load in Different Fragments

Seventeen different nematode morphotypes could be distinguished in 82 individual fecal samples. The NNI varied between no infection and one to three different nematode morphotypes per individual. The most common nematode morphotype, N1, was found in 82.5% of all infected individuals and accounted for 80.2% of the total FEC rate. Individuals from the four forest fragments differed in their infection status (being infected or not), in NNI, and in fecal egg count (FEC) values. The highest number of infected individuals and the highest parasite load in both NNI and FEC was found in the fragment M4-5.

Free-ranging mammals are typically exposed to a diverse array of parasites, and an individual mammal may contain several hundred individual macroparasites, with the host population harboring a community of 40 or more different parasite species (Dobson et al. 1992). For example, feral Soay sheep on the island of St. Kilda, Scotland, harbor 20 different species of helminth alone (Gulland 1992). Many factors have been proposed as determinants of parasite community diversity in primates, such as host body mass and life history, social contact and population density, diet, and habitat diversity. A recent meta-analysis in primates, including 941 host-parasite combinations, supported the importance of body mass and population density on helminth diversity (Nunn et al. 2003). Almost 60% of the investigated host species had between zero and six parasite species. An average of 1.8 parasite species were recorded for each of the 15 prosimian species. Also compared to another study including 131 ver-

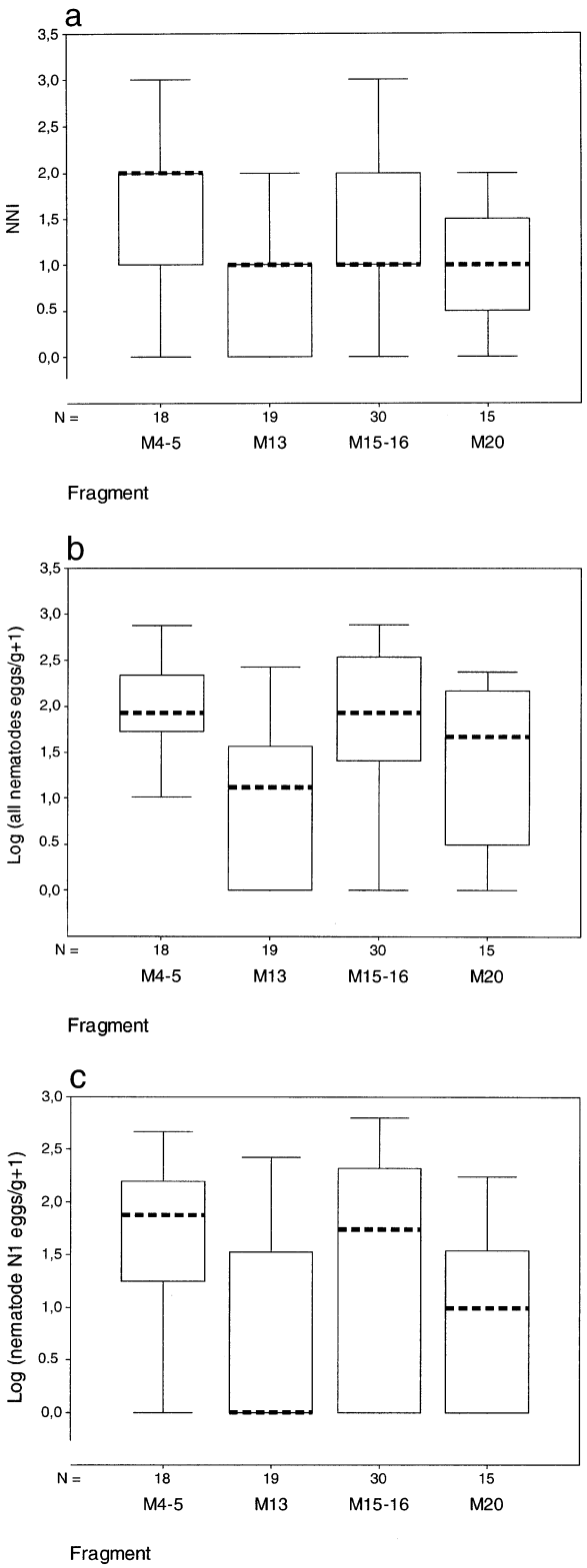

FIG. 2. Differences in (a) the number of nematode morphotypes per individual (NNI), (b) the fecal egg count (FEC) of all nematodes, and (c) the FEC of the most common nematode, N1, of *Microcebus murinus* in the four forest fragments. The box plots indicate medians, quartiles, minima and maxima. n = sample size.

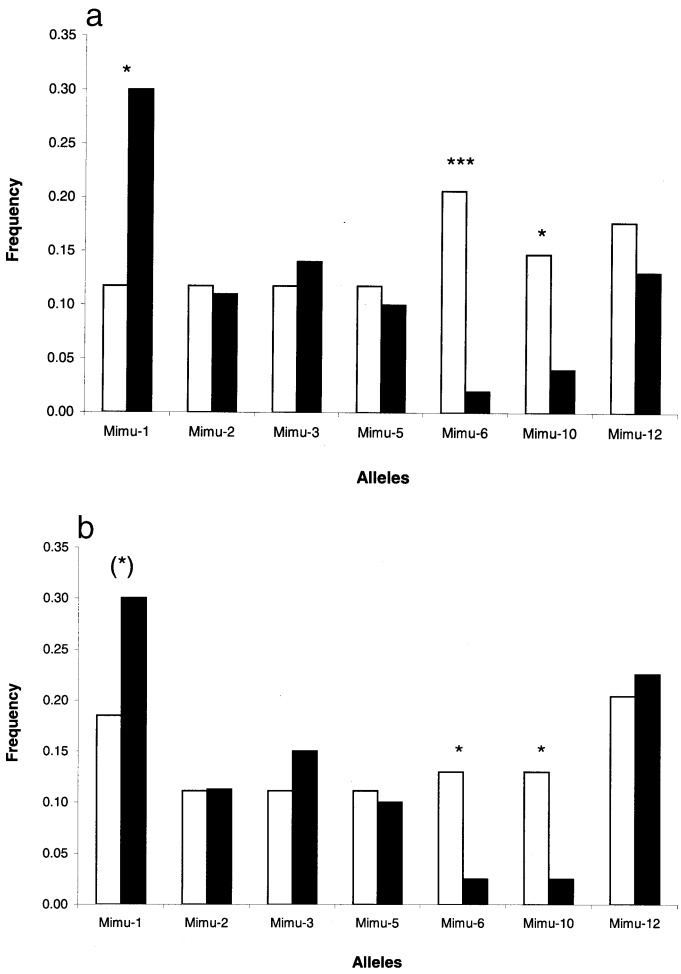

FIG. 3. Frequency of MHC class II DRB-exon 2 alleles in uninfected (open bars) and infected (dark bars) *Microcebus murinus* with respect to (a) all nematodes and (b) the most common nematode, N1. The individuals of all four fragments are pooled (n = 67). Only alleles that occurred in more than two individuals are displayed. * $P \leq 0.05$, *** $P \leq 0.001$.

tebrate host species, the number of infections in *M. murinus* in relation to host mass fits into the linear relationship (Poulin et al. 2003).

MHC Constitution and Parasite Load

We investigated the importance of MHC constitution for resistance to parasites, and the selective mechanisms acting on MHC in the presence of parasites in 67 individuals, where both genetic and parasitic infection data were available. All comparisons indicated no effects of homozygosity or heterozygosity (heterozygosity advantage hypothesis; Doherty and Zinkernagel 1975) on the individual status of being infected or uninfected, NNI, or FEC.

The frequency-dependent selection hypothesis (Takahata and Nei 1990) was investigated by analyzing the effects of specific alleles on the individual parasite load. Whereas the allele *Mimu*-DRB*1 was more frequently found in infected individuals and in individuals with high NNI and FEC values (high parasite burden), the alleles *Mimu*-DRB*6 and 10 were more prevalent in uninfected individuals and in individuals

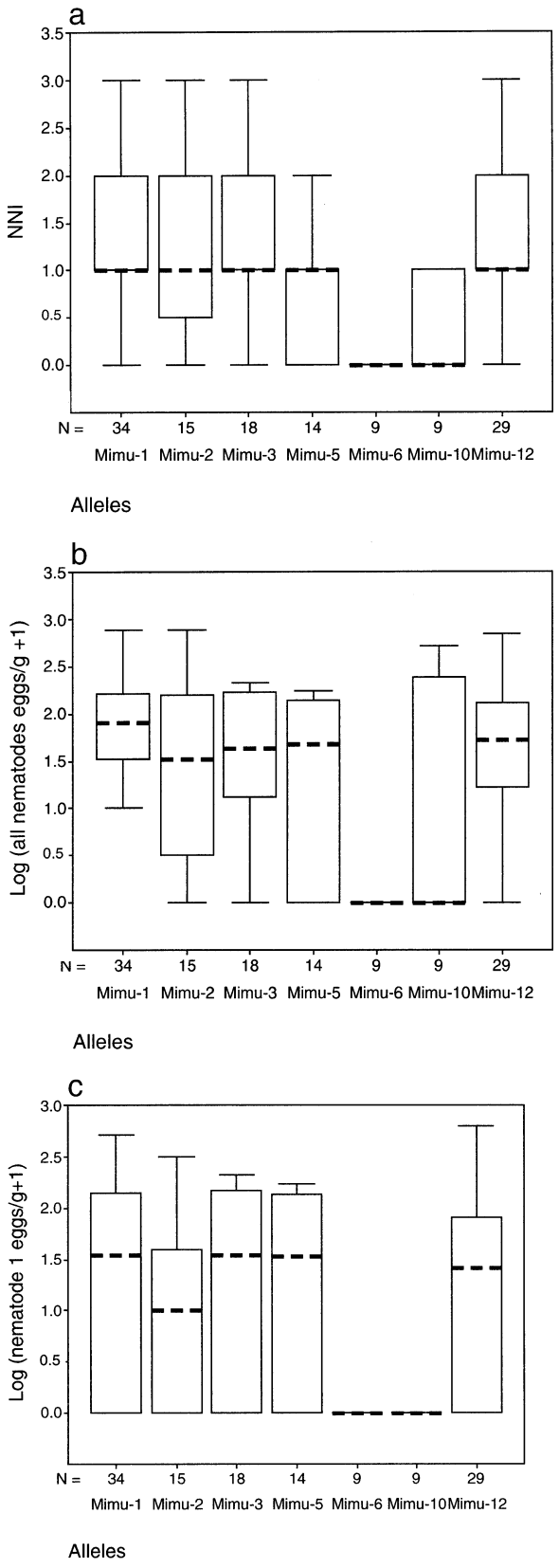

FIG. 4. Effects of specific alleles on (a) number of nematode morphotypes per individual (NNI), (b) fecal egg count of all nematodes (FEC) and (c) FEC of the most common nematode, N1. The individuals of all four fragments were pooled. Only alleles that occurred in more than two individuals are displayed.

with low NNI and FEC values (low parasite burden). Thus, the analyses revealed quite consistent results, irrespective of whether all nematodes or only N1 were considered, whether all fragments or only M13, M15-16, and M20 combined were investigated. Separate statistical analyses of M4-5 were not possible due to the high rate of infected individuals or revealed not significant results probably due to small sample size.

All of these three alleles (*Mimu*-DRB*1, *6, and *10) associated with parasite load had unique amino acid motifs in the antigen binding sites (ABS). *Mimu*-DRB*1 differs from all other alleles by three unique amino acids, all of them located within the ABS (aspartic acid in position 70, glutamic acid in position 71, lysine in position 74). Two of these ABS are mutated in *Mimu*-DRB*6 and *10: the allele *Mimu*-DRB*6 has a unique motif at position 74 (glycine) and *Mimu*-DRB*10 at position 71 (methionine). In addition, only *Mimu*-DRB*6 and *10 possess the amino acid arginine located next to the ABS in position 78 (position numbers after Brown et al. 1993). The presented results indicate strongly that MHC DRB-exon 2 alleles are of importance for the parasite resistance in mouse lemurs. There is increasing evidence that pathogen escape from MHC-dependent immune system recognition may involve changes in only a few amino acids, and small binding-motif differences can lead to large differences in protection (summarized by Frank 2002). Common mechanisms are a change in pathogen antigens (epitopes) that prevents binding to (1) the MHC-encoded cell surface glycoprotein or (2) the T-cell receptor. A third mechanism is molecular mimicry of host proteins that prevent T-cell receptor binding (T-cells that recognize host proteins are destroyed; reviewed by Summers et al. 2003).

Considering the frequency distribution of these relevant alleles in the four different forest fragments, a highly significant accumulation of *Mimu*-DRB*1 was found in the fragment M4–5 (57%), the fragment with the highest parasite burden. The genetic diversity was lower in M4-5 (0.65) compared to the other fragments (genetic diversity: 0.82–0.84). Recent investigations in M4-5 revealed a lower body mass in female mouse lemurs, lower fat deposition in the tail (important during the dry season), and therefore lower survival rates compared to populations of the three other fragments. In addition, the population size declined dramatically between the year 2000 and 2003 (Rüdel 2004). However, whether the higher parasite load in M4-5 is due to the genetic constitution of individuals inhabiting this fragment or to other ecological factors associated with fragment size or degradation needs further investigation.

The multialleles commonly found at the MHC locus result in low allele frequencies and relatively low sample sizes, making it statistically problematic to find associations between MHC alleles and resistance to an infectious disease (Hill 1999). Given the small sample sizes for many of the 14 mouse lemur alleles, it is remarkable that a significant association was found between the *Mimu*-DRB*1 allele and susceptibility to nematode infection, and between the *Mimu*-DRB*6 and *10 alleles and resistance. This provides strong evidence of an actual association between certain MHC alleles and resistance to helminthic infection in mouse lemurs. But then the question arises why favored alleles do not rise

to fixation in the population. In this respect, it is interesting to note that the most common allele, *Mimu*-DRB*1, is associated with high parasite load, whereas the rarer alleles, *Mimu*-DRB*6 and *10, were associated with low parasite load—as might be predicted under negative-frequency dependent selection. It is often assumed that new alleles are lost due to random genetic drift. However, under the theory of negative frequency-dependent selection, rare alleles have a selective advantage and will increase in frequency (May and Anderson 1990). *Mimu*-DRB*6 and *10 might be examples of new alleles that have arisen and are maintained through frequency-dependent selection due to their association with resistance to nematode infection. Association between a gene and a disease can be due to effects of the gene itself, or can arise if the studied gene is in linkage disequilibrium with another gene that causes the resistance (Langefors et al. 2001). Mouse lemurs in the littoral rain forest in southeastern Madagascar seems to have a single MHC-class II DRB locus (Schad et al. 2004; this study), but we cannot exclude the possibility that another linked gene has caused the observed association.

An association between MHC class IIB polymorphism and parasite diversity in different habitats was also recently identified in three-spined sticklebacks (*Gasterosteus aculeatus*), independent of genomewide heterozygosity as measured by microsatellites (Wegner et al. 2003). In this study, intermediate allele numbers were associated with minimal parasite load. In contrary to three-spined sticklebacks, mouse lemurs in the littoral rain forest in southeastern Madagascar have only a single MHC-class II DRB locus. Thus the proposed allele-counting hypotheses (Reusch et al. 2001) as a strategy for optimal pathogen and parasite resistance does not apply to this mouse lemur population. Associations between MHC heterozygosity and infectious diseases in free-ranging animals under natural conditions have been found in chinook salmon (*Oncorhynchus tsawytscha*, Arkush et al. 2002) and in Gila topminnow (*Poeciliopsis occidentalis occidentalis*, Hedrick et al. 2001), while an association between certain MHC alleles and disease resistance or susceptibility was found in Atlantic salmon (*Salmo salar*, Langefors et al. 2001), hairy-footed gerbil (*Gerbillurus paeba*, Harf and Sommer 2005), and Soay sheep (*Ovis aries*), where MHC variants also appear to play a major role in protection against strongyle nematode invasion (Paterson et al. 1998). Most studies deal with single viral, bacterial, or parasitic agents and it was suggested that studies combining two or more pathogens may increase the amount of evidence for heterozygous advantage (Langefors et al. 2001). In our study in mouse lemurs, despite the fact that several nematodes were investigated, none of the comparisons indicated any effects of homozygosity or heterozygosity. Recently, De Boer et al. (2004) studied the degree of MHC polymorphism arising when heterozygote advantage is the only selection pressure by using mathematical models. They revealed that heterozygote advantage on its own is not sufficient to explain the high population diversity of the MHC. Although heterozygote advantage is clearly an important selection pressure, simulations indicate that additional frequency-dependent selection pressure is required. Host-parasite coevolution would be sufficient to explain the large degree of MHC polymorphism (Borghans et al. 2004).

Conclusion

Our study supports the hypothesis that MHC polymorphism in *M. murinus* is maintained through pathogen-driven selection acting by frequency-dependent selection. For further investigation of the selection hypotheses, allele frequencies and parasite burden need to be followed through time to determine whether allele frequencies change in a cycling pattern. This would help to understand the role of MHC diversity and parasite selection in free-ranging populations under natural conditions. Like recent research on Atlantic salmon (Langefors et al. 2001; Lohm et al. 2002), the present study of a free-ranging primate emphasizes the importance of maintenance and, even more important, renewal of genetic variation at the MHC, either from mutation, recombination, or immigration from other populations, when combating new or coevolving virulent pathogens.

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