Heredity (2007) 99, 265–277

www.nature.com/hdy

ORIGINAL ARTICLE

Neutral versus adaptive genetic variation in parasite resistance: importance of major histocompatibility complex supertypes in a free-ranging primate

N Schwensow[1,3], J Fietz[2], KH Dausmann[1] and S Sommer[1,3]
[1]*Department of Animal Ecology and Conservation, University of Hamburg, Hamburg, Germany and* [2]*Department of Experimental Ecology, University of Ulm, Ulm, Germany*

Current discussions in evolutionary ecology and conservation genetics focus on the relative importance of using selective neutral markers or markers of coding genes to identify adaptive and evolutionary relevant processes. Genetic diversity might be particularly important in immune genes (e.g., in genes of the major histocompatibility complex, MHC), which are influencing pathogen and parasite resistance. We investigated the effects of neutral versus adaptive genetic variation in parasite resistance in a natural population of fat-tailed dwarf lemurs (*Cheirogaleus medius*). No association between neutral overall individual genetic diversity and parasite load could be detected. In 149 individuals, we identified 50 MHC class II alleles of the functionally important duplicated DRB locus. The investigation of the functional importance of immune gene (MHC) diversity and parasite selection in natural populations is often problematic due to extensive polymorphism in the MHC genes and restrictions in available sample sizes. Here, for the first time we applied an approach that has been developed in human medical studies. Eleven MHC class II supertypes were identified based on shared antigen-binding similarities. The number of individual MHC supertypes had no influence on the nematode burden. However, we found evidence for a specific MHC supertype (supertype 1) that was linked to infected individuals, a higher number of different nematode infections and high intensity of infection per individual. Moreover, one rare MHC supertype (supertype 7) was revealed to be advantageous with respect to parasite burden. Thus, our results add evidence to the small body of studies that show significant associations between specific MHC constitutions and naturally occurring parasites in the complexity of natural populations.
Heredity (2007) **99,** 265–277; doi:10.1038/sj.hdy.6800993; published online 23 May 2007

Keywords: MHC-supertypes; microsatellites; parasite-driven selection; primate; Madagascar; *Cheirogaleus medius*

Introduction

Many recent studies revealed evidence that genetic diversity plays an important role in protecting animal populations against pathogens and widespread diseases (Altizer *et al*., 2003). Most of them make use of neutral markers such as microsatellites or single nucleotide polymorphisms to assess the level of variability present in individuals and populations (Sunnucks, 2000; Lowe *et al*., 2004). Correlations between low individual heterozygosity at neutral genetic markers and components of individual fitness have been indicated. These can arise in different ways, but the most probable explanations are either inbreeding effects due to a genome-wide reduction of genetic variability (including fitness-relevant loci) or linkage disequilibrium to loci under selection (Balloux and Lugon-Moulin, 2002; Hansson and Westerberg, 2002; Keller and Waller, 2002; DeWoody and DeWoody, 2005). However, variation at neutral loci cannot provide direct information on adaptive selective processes involving the interaction of individuals with their environment (Meyers and Bull, 2002; van Tienderen *et al*., 2002; Sommer, 2005). In contrast to neutral markers, the extant of individual variation at functionally important markers such as loci in the major histocompatibility complex (MHC) is thought to be of adaptive significance. MHC variants influence many important biological traits, including immune recognition, susceptibility to infectious and autoimmune diseases, individual odours, mating preferences, kin recognition, cooperation and pregnancy outcome (e.g., reviewed by Potts and Wakeland, 1990; Hedrick, 1994; Penn, 2002; Bernatchez and Landry, 2003; Sommer, 2005). MHC genes are the most polymorphic loci in the vertebrate nuclear genome (Robinson *et al*., 2003). MHC-encoded molecules are transmembrane glycoproteins that bind antigens derived from pathogens or parasites and present them to T lymphocytes, which in turn initiate appropriate immune responses (Doherty and Zinkernagel, 1975; Klein, 1986). There are two important groups of MHC genes. MHC

Correspondence: Current address. PD Dr S Sommer, Evolutionary Genetics, Leibniz Institute for Zoo- and Wildlife Research (IZW), Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany.
E-mail: sommer@izw-berlin.de
[3]*Current address: Leibniz-Institute for Zoo- and Wildlife Research (IZW), Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany.*
Received 21 September 2006; revised 6 February 2007; accepted 19 February 2007; published online 23 May 2007

class I genes are expressed on virtually all nucleated somatic cells and their products are essential mainly for immune protection from intracellular pathogens. MHC class II genes are only expressed on special antigen-presenting cells such as B cells and macrophages. MHC class II molecules bind and present peptides mainly stemming from extracellular parasites (e.g., bacteria, nematodes, cestodes) (Klein and Horejsi, 1997). In many studies of MHC class II genes, the second exon of the DRB locus is of special interest because it codes for parts of the functionally important antigen-binding sites (ABS) (Ohta, 1998). In a variety of species, the ABS are highly variable in both the overall number of alleles and the extent of sequence variation between alleles (Hughes and Yeager, 1998), and this has been attributed to balancing selection (Bergström and Gyllensten, 1995; Jeffery and Bangham, 2000; Bernatchez and Landry, 2003; Garrigan and Hedrick, 2003). The subsequent alteration in the ABS allows binding of a diverse array of antigens (Brown *et al*., 1988, 1993; Janeway and Travers, 2002). Thus, MHC variability is believed to be maintained by pathogen-driven selection (reviewed by Hedrick and Kim, 2000; Jeffery and Bangham, 2000; Bernatchez and Landry, 2003; Sommer, 2005).

One of the most debated selection mechanisms is the 'heterozygote advantage hypothesis' (Hughes and Nei, 1989; Takahata and Nei, 1990). It is based on the suggestion that heterozygotes are able to recognise two suits of pathogens, one for each allele, and are therefore favoured due to their ability to resist a broader array of pathogens than homozygotes. The 'rare allele advantage hypothesis' (also known as 'frequency-dependent selection hypothesis' or 'Red Queen hypothesis') (Clarke and Kirby, 1966; Doherty and Zinkernagel, 1975) presumes a coevolutionary arms race between hosts and parasites. MHC alleles that provide more resistance to parasites cause an advantage to the host and spread out through the population. This increases selection on parasites to evade recognition by these common alleles. As the parasite antigenicity changes, the relative fitness of the common host genotypes decreases and provides a selective advantage to new, rare MHC alleles to which the parasites are not yet adapted. This hypothesis is supported by both mathematical models (Takahata and Nei, 1990; Borghans *et al*., 2004) and several studies that showed associations between specific MHC alleles and pathogen resistance (Langefors *et al*., 2001; Lohm *et al*., 2002; Froeschke and Sommer, 2005; Harf and Sommer, 2005; Meyer-Lucht and Sommer, 2005; Schad *et al*., 2005).

Even though the highly polymorphic MHC genes clearly play a crucial role in immune response, their great diversity is a major obstacle in distinguishing allele-specific effects and complicates the attribution of specific alleles with the outcome of diseases. Collecting sample numbers sufficient for definitive results is often not feasible. In humans, a new approach to circumvent this problem was recently proposed by classifying MHC alleles to supertypes based on similar antigen-binding motifs (Southwood *et al*., 1998; Sette and Sidney, 1999; Trachtenberg *et al*., 2003; Lund *et al*., 2004). The biological relevance of this kind of classification is supported by a growing body of evidence of cross-presentation of specific antigen-binding motifs by different human leukocyte antigen (HLA) molecules assigned to discrete supertypes (Bertoni *et al*., 1997; Trachtenberg *et al*., 2003). For example, a study by Trachtenberg *et al*. (2003) indicated that HLA supertypes are highly predictive of viral load and showed the advantage of a rare HLA-supertype in HIV disease progression consistent with the rare-allele advantage model. This classification approach of common functional traits does not only provide a new approach to investigate selection mechanisms in natural populations, where high allelic diversity otherwise causes problems in obtaining statistically sufficient large sample sizes. It might also represent a new avenue in the development of epitope-based vaccines (Lund *et al*., 2004 and references herein).

In this study, we investigated the role of neutral versus adaptive genetic variation in parasite resistance in a free-ranging animal population under natural selection conditions. We used a fat-tailed dwarf lemur population (*Cheirogaleus medius*; cheirogaleidae, primates) of the dry deciduous forest of western Madagascar as a model, which has been the focus of long-term population and behavioural ecological investigations (Fietz and Ganzhorn, 1999; Fietz *et al*., 2000; Fietz and Dausmann, 2003; Dausmann *et al*., 2004, 2005). The main objectives of our study were: (1) to examine associations between microsatellite variability and the diversity of a functionally important region of an immune gene (MHC class II DRB exon2) with parasite burden and (2) to identify possible parasite-driven selection acting on the MHC under natural selection conditions. For this purpose, we applied an *in silico* method to define MHC-DRB exon 2 supertypes by similarities in the antigen-binding motifs (Doytchinova and Flower, 2005). Our third goal was to investigate the functional importance of MHC supertypes. To our best knowledge, this is the first time that this approach, developed for human malaria and HIV studies, is used to examine MHC supertype-specific selection and the functional importance of MHC supertype variation in a free-ranging primate population.

Materials and methods

Study area and sample collection

Sampling was carried out in western Madagascar, in the dry deciduous Kirindy forest located about 60 km northeast of Morondava. The research area of about 25 Ha (500×500 m) is part of a forestry concession of the 'Centre de Formation Professionelle Forestière de Morondava, CFPF'. The region is characterized by a strong seasonality, with a dry season of about 8 months and a rainy season. The hibernating *C. medius* is only active during the rainy season (about November/December to April). A detailed description of the forest and trapping conditions is given elsewhere (Ganzhorn and Sorg, 1996; Fietz 1999a, b; Fietz and Dausmann, 2003). Briefly, captures were carried out for four consecutive nights per month using 200 Sherman traps ($7.7 \times 7.7 \times 30.5$ cm), which were placed in 50-m intervals at the intersections of a grid system of trails. The traps were opened in late afternoon, baited with banana and checked early the next morning. Captured individuals were sexed, individually marked and anaesthetized for tissue collection. The animals were released at their capture sites in late afternoon of the same day. Individual faecal samples for later parasitological examinations were collected from each trap or directly from the individual during

handling. The traps were cleaned before reuse. In total, tissue samples for genetic analyses from 149 individuals were taken and 115 faecal samples were collected. From 60 individuals, both genetic and faecal samples were collected (rainy seasons 1995/1996, 1999/2000 and 2000/2001).

Parasite screening

The gastrointestinal parasite burden was investigated by faecal egg counts (FECs, eggs/g faeces). This is a noninvasive and appropriate technique to assess the dimension of nematode infections (Soulsby, 1982) and has been used in many recent studies (Paterson *et al*., 1998; Coltman *et al*., 1999; Cassinello *et al*., 2001; Irvine *et al*., 2001). We applied a modification of the McMaster flotation technique (Gordon and Whitlock, 1939) by using a flotation-dilution of potassium iodide, which enhances the detectability of eggs due to its high specific weight (Meyer-Lucht and Sommer, 2005). Faeces samples were screened for helminthic parasite eggs by counting four chambers of McMaster for each sample. Helminth eggs were assigned to morphotypes based on size and morphological characteristics. Photographs of all morphotypes were taken for later taxonomic classification. Owing to the prevalence of nematodes, we used the number of different nematode morphotype infections per individual (NNI) and the FEC value as measurements of the intensity of the parasite burden. Both measurements reflect the worm burden and fecundity, which in turn are influenced by the immune state of the host (Stear *et al*., 1995, 1997).

Molecular techniques

Overall genetic variability was assessed on the basis of seven microsatellite loci. The PCR analysis of the microsatellite loci was conducted using an extended data set with the primers and conditions as described in Fietz *et al*. (2000). Adaptive variability was studied in the highly polymorphic MHC-DRB exon2 (171 bp, without primer), which includes the functionally important ABS (Brown *et al*., 1988, 1993). The PCR amplification was carried out using the primers JS1 and JS2 as described in Schad *et al*. (2004), which was originally designed for the closely related lemur species *Microcebus murinus* but were also successfully applied in different rodent species (Froeschke and Sommer, 2005; Harf and Sommer, 2005; Meyer-Lucht and Sommer, 2005). To genotype the individuals, we used single-strand conformation polymorphism analysis (Orita *et al*., 1989a, b). PCR products were loaded on 15% polyacrylamide gels following the manufacturer's instructions (ETC, Elektrophoresetechnik, Kirchentellinsfurt, Germany). Runs were carried out using a horizontal cooling electrophoresis system (Amersham Pharmacia Biotech, Freiburg, Germany). After electrophoresis, the gels were fixed and silver stained to visualize the resulting bands. Bands were rearranged and classified into alleles. Alleles were cut out of the gel, dissolved in $1\times$ tris–borate–EDTA buffer and always sequenced bidirectionally. We controlled allele identity or PCR artefacts by sequencing bands classified into an identical allele from different individuals as well as from the same individual. The reamplification was carried out under the same PCR conditions as before but with a reduced number of cycles. Cycle sequencing was performed using a dye-terminator kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems sequencer (Model 3100) following the manufacturer's protocol. Details on the molecular techniques are outlined in Sommer *et al*. (2002), Sommer (2003) and Schad *et al*. (2004).

Analyses and statistical treatment

As measuring units for the overall individual genetic diversity, we used the observed individual microsatellite heterozygosity (H_{obs}) and the genetic distance between microsatellite alleles (d^2 value). H_{obs} was calculated by dividing the number of heterozygous microsatellite loci per individual by the total number of typed loci. d^2 results from the squared difference in repeat units between two alleles at a locus averaged over all typed loci and was calculated as $d^2 = 1/n\Sigma^n(a_i - a_j)^2$, where a_i and a_j are the length in repeat numbers of each allele at a locus averaged over n-typed loci (Slate and Pemberton, 2002; Coltman and Slate, 2003). The overall expected heterozygosity (H_{exp}) was calculated using Cervus (Marshall *et al*., 1998). We calculated deviations from Hardy–Weinberg and linkage disequilibrium using Arlequin 3.0 (Excoffier *et al*., 2005). The null-allele probability was proved by Microchecker (van Oosterhout *et al*., 2004).

MHC class II DRB sequences were edited and aligned using Mega 3 (Kumar *et al*., 2004). Individual levels of MHC diversity were calculated by the minimal, mean and maximal number of amino-acid differences between alleles of an individual. Two different approaches were used to reveal evidence for selection processes in the MHC. Mega 3 was employed to calculate the relative rates of nonsynonymous (d_N) and synonymous (d_S) base pair substitutions according to Nei and Gojobory (1986) applying the Jukes–Cantor correction for multiple hits (Jukes and Cantor, 1969). All calculations were carried out separately for putative ABS and non-ABS assuming concordance to human ABS (Brown *et al*., 1988, 1993). The rates d_N/d_S were tested for significant differences with a Z-test.

Furthermore, we checked for the presence of codon sites affected by positive selection (positively selected sites, PSS) with the help of a maximum-likelihood analysis by using the programme CODEML (included in PAML version 3.14 software package) (Yang, 1997). PSS are indicated by a ratio $\omega = d_N/d_S > 1$. We used the models M7 (β) and M8 (β and ω) (Yang *et al*., 2000), which have been shown to be more robust against recombination in the sequences than the other models implemented in CODEML (Anisimova *et al*., 2003 but see Shriner *et al*., 2003). M7 served as null model where the ω ratio varies according to the β distribution and does not allow PSS ($0 < \omega < 1$). M8 adds a class of sites to account for the possible occurrence of PSS ($\omega > 1$). Both models can be compared by a likelihood-ratio test (LRT) where twice the log-likelihood difference is compared to a χ^2 distribution with degrees of freedom equal to the difference in the number of parameters between the compared models (Yang *et al*., 2000). In the next step, the Bayesian approach integrated in CODEML identifies codon sites under positive selection. In case these species-specific PSS overlap with the human ABS (Brown *et al*., 1988, 1993), this is an indication for the concordance

of selection in the investigated lemur DRB fragment with the human HLA-DR1 sequence. Possible recombination was estimated from the sequences using the likelihood permutation test implemented in the programme LDhat (McVean *et al*., 2002).

MHC-supertypes were defined applying amino-acid sequence-based clustering as proposed by Doytchinova and Flower (2005). The amino-acid sequences of all PSS (see above) were aligned. All amino acids outside PSS were excluded. Each amino acid was described by five z-descriptors: z_1 (hydorphobicity), z_2 (steric bulk), z_3 (polarity), z_4 and z_5 (electronic effects) (Sandberg *et al*., 1998) and thus translated into a mathematical matrix. This matrix was imported into Genesis 1.6.0 Beta 1 (Sturn *et al*., 2002). Hierarchical clustering was applied using average linkage clustering and the Euclidian distance method.

For statistical analyses of an association of genetic variability and parasite load, FEC values were log-transformed to improve normality. The relative risk of being infected was estimated by odds ratio tests, a common method in epidemiological studies to evaluate the exposition of individuals carrying a risk factor. The ratio of the odds of an event occurring in one group is compared to the odds of it occurring in another group by using a 2×2 cross-classification table (Sachs, 1992). All additional statistical tests were performed by SPSS version 11.5. Calculations are two-tailed and based on a significance level of $\alpha = 0.05$. Bonferroni-corrected significance levels and the Tukey *post hoc* test were used for multiple comparisons (Rize, 1989; Sachs, 1992).

Results

Parasite load

From 21 individuals, up to nine faecal samples were available. Neither the sampling day (analysis of variance (ANOVA): $F_{8,67} = 1.025$, $P = 0.43$) nor the sampling year (ANOVA: $F_{2,112} = 0.083$, $P = 0.92$) influenced the FECs. The mean FEC values per individual were used for subsequent analyses. Nine out of 60 individuals were subadult and due to the fact that worm burden is known to be also dependent on host age, these individuals were excluded from further analysis. The sex (31 males and 20 females) had no influence on the infection status ($\chi^2 = 0.631$, d.f. = 1, NS), NNIs (Mann–Whitney *U*-test: $Z = -0.800$, NS) and on the intensity of infection (FEC) ($t = -0.787$, d.f. = 49, NS) values. Therefore, data of adult males and females were pooled.

Nine different helminth egg morphotypes could be distinguished. Seven egg morphotypes were assigned to nematode species. Additionally, one cestode egg morphotype and one trematode egg morphotype were identified. A total of 40.0% individuals were not infected, whereas 60.0% were infected with one to five different helminths. Of all infections, 86.1% were caused by nematodes whereas only 8.3 and 5.6% of the infected individuals suffered only from cestodes or trematodes, respectively. Owing to the high frequency of nematode infections and the minor prevalence of cestode and trematode infections, subsequent statistical analysis focused on nematodes. Individuals carried between one to four different nematode species.

Molecular variability

The seven microsatellites had a total length between 12 and 25 bp and exhibited between five and 13 different alleles. The individual heterozygosity (H_{obs}) ranged from 0.429 to 1. No completely homozygous individual was found, all animals had between two and seven heterozygous microsatellite loci. The individual d^2 values ranged from 1.71 to 181. Thereby, locus 18 showed evidence for linkage disequilibrium with both locus 49 and 93 ($P < 0.05$) (Table 1). Three loci showed deviations from Hardy–Weinberg disequilibrium. One locus showed slight evidence for an increased null-allele probability (locus 54: 0.08) (Table 1).

In a total of 149 individuals, we found a high degree of variability with 50 MHC class II DRB exon 2 alleles differing at one to 42 positions (average = 21.67, s.e. = 2.46) from each other. Seventy-four nucleotide positions of the investigated fragment were variable. Nucleotide sequences are available at GenBank (accession nos. EF194225–EF194272). As we found no indels or evidence for a pseudogene but evidence for selection processes (see below), we assume all alleles to be functional. The alleles *Chme-DRB**Wa03 and Chme-DRB*Wb03 have already been identified in a previous study (Go *et al*., 2002). Within one individual, we found between two and four different alleles, which indicated a duplication of the DRB gene in this species. All nucleotide sequences could be transformed into unique amino-acid sequences with 33 out of 57 amino-acid positions being variable. Alleles differed at the amino-acid level at one to 24 sites (average = 14.42, s.e. = 2.05) (Figure 1). The population recombination rate was estimated as 4Ner = 30, which significantly differed from 0 ($P < 0.001$), indicating that recombination probably occurred.

Evidence for selection processes in the MHC

Two different approaches were used to reveal evidence for selection maintaining polymorphism in the investigated functional important part of the MHC. First, the rates of synonymous (d_S) and nonsynonymous (d_N) substitutions were calculated separately for putative ABS and non-ABS of all identified alleles ($n = 50$) assuming concordance to human ABS (Brown *et al*., 1988, 1993).

In the ABS, the rate of nonsynonymous substations ($d_N = 0.42$ was significantly higher than the rate of

Table 1 H_{obs} and H_{exp}, the N_a of seven microsatellites in 60 individuals of *C. medius* included in both genetic and parasite analyses

Locus	N_a	H_{obs}	H_{exp}
18	13	0.80	0.87*
49	7	0.70	0.80*
54	6	0.61	0.75*
84	9	0.87	0.84
86	11	0.70	0.75
93	5	0.82	0.70
110	7	0.67	0.75
Mean	8.29	0.74	0.78

Abbreviations: H_{exp}, expected heterozygosity; H_{obs}, observed heterozygosity; N_a, number of alleles.
*Significant deviations from Hardy–Weinberg disequilibrium ($P < 0.05$).

```
Chme_01    ERVRLLDRFY YSGEEYVRFD SDVGEFRAVT ELGRPVAENL NSRQDLLEQR RAEVDTV
Chme_02    ........YF HN....A... .......... .....A..YW ..QK.F..RK .......
Chme_03    Q...SVN.YI .NR...A... ...D.Y..L. P....D..YW ..QK.I..RT ...A...
Chme_04    ....F...Y. .N........ .......... .....D.KYW .....I..D. ..V...A
Chme_05    ....Y.E.YI .......... .....Y.... ....RS...W ..QK....R. ..A...Y
Chme_06    ........YF HN...F.... .......... .....D..YW ..QK.I..DE ..Q....
Chme_07    ....F...YF .NR....... .......... ....RS..YW .....I..DE ..S...Y
Chme_08    ....F...YF .NR....... .......... ....RN..YW ...K.I..DK ..K...F
Chme_09    Q.....E.YI ..R...A... .....Y.... A....D.KYW ..QK...... ..A...Y
Chme_10    Q...Y.E.YI HN....A... .......... .........W ..QK....D. ..S...Y
Chme_11    Q.....E.HI .......... .......... ....RS..YF ..QK.Y.... ..A...Y
Chme_12    ........YF .NR...A... .....Y.... .....D.KYW ..QK....R. ......Y
Chme_13    ........YI SN...T.... .....Y.... .....D..YW .....I..R. .......
Chme_14    ........YI SN...T.... .....Y.... .....D..YW .....I..RK .......
Chme_15    Q...HVT.RF .NR..F.... .....H.... P....A..YF ..QK.Y...M ..A....
Chme_16    Q...GVT.RI .NR..F.... .....H..L. P..QQI..D. ..NK.Y...M ..A....
Chme_17    Q...Y.E.HI .....FL... .....Y.... .....D.KYW ..QK.I..DA ..S...Y
Chme_18    Q...Y.E.RI HNR...A... .....Y.... .....D.KYW ..QK....D. ..S...Y
Chme_19    Q....VT.YI .NQ...A... ...D.Y..L. P....D..YW ..QK.I..RT ...A...
Chme_20    Q...Y.E.YI HN........ .....Y.... .....D...W .....I..DE ..A...Y
Chme_21    ....Y.E.RI HNR...A... ..L..Y.... .....D..YW .....I.... .......
Chme_22    Q...Y.E.HI .....FL... .......... .....E...W ..QK.Y.... ..A...F
Chme_23    Q...Y.A.YF .NR....... .....Y.... ....RS..YW ..QK.I..R. ..A...Y
Chme_24    Q..Q..V.YI HNQ..N.... .......... .....D..YW ..QKEI...K .......
Chme_25    ....F...Y. .N........ .......... .....A.... .....I..DK ..S...Y
Chme_26    ....F.V.YF .N...FA... .....Y.... .....D.KYW ..QK...... ..A...F
Chme_27    Q....VT.YI .NQ....... ...D.Y.... P...RS...W ..QK.I..RT ...A...
Chme_28    Q.....E.YI ..R...A... .....Y.... ....RS...W ..QK...... ..A...Y
Chme_29    Q...VVT.HI .NR...A... .....Y..L. P....D..YW ..QK.....T ..A...A
Chme_30    Q...HVT.YI .NR..F.... .....Y.... P....D..YW ..QK.I...T ...A...
Chme_31    ....F...YF .N........ .......... .....A.... .....I..DE ..S...Y
Chme_32    Q...FVT.YI .NR..F.... ...W.H.... P....D..Y. ..QK.F...T ..A...L
Chme_33    Q...Y.E.HI SN...N.... .....Y.... .....D..YW ..QK.Y...T ..A...F
Chme_34    Q...FVT.YI .NQ...A... ...D.Y.... P....D..YW ..QK.I..RT .......
Chme_35    Q....VT.YI .NR..F.... ...W.H.... P....A..D. ..QK.F...T ..A...R
Chme_36    ....F...Y. .N...F.... .....Y.... .........W .....I..R. .......
Chme_37    Q...SVT.HI .NQ...A... .....Y.... P....A..YW ..QK.F.... ...A...
Chme_38    ....Y.E.YI .......... .....Y.... ....RS...W ..QK....R. ......Y
Chme_39    Q...SVN.YI .NQ..F.... ...D.Y.... P....D..YW ..QK.I..RT ...A...
Chme_40    Q.......Y. .N........ .......... ....RS..YF ..QK....D. ..A...Y
Chme_41    ....F...Y. .N........ .......... .....A.... .....I..DE ..S...Y
Chme_42    ....F...YF .NQ....... .....Y.... .....D..YW ..QK.F..D. ..Q....
Chme_43    Q...Y.A.YF .NR....... .......... ....RS..YW ..QK.I..RK ...A...
Chme_44    Q...SVN.YI .NQ...A... ...D.Y.... P....D..YW ..QK.I..RT ...A...
Chme_45    Q....VT.YI .NR...A... ...D.Y.... P...RS..YW ..QK.I...T ...A...
Chme_46    ....F.E.QI HN...NL... .....Y.... .....D...W ..QK.F..D. ..A...F
Chme_47    Q....VT.YI .NQ...A... ...D.Y.... P....D..YW ..QK.I..RT ...A...
Chme_48    Q.....A.GI .NQ..FT... .......... ....DI..YY .NQKE....A ..A...C
Chme_49    Q.......Y. .N........ .......... ....RS..YF ..QK.F..D. ..A...Y
Chme_50    .......... .......... .......... .......... ........RK ...A...
human ABS        A A  A    AA         A         A A  A    A  A AA   A   A
               P   PP P P  PP         P         PP  PP      P  PP   P   P
                   ↑  ↑    ↑↑        ↑         ↑    ↑         ↑↑   ↑   ↑
```

Figure 1 Alignment of all identified MHC class II DRB exon 2 amino-acid sequences of *Cheirogaleus medius*. Dots indicate identity with the amino-acid sequence of allele *Chme*_1. 'A' indicates human antigen-binding sites according to Brown *et al*. (1988, 1993). 'P' indicates species-specific sites under positive selection identified by likelihood analysis (all significant except for position 11, see text for details). Arrows indicate concordance of human ABS and positively selected sites in *C. medius*.

synonymous substations ($d_S = 0.091$) with a ratio of 4.56 (Table 2, $Z = 3.60$, $P < 0.001$). Also, in the non-ABS we found a significant deviation from unity but with a lower ratio of 2.19 (Table 2, $Z = 2.00$, $P = 0.048$).

Second, the species-specific codon sites affected by positive selection (PSS) were analysed with the help of a maximum-likelihood analysis. The LRT statistic for comparing M7 (β) and M8 (β and ω) is $2\Delta\ln L = 2 \times ((-1836.205) - (-1860.851)) = 49.29$ ($\ln L$ = log-likelihood value). The comparison with a χ^2 distribution indicated that our data fitted the M8 model (occurrence of PSS possible), significantly better than the M7 model (PSS not allowed) (d.f. = 2, $P < 0.001$). M7 is thus rejected in favour of M8. In Malagasy fat-tailed dwarf lemurs, the maximum-likelihood approach detected 29.82% of all investigated amino-acid sites as positively selected, with 16 out of the 17 identified PSS being statistically significant after Bayes empirical analysis (Table 3). Eleven PSS were identical with the ABS reported in the corresponding human sequence (Brown *et al*., 1988, 1993) (Table 3, Figure 1). The identified but nonsignificant PSS (position 11, Table 3, Figure 1) is an ABS in humans. It was excluded from MHC-supertype analyses to avoid conclusions based on possible false positives. Four PSS (amino-acid positions 10, 36, 39 and 46) were situated right next to a human ABS. Additionally, we identified position 5 and 13 as PSS with a distance of two amino acids to a human ABS. Positions 7, 37, 44 and 47 are ABS

Table 2 The estimated rates (±s.e.) of d_N and d_S substitutions for ABS and non-ABS (ABS according to the human sequence, Brown *et al*. (1988, 1993)), and their ratio for MHC class II exon 2 sequences in *C. medius*

Position	n	d_N	d_S	d_N/d_S	P
ABS	15	0.42±0.10	0.09±0.04	4.56	<0.001
Non-ABS	42	0.11±0.03	0.05±0.02	2.19	<0.05
All	57	0.17±0.03	0.06±0.02	2.95	<0.001

Abbreviations: ABS, antigen-binding sites; d_N, nonsynonymous; d_S, synonymous; MHC, major histocompatibility complex.
n is the number of codons in each category and *P* is the probability that d_N and d_S are different using a Z-test.

Table 3 Evidence for selection on amino-acid positions of MHC class II sequences in *C. medius* assuming concordance with of the human ABS (Brown *et al*., 1988, 1993) and by species-specific PSS identified by likelihood analysis

Amino-acid position	*ABS*	*PSS*	P	*Concordance*	*Distance (amino acids) to nearest human ABS*
5		X	<0.001		2
7	X				
9	X	X	<0.001	X	0
10		X	0.002		1
11	X	X	0.113 (NS)	(X)	0
13		X	0.002		2
16	X	X	<0.001	X	0
17	X	X	0.001	X	0
26	X	X	0.021	X	0
35	X	X	0.022	X	0
36		X	<0.001		1
37	X				
39		X	0.012		1
40	X	X	0.027	X	0
44	X				
46		X	<0.001		1
47	X				
49	X	X	<0.001	X	0
50	X	X	<0.001	X	0
53	X	X	0.001	X	0
57	X	X	<0.001	X	0

Abbreviations: ABS, antigen-binding sites; MHC, major histocompatibility complex; PSS, positively selected sites.
X represents present.

in human but are not identified to be under positive selection in our data. Moreover, positions 37 and 47 are invariable in *C. medius* (Figure 1).

In *C. medius*, eleven MHC class II supertypes were defined based on significant PSS using a hierarchical amino-acid sequence-based clustering considering physiochemical similarities between all identified MHC class II alleles ($n = 50$) (Doytchinova and Flower, 2005) (Figure 2). As only one faecal sample was available from an individual carrying a MHC supertype 2 allele and no parasitological data were available from individuals carrying MHC supertype 10, both MHC-supertypes were excluded from further analysis.

Microsatellite variability and parasite load

No association between neutral overall individual genetic diversity and parasite load could be detected (Figure 3a–f). The observed individual heterozygosity (H_{obs}) of infected and noninfected individuals did not differ (*t*-test: $t = -0.262$, d.f. = 49, NS; Figure 3a). The infection status was also not effected by the mean genetic distance between alleles (d^2 values; *t*-test: $t = -0.623$, d.f. = 49, NS; Figure 3b).

We found no evidence for an effect of H_{obs} on the NNIs (linear regression: $R = 0.105$, NS; Figure 3c). Also, the d^2 value had no influence on the detected numbers of nematodes (linear regression: $R = 0.057$, NS; Figure 3d). Furthermore, we found no effect of H_{obs} on the intensity of infection (FEC values) (linear regression: $R = 0.072$, NS; Figure 3e). Also, no correlation of the d^2 on FEC values (linear regression: $R = 0.111$, NS; Figure 3f) were detectable.

MHC variability and parasite load – importance of supertypes

To identify possible parasite-driven selection, we investigated the importance of individual MHC-DRB diversity and MHC supertypes in parasite resistance. The individual MHC-DRB diversity was measured by three values: the minimal, the mean and the maximal possible sum of pair-wise amino-acid differences between all alleles of a single individual. The minimal distances ranged from 5 to 22, the mean distances ranged from 10 to 22 and the maximal distances ranged from 9 to 23 amino-acid differences between the alleles. The individual distance between the alleles had neither an effect on the infection status (*t*-test: minimal distance $t = 1.270$, NS; mean distance $t = 0.732$, NS; maximal distance $t = 0.319$, NS) or on the NNI (Spearman's rank correlation: minimal distance $R = 0.114$, NS; mean distance $R = 0.002$, NS; maximal distance $R = -0.001$, NS) nor on the intensity of infection (Pearson's correlation: minimal distance $R = 0.152$, NS; mean distance $R = 0.015$, NS; maximal distance $R = -0.029$, NS) (figures not shown).

All individuals carried alleles from between two and four MHC supertypes. The individual number of MHC supertypes had no significant effects on the individual status of being infected or not ($\chi^2 = 0.988$, d.f. = 2, NS). Similarly, there was no effect of the individual number of MHC supertypes on the NNI (Kruskal–Wallis test: $\chi^2 = 0.123$, d.f. = 2, NS) or on the intensity of infection (FEC values, ANOVA: $F = 0.030$, NS) (figures not shown).

However, our analysis displayed significant differences in the functional importance of certain MHC supertypes in parasite resistance (Figure 4). Supertype 1 was significantly linked to the individual infection status (odds ratio test: $\chi^2 = 5.542$, $P < 0.02$) with a total of 81.3% of the individuals carrying an allele from this supertype being infected. The relative risk of being infected is 1.8-fold higher in supertype 1 individuals than in individuals carrying alleles from other MHC supertypes (Figure 4a). Individuals carrying alleles from supertype 1 also displayed less variation in the number of infections leading to significant higher NNI (Mann–Whitney *U*-test: $Z = -2.071$, $P < 0.04$, Bonferroni correction not significant) (Figure 4b). Additionally, supertype 1 individuals exposed significantly higher FEC values than individuals not carrying this supertype (*t*-test: $t = 2.290$, $P < 0.03$) (Figure 4c). In contrast, individuals carrying alleles from the supertype 7 were more often observed in the category 'not infected' (odds ratio tests:

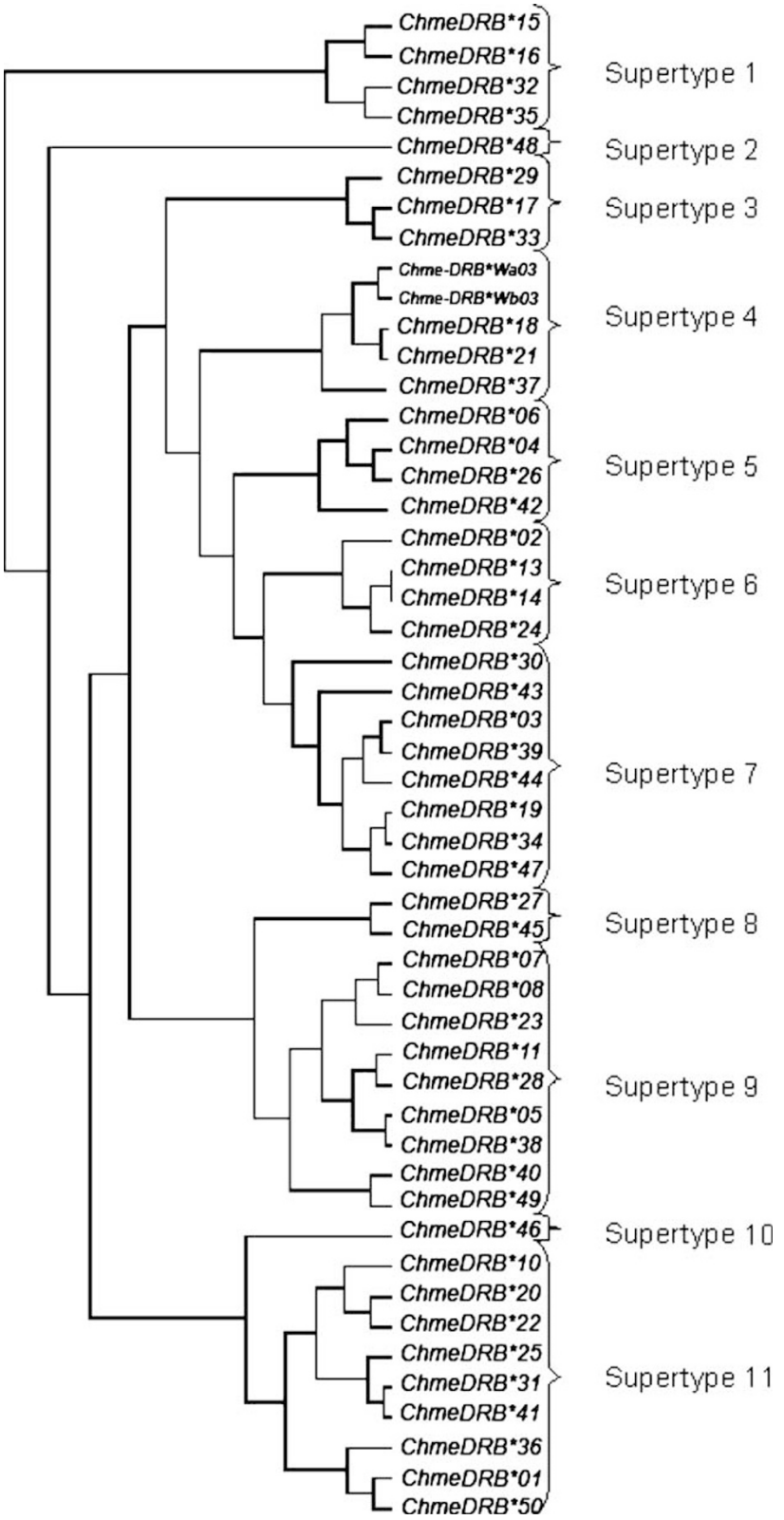

Figure 2 Tree constructed by hierarchical clustering. All 50 identified *Cheirogaleus medius* MHC class II alleles were grouped into 11 supertypes using a hierarchical amino-acid sequence-based clustering approach based on physiochemical similarities (see text for details).

$\chi^2 = 3.850$, $P < 0.05$) (Figure 4a) and tended to display lower NNI, although the latter value slightly missed significance (Mann–Whitney *U*-test: $Z = -1.830$, $P = 0.067$) (Figure 4b). Supertype 7 was significantly linked to lower intensity of infection (*t*-test: $t = -2.048$, $P < 0.05$) (Figure 4c). Individuals carrying supertype 7

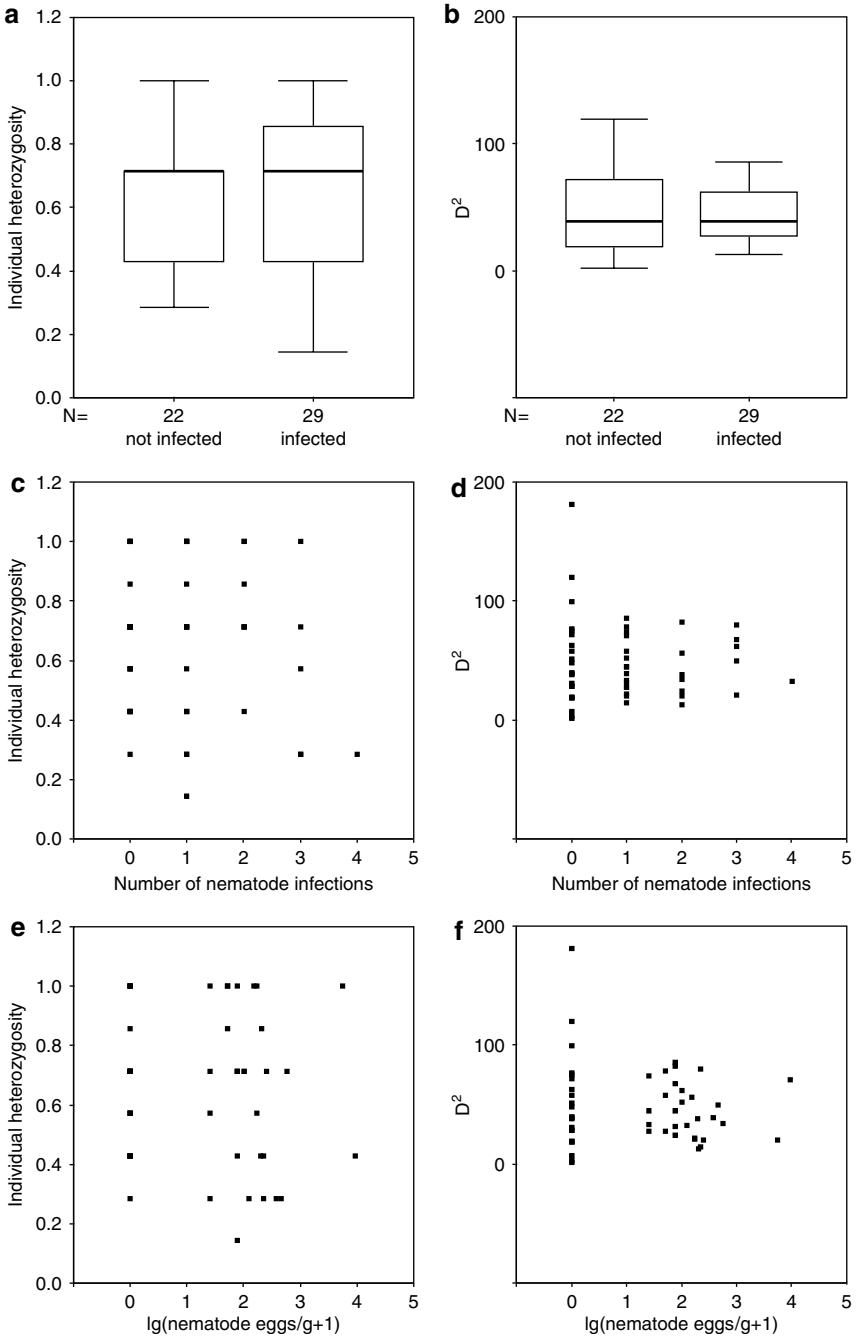

Figure 3 Microsatellite variability and parasite load. No effects of the (**a**) individual heterozygosity (H_{obs}) and (**b**) genetic distance between alleles (d^2) on the infection status; (**c**) H_{obs} and (**d**) d^2 on the numbers of different nematode infections per individual and (**e**) H_{obs} and (**f**) d^2 on the individual intensity of infection were observed. n = sample size. Box plots represent medians and quartiles, whiskers signify the 10th and 90th percentiles.

had a 3.7-fold lower FEC value than supertype 1 individuals.

Discussion

Many recent studies report that individual heterozygosity at apparently neutral microsatellite markers is correlated with key components of individual fitness such as survival (Coulson *et al*., 1999), fecundity (Amos and Balmford, 2001), disease resistance (Coltman *et al*., 1999; Cassinello *et al*., 2001; Acevedo-Whitehouse *et al*., 2003) and lifetime reproductive success (Slate *et al*., 2000). Others do not find such correlations (Côté *et al*., 2005) and null results are likely to be underrepresented in the literature because of publication bias in favour of significant correlations (Hansson and Westerberg, 2002). A recent review and meta-analysis of both published and unpublished studies of the association between neutral marker heterozygosity and traits or components of individual fitness reported that associations were com-

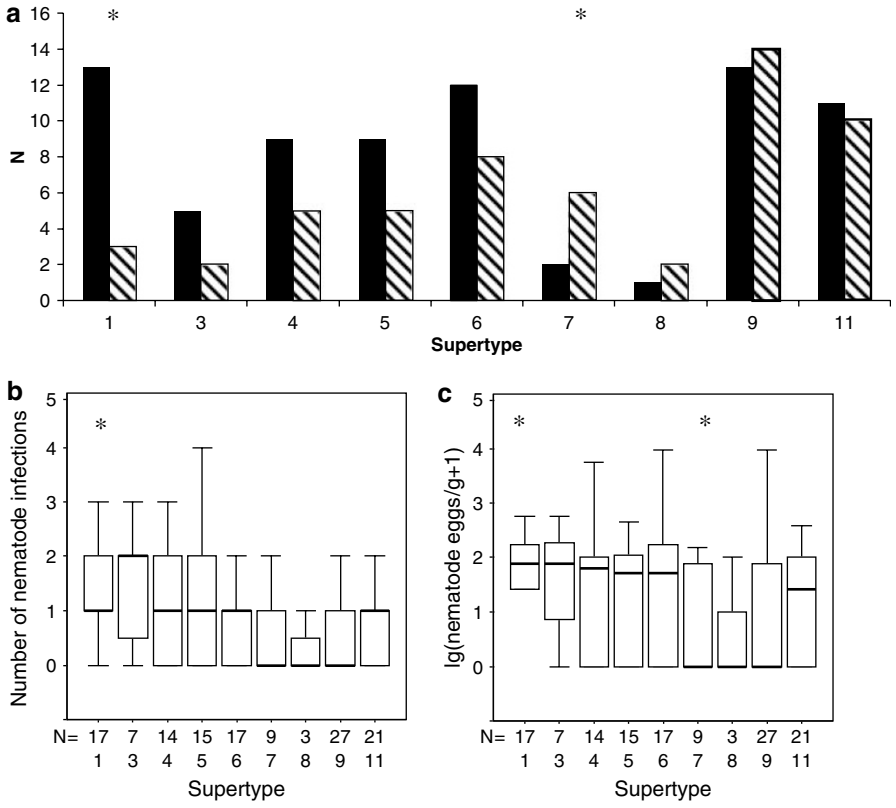

Figure 4 MHC variability and parasite load – importance of MHC-supertypes. (**a**) infection status, (**b**) number of nematode infections per individual and (**c**) intensity of infection. n = sample size; $^*P < 0.05$. Box plots represent medians and quartiles, whiskers signify the 10th and 90th percentiles. Black bars represent infected individuals and hatched bars represent not infected individuals.

mon, yet typically weak (Coltman and Slate, 2003). The variation at neutral loci cannot provide direct information on selective processes involving the interaction of individuals with their environment or on the capacity for future adaptive changes (Meyers and Bull, 2002; van Tienderen *et al*., 2002). However, these are issues of particular relevance in evolutionary ecology and conservation (Crandall *et al*., 2000; Stockwell *et al*., 2003). Contrary to neutral markers, MHC variability reflects evolutionary relevant and adaptive processes within and between populations and is very suitable to investigate a wide range of open questions in evolutionary ecology and conservation. The comparison with neutral markers allows the construction of null hypotheses concerning the diversity at selectively relevant genes and conclusions on the relevance of MHC polymorphism. A candidate-gene approach might be more fruitful for the research on gene–resistance correlations (Côté *et al*., 2005) and is therefore probably more suitable for an investigation of selective processes.

We used the free-ranging fat-tailed dwarf lemur population as a model to investigate the role of neutral versus adaptive genetic variation in parasite resistance and to identify possible parasite-driven selection acting on the MHC under natural selection conditions. In 149 genetically investigated Malagasy fat-tailed dwarf lemurs (*C. medius*), 50 distinct MHC class II DRB exon 2 alleles were detected. Each individual showed between two and four different alleles, indicating a duplication of the locus. All alleles had a unique amino-acid sequence. There was no evidence that either locus was a pseudogene; thus, all loci are assumed to be functional. Gene duplications are common in the MHC (Kasahara, 1999; Go *et al*., 2003; Kelley *et al*., 2005) and coexpression of duplicated loci might be selectively favoured due to increased parasite recognition (Nuismer and Otto, 2004). Duplication events seem to be a common feature in lemurs (Go *et al*., 2003). For example, in aye-ayes (*Daubetonia madagascariensis*), 12 DRB genes have been found (Go *et al*., 2005). Interestingly, also in a closely related mouse lemur species (*Microcebus murinus*), which lives sympatrically with *C. medius* in western Madagascar, the DRB locus was duplicated (Schwensow and Sommer, unpublished data). However, no evidence for a MHC-DRB gene duplication was observed in a distant southern *Microcebus murinus* population (Schad *et al*., 2005). The fact that duplication events are present in some but not all populations of a species might support the idea that under certain local conditions duplication and coexpression might be advantageous and that locally different selection pressure might affect the evolution of MHC polymorphism.

Genes within the MHC most often show extensive polymorphism. In a variety of species, sites, especially the ABS, 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). The subsequent alteration in the ABS allows binding of a diverse array of antigens (Brown *et al*., 1988, 1993; Janeway and Travers, 2002). The vast majority of studies that have tested for the possible role of selection report results for the standard d_N/d_S test ratio only (Bernatchez

and Landry, 2003), assuming concordance of the functionally important ABS with human sequences (Brown *et al*., 1988, 1993). Recently, alternative approaches based on maximum-likelihood models have been suggested that potentially provide more accurate estimates of d_N/d_S ratios and allow the identification of species-specific PSS (Yang and Bielawski, 2000; Yang *et al*., 2000). In simulations, Wong *et al*. (2004) demonstrated that the maximum-likelihood method has a good power and accuracy in detecting positive selection. It is particularly appropriate for detecting selection in MHC genes, where positive selection could be acting simultaneously in groups of codons (Suzuki and Nei, 2004). Recently, this approach was also successfully applied in MHC studies of natural vertebrate populations. Kundu and Faulkes (2004) were able to identify PSS in four species of African mole-rats (Bathyergidae: *Heliophobius argenteocinereus, Heterocephalus glaber, Cryptomys hottentotus hottentotus, Cryptomys damarensis*). As in our study in Malagasy fat-tailed dwarf lemurs, the comparisons of PSS in Atlantic salmon (*Salmo salar*) (Consuegra *et al*., 2005) and in two chamois subspecies (*Rupicapra* spp.) (Schaschl *et al*., 2005) revealed high but not complete coincidence with the human ABS. In *C. medius*, about 30% of all investigated amino-acid sites were PSS. Bayes empirical analysis revealed 17 PSS under positive selection, 16 of them with significance. In comparison, 15 ABS are reported in the corresponding human fragment (Brown *et al*., 1988, 1993). Eleven PSS match with the human ABS. Two human ABS (position 37 and 47) were invariable in *C. medius* and are therefore probably not involved in antigen recognition and binding in this species. However, directly next to these sites (position 36 and 46) highly significant PSS were identified. Also, all other species-specific PSS were in close proximity to ABS, indicating a slight displacement in antigen recognition in *C. medius* compared to humans. Moreover, positions 5, 36 and 46 were also identified as PSS in a sympatric population of the closely related mouse lemurs, *Microcebus murinus* (Schwensow and Sommer, unpublished data), which suggests that they might be fixed substitutions, which is another footprint of balancing selection. The results indicate that maximum-likelihood analyses might potentially offer higher resolution in detecting the effects of selection than assuming concordance with human ABS *per se*. We cannot exclude the possibility of an overestimation of the d_N/d_S due to recombination (Shriner *et al*., 2003). Recombination has been found to be important for the generation of polymorphism within the MHC (Richman *et al*., 2003).

To identify possible parasite-mediated selection acting on the MHC, we tested for associations the individual MHC-DRB supertype constitution and different measures of parasite burden. To the best of our knowledge, this is the first time that this approach, primarily developed in the context of human vaccine design, has been applied to a natural population of a nonmodel organism to examine MHC supertype-specific selection and the functional importance of MHC-supertype variation in an ecological context. In our study, the 50 *C. medius* alleles could be grouped into 11 distinct MHC supertypes. We found no direct associations between MHC-DRB alleles and parasite load (data not shown) but the individual number of MHC alleles was significantly correlated with the individual number of MHC supertypes. This means that individuals with a higher number of alleles most often had alleles that differed strongly in their PSS, which lead to grouping into different supertypes. Thereby, the number of individual MHC supertypes (and also the individual number of MHC alleles) had no influence on the nematode burden. However, we found evidence for a specific MHC supertype (supertype 1) that was significantly linked to infected individuals, a higher number of different nematode infections and high intensity of infection per individual. Individuals carrying an allele belonging to this MHC supertype carried a 1.8-fold higher risk of belonging to the group of infected individuals than individuals carrying alleles from other MHC supertypes. Interestingly, supertype 1 alleles differ from all other alleles in a unique amino-acid motive (histidine) at the putative antigen-binding site at position 26. There is increasing evidence that pathogen escape from host immune system recognition may occur due to the exchange of only few amino acids and that small binding motive differences in MHC molecules can lead to large differences in protection (summarized in Frank, 2002). For instance, one amino-acid difference in the human DRB peptide-binding region abrogates protection to malaria (Davenport *et al*., 1995). Also, in Malagasy mouse lemurs (*Microcebus murinus*), certain amino-acid motifs in the ABS were correlated with high or low parasite burden (Schad *et al*., 2005). In addition, MHC supertype 7 was detected to be advantageous with respect to infection status and the intensity of infection. Thereby, it is interesting to note that it is one of the most frequent supertypes (supertype 1) associated with high parasite load, whereas the rather rare supertype 7 was associated with lower parasite burden, which meets the prediction of frequency-dependent selection (Clarke and Kirby, 1966; Doherty and Zinkernagel, 1975). Although our data do not support the heterozygote advantage hypothesis, they add empirical evidence to the small body of studies in free-ranging animal populations investigated under the complexity of natural selection conditions that show significant disease associations with certain MHC alleles or supertypes.

Contrary to many other studies, we found no association between neutral overall individual genetic diversity and parasite load. Thus, we consider a linkage to immunorelevant loci or an immunofunctional importance of the microsatelite loci as unlikely. Possible explanations of heterozygosity–fitness correlation based on neutral markers have been summarized by Hansson and Westerberg (2002). The heterozygosity in the investigated microsatellite loci also does not correlate with the number of MHC alleles or MHC supertypes (Schwensow, unpublished data). Overall, heterozygosity might not be sufficiently reflected by the seven microsatellite loci investigated and future studies with increased numbers of microsatellites are desirable. We are aware that this study is based on a relatively small sample size that might have influenced the results and prevented more in-depth statistical analysis. However, one advantage of studies in natural populations of nonmodel organisms is that it allows examination of selection acting in an ecological context. This enhances the potential for identifying sources of selective pressure, which are important topics in evolutionary and conservation genetics.

Acknowledgements

We thank the 'Commission Tripartite' of the Malagasy Government, the 'Laboratoire de Primatologie et des Vertébrés de ÍUniversité ďAntananarivo', the 'Parc Botanique et Zoologique de Tsimbazaza', the 'Ministère pour la Production Animale' and the 'Département des Eaux et Forêts' for their collaboration and permission to work in Madagascar. We also thank the 'Centre de Formation Professionnelle Forestière de Morondava', B Rakotosamimanana, R Rasoloarison and L Razafimanantsoa for logistical support. We thank the German Primate Centre (DPZ) and PM Kappeler for the opportunity to work at the field station. We thank I Tomaschweski for technical assistance in the lab, A Hapke and H Zischler for introducing microsatellite analyses, J Ganzhorn for unflagging support and three anonymous reviewers for helpful comments on a former version of this paper. This study was made possible by the German Science Foundation (So 428/4-1, So 428/4-2).

References

Acevedo-Whitehouse K, Gulland F, Greig D, Amos W (2003). Inbreeding: disease susceptibility in California sea lions. *Nature* **422**: 35.

Altizer S, Harvell D, Friedle E (2003). Rapid evolutionary dynamics and disease threats to biodiversity. *Trends Ecol Evol* **18**: 589–596.

Amos W, Balmford A (2001). When does conservation genetics matter? *Heredity* **87**: 257–265.

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

Balloux F, Lugon-Moulin N (2002). The estimation of population differentiation with microsatellite markers. *Mol Ecol* **11**: 155–165.

Bergström T, Gyllensten U (1995). Evolution of MHC class II polymorphism: the rise and fall of class II gene function in primates. *Immunol Rev* **143**: 13–31.

Bernatchez L, Landry C (2003). MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? *J Evol Biol* **16**: 363–377.

Bertoni R, Sidney J, Fowler P, Chesnut RW, Chisari F, Sette A (1997). Human histocompatibility leukocyte antigen-binding supermotifs predict broadly cross-reactive cytotoxic T lymphocyte responses in patients with acute hepatitis. *J Clin Invest* **100**: 503–513.

Borghans JAM, Beltman JB, de Boer RJ (2004). MHC polymorphism under host–pathogen coevolution. *Immunogenetics* **55**: 732–739.

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL *et al.* (1993). Three-dimensional structure of the human class II histocompatibility antigen HLA DR1. *Nature* **364**: 33–39.

Brown JH, Jardetzky TS, Saper MA, Samraoui B, Bjorkman PJ, Wiley DC (1988). A hypothetical model of foreign antigen binding site of class II histocompatibility molecules. *Nature* **332**: 845–850.

Cassinello J, Gomendio M, Roldan ES (2001). Relationship between coefficient of inbreeding and parasite burden in endangered gazelles. *Conser Biol* **15**: 1171–1174.

Clarke B, Kirby DR (1966). Maintenance of histocompatibility polymorphisms. *Nature* **211**: 999–1000.

Coltman DW, Pilkington JG, Smith JA, Pemberton JM (1999). Parasite-mediated selection against inbred Soay sheep in a free-living, island population. *Evolution* **53**: 1259–1267.

Coltman DW, Slate J (2003). Microsatellite measures of inbreeding: a meta-analysis. *Evolution* **57**: 971–983.

Consuegra S, Megens HJ, Schaschl H, Leon K, Stet RJM, Jordan WC (2005). Rapid evolution of the MH class I locus results in different allelic compositions in recently diverged populations of Atlantic salmon. *Mol Biol and Evol* **22**: 1095–1106.

Côté SD, Stien A, Irvine RJ, Dallas JF, Marshall F, Hovorsen O *et al.* (2005). Resistance to abomasal nematodes and individual genetic variability in reindeer. *Mol Ecol* **14**: 4159–4168.

Coulson T, Albon S, Slate J, Pemberton J (1999). Microsatellite loci reveal sex-dependent responses to inbreeding and outbreeding in red deer calves. *Evolution* **53**: 1951–1960.

Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000). Considering evolutionary processes in evolutionary biology. *Trends Ecol Evol* **15**: 290–295.

Dausmann KH, Glos J, Ganzhorn JU, Heldmaier G (2004). Hibernation in a tropical primate. *Nature* **429**: 825–826.

Dausmann KH, Glos J, Ganzhorn JU, Heldmaier G (2005). Hibernation in the tropics: lessons from a primate. *J Comp Physiol B* **175**: 147–155.

Davenport MP, Quinn CL, Chicz RM, Green BN, Willis AC, Lane WS *et al.* (1995). Naturally processed peptides from two disease-resistance-associated HLA-DR13 alleles show related sequence motifs and the effects of the dimorphism at position 86 of the HLA-DRß chain. *Proc Natl Acad Sci USA* **92**: 6567–6571.

DeWoody YD, DeWoody JA (2005). On the estimation of genome-wide heterozygosity using molecular markers. *J Hered* **96**: 85–88.

Doherty PC, Zinkernagel RM (1975). Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. *Nature* **256**: 50–52.

Doytchinova IA, Flower DR (2005). *In silico* identification of supertypes for class II MHCs. *J Immunol* **174**: 7085–7095.

Excoffier L, Laval G, Schneider S (2005). Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol Bioinformatics* (Online) **1**: 47–50.

Fietz J (1999a). Monogamy as a rule rather than exception in nocturnal lemurs: the case of the fat-tailed dwarf lemur, *Cheirogaleus medius*. *Ethology* **105**: 259–272.

Fietz J (1999b). Mating system of *Microcebus murinus*. *Am J Primatol* **48**: 127–133.

Fietz J, Dausmann KH (2003). Costs and potential benefits of parental care in the nocturnal fat-tailed dwarf lemur (*Cheirogaleus medius*). *Folia Primatologica* **74**: 246–258.

Fietz J, Ganzhorn JU (1999). Feeding ecology of the hibernating primate *Cheirogaleus medius*: How does it get so fat? *Oecologia* **121**: 157–164.

Fietz J, Zischler H, Schwiegk C, Tomiuk J, Dausmann KH, Ganzhorn JU (2000). High rates of extra-pair young in the pair-living fat-tailed dwarf lemur, *Cheirogaleus medius*. *Behav Ecol Sociobiol* **49**: 8–17.

Frank SA (2002). *Immunology and the Evolution of Infectious Disease*. Princeton University Press: Princeton.

Froeschke G, Sommer S (2005). MHC Class II DRB constitution and parasite load in the striped mouse, *Rhabdomys pumilio*, in the Southern Kalahari. *Mol Biol Evol* **22**: 1254–1259.

Ganzhorn JU, Sorg JP (1996). Ecology and economy of a tropical dry forest in Madagascar. *Primate Report 46*. Goltze, Göttingen.

Garrigan D, Hedrick PW (2003). Detecting adaptive molecular polymorphism: lessons from the MHC. *Evolution* **57**: 1707–1722.

Go Y, Rakotoarisoa G, Kawamoto Y, Shima T, Koyama N, Randrianjafy A *et al.* (2005). Characterization and evolution of major histocompatibility complex class II genes in the aye-aye, *Daubentonia madagascariensis*. *Primates* **46**: 135–139.

Go Y, Satta Y, Kawamoto Y, Rakotoarisoa G, Randrianjafy A, Koyama N *et al.* (2002). Mhc-DRB genes evolution in lemurs. *Immunogenetics* **54**: 403–417.

Go Y, Satta Y, Kawamoto Y, Rakotoarisoa G, Randrianjafy A, Koyama N *et al.* (2003). Frequent segmental sequence

exchanges and rapid gene duplication characterize the MHC class I genes in lemurs. *Immunogenetics* **55**: 450–461.
Gordon HM, Whitlock HV (1939). A new technique for counting nematode eggs in sheep faeces. *J Counc Sci Ind Res Australia* **12**: 50–52.
Hansson B, Westerberg L (2002). On the correlation between heterozygosity and fitness in natural populations. *Mol Ecol* **11**: 2467–2474.
Harf R, Sommer S (2005). Association between major histocompatibility complex class II DRB alleles and parasite load in the hairy-footed gerbil, *Gerbillurus paeba*, in the southern Kalahari. *Mol Ecol* **14**: 85–91.
Hedrick PW (1994). Evolutionary genetics of the major histocompatibility complex. *Am Nat* **143**: 945–964.
Hedrick PW, Kim KJ (2000). Genetics of complex polymophisms: parasites and maintenance of the major histocompatibility complex variation. In: Singh RS, Krimbas CB (eds). *Evolutionary Genetics: from Molecules to Morphology*. Cambridge University Press: Cambridge. pp 204–234.
Hughes AL, Nei M (1989). Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. *Proc Natl Acad Sci USA* **86**: 958–962.
Hughes AL, Yeager M (1998). Natural selection at major histocompatibility complex loci of vertebrates. *Ann Rev Genet* **32**: 415–435.
Irvine JI, Stien A, Dallas JF, Halvorsen O, Langvatn R, Albon SD (2001). Contrasting regulation of fecundity in two abomasal nematodes of Svalbard reindeer (*Rangifer tarandus platyrhynchus*). *Parasitology* **122**: 673–681.
Janeway CA, Travers P (2002). *Immunology*. Spektrum Akademischer Verlag GmbH: Heidelberg, Berlin, Oxford.
Jeffery KJ, Bangham CR (2000). Do infectious diseases drive MHC diversity? *Microbes Infect* **2**: 1335–1341.
Jukes TH, Cantor CR (1969). Evolution of protein molecules. In: Munroe HN (ed). *Mammalian Protein Metabolism*. Academic Press: New York. pp. 21–132.
Kasahara M (1999). The chromosomal duplication model of the major histocompatibility complex. *Immunol Rev* **167**: 17–32.
Keller L, Waller D (2002). Infreeding effects in wild populations. *Trends Ecol Evol* **17**: 230–241.
Kelley J, Walter L, Trowsdale J (2005). Comparative genomics of major histocompatibility complexes. *Immunogenetics* **56**: 683–695.
Klein J (1986). *Natural History of the Major Histocompatibility Complex*. Wiley & Sons: New York.
Klein J, Horejsi V 1997. *Immunology*. Blackwell Science: Oxford.
Kumar S, Tamura K, Jacobsen IB, Nei M (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.
Kundu S, Faulkes CG (2004). Patterns of MHC selection in African mole-rats, family Bathyergidae: the effects of sociality and habitat. *Proc R Soc Lond B* **268**: 479–485.
Langefors A, Lohm J, Grahn M, Andersen O, von Schantz T (2001). Association between major histocompatibility complex class IIB alleles and resistance to *Aeromonas salmonicida* in Atlantic salmon. *Proc R Soc Lond B* **268**: 479–485.
Lohm J, Grahn M, Langefors A, Andersen O, Storset A, von Schantz T (2002). Experimental evidence for major histocompatibility complex-allele-specific resistance to a bacterial infection. *ProcR Soc Lond B* **269**: 2029–2033.
Lowe A, Harris S, Ashton P (2004). *Ecological Genetics: Design, Analysis and Application*. Blackwell Publishing Ltd: Oxford, UK.
Lund O, Nielsen M, Kesmir C, Petersen AG, Lundegaard C, Worning P *et al*. (2004). Definition of supertypes for HLA molecules using clustering of specifity matrices. *Immunogenetics* **55**: 797–810.
Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Mol Ecol* **7**: 639–655.
McVean G, Awadalla R, Fearnhead P (2002). A coalescent-based method for detecting and estimation recombination from gene sequences. *Genetics* **160**: 1231–1241.
Meyer-Lucht Y, Sommer S (2005). MHC diversity and the association to nematode parasitism in the yellow-necked mouse (*Apodemus flavicollis*). *Mol Ecol* **14**: 2233–2243.
Meyers LA, Bull JJ (2002). Fighting change with change: adaptive variation in an uncertain world. *Trends Ecol Evol* **17**: 551–557.
Nei M, Gojobory T (1986). Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. *Mol Biol Evol* **3**: 418–426.
Nuismer SL, Otto SP (2004). Host–parasite interactions and the evolution of ploidy. *Proc Natl Acad Sci USA* **101**: 11036–11039.
Ohta T (1998). On the patterns of polymorphisms at major histocompatibility complex loci. *J Mol Evol* **46**: 633–638.
Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989a). Detection of polymorphism of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc Natl Acad Sci USA* **86**: 2766–2770.
Orita M, Sekiya T, Hayashi K (1989b). Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics* **5**: 874–879.
Paterson S, Wilson ACC, Pemberton JM (1998). Major histocompatibility complex variation associated with juvenile survival and parasite resistance in a large unmanaged ungulate population (*Ovis aries*). *Evolution* **95**: 3714–3719.
Penn DJ (2002). The scent of genetic compatibility: sexual selection and the major histocompatibiliy complex. *Ethology* **108**: 1–21.
Potts WK, Wakeland EK (1990). Evolution of diversity at the major histocompatibility complex. *Trends Ecol Evol* **5**: 181–186.
Richman A, Herrera LG, Nash D, Schierup MH (2003). Relative roles of mutation and recombination in generating allelic polymorphism at an MHC class II locus in Peromyscus mainculatus. *Genet Res* **82**: 89–99.
Rize WR (1989). Analysing tables of statistical tests. *Evolution* **43**: 223–225.
Robinson J, Waller MJ, Parham P, de Groot N, Bontrop R, Kennedy LJ *et al.* (2003). IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. *Nucleic Acids Res* **31**: 311–314.
Sachs I (1992). *Angewandte Statistik*. Springer Verlag: Berlin.
Sandberg M, Eriksson L, Jonsson J, Sjöström M, Wold S (1998). New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. *J Med Chem* **41**: 2481–2491.
Schad J, Ganzhorn JU, Sommer S (2005). Parasite burden and constitution of major histocopatibility complex in the Malagasy mouse lemur, *Microcebus murinus*. *Evolution* **59**: 439–450.
Schad J, Sommer S, Ganzhorn JU (2004). MHC variability of a small lemur in the littoral forest fragments of southeastern Madagascar. *Conserv Genet* **5**: 299–309.
Schaschl H, Suchentrunk F, Hammer S, Goodman SJ (2005). Recombination and the origin of sequence diversity in the DRB class II locus in chamois (*Rupicapra ssp.*). *Immunogenetics* **57**: 108–115.
Sette A, Sidney J (1999). Nine major HLA class I supertypes account for the vast preponderance of HLA-A and -B polymorphism. *Immunogenetics* **50**: 201–212.
Shriner D, Nickle DC, Jensen MA, Mullins JI (2003). Potential impact of recombination on sitewise approaches for detecting positive natural selection. *Genet Res Camb* **81**: 115–121.
Slate J, Kruuk L, Marshall TC, Pemberton J, Clutton-Brook T (2000). Inbreeding depression influences lifetime breeding success in a wild population of red deer (*Cervus elephus*). *Proc R Soc Lond B* **267**: 1657–1662.
Slate J, Pemberton JM (2002). Comparing molecular measures for detecting inbreeding depression. *J Evol Biol* **15**: 20–31.

Sommer S (2003). Effects of habitat fragmentation and changes of dispersal behaviour after a recent population decline on the genetic variability of noncoding and coding DNA of monogamous Madagasy rodent. *Mol Ecol* **12**: 2845–2851.

Sommer S (2005). The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Front Zool* **2**: 16 (doi:10.1186/1742-9994-2-16).

Sommer S, Schwab D, Ganzhorn JU (2002). MHC diversity of endemic Malagasy rodents in relation to range contraction and social system. *Behav Ecol and Sociobiol* **51**: 214–221.

Soulsby EJL (1982). *Helminths, Arthropods and Protozoa of Domesticated Animals*. Lea & Febiger: Philadelphia.

Southwood S, Sidney J, Kondo A, del Guercio M-F, Appella E, Hoffman S *et al.* (1998). Several common HLA-DR types share largely overlapping peptide binding repertoires. *J Immunol* **160**: 3363–3373.

Stear MJ, Bairden K, Duncan JL, McKellar QA, Park M, Strain S *et al.* (1997). How hosts control worms. *Nature* **389**: 27.

Stear MJ, Bishop SC, Doligalska M, Duncan JL, Holmes PH, Irvine JI *et al.* (1995). Regulation of egg production, worm burden, worm length and worm fecundity by host responses in sheep infected with *Ostertagia circumcincta*. *Parasite Immunol* **17**: 643–652.

Stockwell CA, Hendry AP, Kinnison MT (2003). Contemporary evolution meets conservation biology. *Trends Ecol Evol* **18**: 94–101.

Sturn A, Quackenbush J, Trajanoski Z (2002). Genesis: cluster analysis of microarray data. *Bioinformatics* **18**: 207–208.

Sunnucks P (2000). Efficient genetic markers for population biology. *Trends Ecol Evol* **15**: 199–203.

Suzuki Y, Nei M (2004). False positive selection identified by ML-based methods: examples from the *Sig1* gene of the diatom *Thalassiosira weissflogii* and the *tax* gene of a human T-cell lymphotropic virus. *Mol Biol Evol* **21**: 914–921.

Takahata N, Nei M (1990). Allelic genealogy under overdominant and frequency-dependent selection and polymorphism of major histocompatibility complex loci. *Genetics* **124**: 967–978.

Trachtenberg E, Korber B, Sollars C, Kepler TB, Hraber PT, Hayes E *et al.* (2003). Advantage of rare HLA supertype in HIV disease progression. *Nat Med* **9**: 928–935.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004). MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol Ecol Notes* **4**: 535–538.

van Tienderen PH, de Haan AA, van der Linden G, Vosman B (2002). Biodiversity assessment using markers for ecologically important traits. *Trends Ecol Evol* **17**: 577–582.

Wong WSW, Yang Z, Goldman N, Nielsen R (2004). Accuracy and power of statistical methods for detecting adaptive evolution in protein coding sequences and for identification of positively selected sites. *Genetics* **168**: 1041–1051.

Yang Z (1997). PAML: a program package for phylogenetic analysis by maximum likelihood. *Cabios* **13**: 555–556.

Yang Z, Bielawski JP (2000). Statistical methods for detecting molecular adaption. *TREE* **15**: 496–503.

Yang Z, Nielsen M, Goldman N (2000). Codon-substitution models for heterogeneous selection pressure at amino acid sides. *Genetics* **155**: 431–449.