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Major histocompatibility complex and microsatellite variation in two populations of wild gorillas

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Abstract

In comparison to their close relatives the chimpanzees and humans, very little is known concerning the amount and structure of genetic variation in gorillas. Two species of gorillas are recognized and while the western gorillas number in the tens of thousands, only several hundred representatives of the mountain gorilla subspecies of eastern gorillas survive. To analyse the possible effects of these different population sizes, this study compares the variation observed at microsatellite and major histocompatibility complex (MHC) loci in samples of wild western and mountain gorillas, collected using a sampling scheme that targeted multiple social groups within defined geographical areas. Noninvasive samples proved a viable source of DNA for sequence analysis of the second exon of the DRB loci of the MHC. Observed levels of variation at the MHC locus were similar between the two gorilla species and were comparable to those in other primates. Comparison of results from analysis of variation at multiple microsatellite loci found only a slight reduction in heterozygosity for the mountain gorillas despite the relatively smaller population size.

Keywords: demography, gorilla, major histocompatibility complex, microsatellite, noninvasive sampling, PCR errors

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Introduction

Genetic studies of wild animals often employ neutral markers, such as mitochondrial DNA (mtDNA) or microsatellites, to estimate the amount of variation present in both individuals and populations (Bruford & Wayne 1993; Avise 2000). This information can be used to examine the dispersal patterns of individuals (Fabiani *et al*. 2003), the structure of genetic variation among populations of a species (Paetkau *et al*. 1997), and the distribution of reproduction and relatedness among individuals within populations (Avise *et al*. 2002), topics that help in the formation of a comprehensive understanding of a species' biology. However, 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 change (Meyers & Bull 2002; van Tienderen *et al*. 2002), issues of particular relevance for conservation (Crandall *et al*. 2000). In some cases, the time span between the separation of populations might even be too short to leave a signal at neutral loci so that differences between populations are only detectable at genes under selection (Cohen 2002; Koskinen *et al*. 2002), such as those of the major histocompatibility complex (MHC).

The genes of the MHC produce molecules that are an integral part of the vertebrate immune system (Doherty & Zinkernagel 1975). They are the most polymorphic loci in the vertebrate nuclear genome (Robinson *et al*. 2003). Studies on the evolution of MHC genes in humans and a variety of other species have found significantly more nonsynonymous than synonymous substitutions at amino acids of functional importance in these loci, and this high occurrence of amino acid replacement has been attributed to positive (diversifying) selection (Hedrick & Thomson 1983; Hughes & Nei 1989; Bernatchez & Landry 2003; Garrigan & Hedrick 2003). Pathogen pressure is presumed to be the selective force promoting MHC diversity, and although a

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general link between levels of MHC variation and disease resistance has been difficult to demonstrate (Hedrick & Miller 1991; Gutierrez-Espeleta *et al*. 2001), some studies in humans have shown associations between particular variants and resistance to malaria (Hill *et al*. 1991; Hill 1999), hepatitis C (Thursz *et al*. 1999), tuberculosis (Jepson *et al*. 1997) and acquired immune deficiency syndrome (Carrington *et al*. 1999). More generally, the fitness benefits associated with variation at the MHC are underscored by studies suggesting a higher prenatal loss of offspring with reduced MHC heterozygosity (Knapp *et al*. 1996; Black & Hedrick 1997; Dorak *et al*. 2002), as well as increased reproductive success of male macaques that are heterozygous at an MHC locus (Sauermann *et al*. 2001). It has been suggested that species exhibiting low MHC variation might have a higher susceptibility to infectious disease (Evermann *et al*. 1988; Mikko *et al*. 1999), which is of particular concern for endangered species living in small, isolated populations which already face a significant threat of extinction from exposure to pathogens and parasites (Lyles & Dobson 1993; Murray *et al*. 1999; Lafferty & Gerber 2002).

While much is known concerning the process of evolution of MHC loci in humans and other primate species from studies using captive individuals (Klein *et al*. 1993; Bergström *et al*. 1999; Bontrop *et al*. 1999; Vogel *et al*. 1999), comparatively little research has focused on the amount and pattern of variation found within wild primate populations (but see: Alberts 1999; Nino-Vasquez *et al*. 2000). This is mostly because of problems associated with obtaining suitable samples from wild animals. The impossibility of taking blood samples without disturbing the behaviour and possibly compromising the welfare of wild great apes and other endangered animals has led to the development of techniques utilizing DNA obtained from noninvasively collected samples such as faeces or hair (Höss *et al*. 1992; Morin *et al*. 1994). However, such methods have not yet led to many comprehensive studies of variation at nuclear loci (Taberlet *et al*. 1999), as it has become clear that accurate results can only be obtained after the application of painstaking criteria that add appreciably to the duration and cost of analyses (Taberlet *et al*. 1996; Vigilant 2002). In this study we used DNA obtained from faecal samples to characterize variation at the DRB loci of the MHC class II complex as well as at multiple microsatellite loci in a sampling of individuals from two populations of wild gorillas.

Although considered for most of the 20th century to be a single species exhibiting subspecific variation, gorillas have recently been taxonomically re-classified as two species (Groves 2001). The western gorilla (*Gorilla gorilla*) occurs over a large area in west-central Africa and was estimated to number some 90 000 individuals (Harcourt 1996), although new estimates indicate a tremendous decline in numbers (Walsh *et al*. 2003). In contrast, the eastern gorilla (*Gorilla beringei*) is much less numerous, and the mountain gorilla subspecies (*Gorilla beringei beringei*) occurs in only two isolated localities, each containing fewer than 400 individuals (McNeilage *et al*. 2001; Kalpers *et al*. 2003). Analyses of the variation in mtDNA sequences have reported nucleotide diversity that is an order of magnitude higher in western gorillas than in either of the eastern gorilla species (Garner & Ryder 1996; Jensen-Seaman & Kidd 2001), perhaps reflecting very different demographic histories for gorillas in west and east central Africa. However, those results are probably biased by use of samples from captive western gorillas of unknown, but probably diverse, origins in contrast to intense sampling of geographically restricted populations of eastern gorillas. In fact, a species-level comparison of mtDNA control region sequence variation in western and eastern gorillas produces similar diversity estimates (Bradley 2003; Vigilant & Bradley in press), but the recent finding that application of typical methods of mtDNA sequence analysis to gorillas is likely to produce artefactual results means that too few authenticated gorilla mtDNA control region sequences are available to allow any reliable inter- or intraspecies comparisons (Jensen-Seaman 2000; Thalmann *et al*. 2004).

Very few data exist on comparative levels of variation at nuclear-encoded loci in western and eastern gorillas, with the broadest study to date including only captive western gorillas (Yu *et al*. 2004), and the limited results available do not differentiate the two species (Kaessmann *et al*. 2001; Jensen-Seaman *et al*. 2003). Our goal in this study was to examine multiple highly variable segments of the nuclear genome and contrast the levels of microsatellite and MHC variation observed. To avoid biases caused by population structure, for both species a limited geographical sampling of social groups was used to examine whether the current small size of the mountain gorilla population was associated with a reduced level of diversity at neutral or selected nuclear loci. In this study, we conducted what is, to our knowledge, the first analysis of sequence variation in a multigene family such as the MHC using DNA from noninvasive samples and we evaluated the feasibility and reliability of MHC analysis using such samples.

Materials and methods

Sampling and DNA extraction

Except for the validation study (see below), the DNAs used were derived from samples of wild gorilla faeces. Western gorilla samples were collected at the Mondika Research Station in the Central African Republic and Republic of Congo (site description in: Doran *et al*. 2002). Samples from mountain gorillas were collected in the Bwindi Impenetrable National Forest in Uganda (site description in: Robbins & McNeilage 2003). Mountain gorillas occur in two separate populations, and the Bwindi population is estimated to total some

320 individuals living in a 330-km² area (A. McNeilage, personal communication), while the western gorillas occupy a mostly continuous area of 35 000 km² (Harcourt 1996). The population at Mondika is within this contiguous distribution. The western gorillas analysed using microsatellites include members of 11 social groups and four additional males, all sampled within an area of approximately 50 km², while the mountain gorillas represent eight social groups and five additional males collected from an area of approximately 50 km², plus one additional social group with a home-range of 40 km². Therefore, the numbers of social groups and areas sampled are broadly similar.

Collection of samples occurred primarily at recently vacated nesting sites and occasionally when following gorilla groups. Fresh faecal samples weighing ~5 g were either placed in 50-mL tubes containing 20 g silica gel beads or suspended in 10 mL RNALater (Ambion). Genomic DNA was extracted from 100 mg of dried faeces or 1 mL of faeces solution using the QIAmp DNA Stool kit (Qiagen) as previously described (Morin *et al*. 2001; Nsubuga *et al*. 2004). To monitor contamination, two negative extraction controls were processed along with each set of 10–12 faecal samples. Since faecal samples provide varying amounts of genomic DNA derived from the sampled individual, all DNA extracts and negative controls were subjected to quantitative polymerase chain reaction (PCR) for estimation of the concentration of amplifiable nuclear DNA (Morin *et al*. 2001). This allowed preferential use of extracts containing relatively higher amounts of DNA and adjustment of the number of independent repetitions conducted in the microsatellite analysis. Specifically, all PCRs contained > 25 pg of genomic DNA, an amount shown earlier to be the minimum needed to produce reliable genotypes in microsatellite typing of chimpanzees as well as gorillas (Morin *et al*. 2001). All the samples characterized produced unambiguous, unique composite microsatellite genotypes and were therefore considered to represent distinct individuals. Of these, a subset of samples from 17 western and 19 mountain gorillas was used in the MHC analysis. Some of the individuals chosen for the MHC analysis were known parent–offspring pairs, to facilitate comparisons of observed results with those expected according to Mendelian inheritance patterns. The western gorilla sample included four mother–offspring and three father–offspring pairs while the mountain gorillas included six mother–offspring and six father–offspring pairs. We therefore analysed at the MHC-DRB loci a total of 27 and 26 independent chromosomes from western and mountain gorillas, respectively.

For the purpose of method validation, matched blood and faecal samples were obtained from two captive western lowland gorillas that were resident at the Leipzig Zoo. Their faecal samples were processed as described above, while the blood samples were extracted using the QIAmp Blood Mini Kit (Qiagen) following the manufacturer's instructions.

Amplification of DRB loci

PCR amplification was carried out in a total volume of 20 μL consisting of 1 × PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl) 2 mM $MgCl_2$, 6 μg bovine serum albumin, 200 μM each dNTP, 400 nM each primer, 1 U Ampli*taq* Gold (Applied Biosystems) and sufficient DNA extract (2–4 μL) to ensure a template amount greater than 25 pg. To monitor for contamination, two or more negative controls were included in each set of PCRs. To compare results obtained using different primers, MHC-DRB exon 2 sequences were amplified with three different sets of primers, using for each amplification approximately 50 ng of DNA extracted from blood samples of the two captive individuals. The three primer sets used were: (i) GH46 and 2DRBAMP-B, which were recommended by the International Histocompatibility Working Group for analysis of human DRB polymorphism (Hurley *et al*. 2001) and produce a product of 289 base pairs (bp); (ii) TU68 and TU139, which have been used in a study of gorilla MHC-DRB (Kupfermann *et al*. 1992) and produce a product of 332 bp, and (iii) GH46 and GH50 (Scharf *et al*. 1988), which were shown previously to amplify MHC-DRB in a range of primates (Gyllensten *et al*. 1991a) and produce a 271-bp product. The primers GH46 and GH50 were used for all analyses of wild gorillas. Amplification conditions for all MHC PCRs consisted of an initial denaturation at 95 °C for 3 min, 40 cycles of 90 s at 95 °C, 45 s at 52 °C, and 45 s at 72 °C, followed by 30 min at 72 °C. Following typical practice to avoid spurious results (Fattorini *et al*. 2000; Nino-Vasquez *et al*. 2000), the products of three independent successful amplifications were analysed per individual.

Cloning and sequence analysis

PCR products were separated on a 2.5% agarose gel, then excised and extracted using the QIAEX II Agarose Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. To facilitate ligation into the TA cloning vector, 5 μL of the purified PCR products was mixed with 5 μL of 1 × PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl) 100 μM of adenine deoxyribonucleotide triphosphates and 0.375 U of Ampli*taq* Gold (Perkin-Elmer) and the mixture was incubated for 1 min at 95 °C followed by 30 min at 72 °C. Then 2 μL of the product was cloned directly using the TA Cloning Kit (Invitrogen). The clones were analysed on an ABI 3700 capillary sequencer (Applied Biosystems) after plasmid preparation (colony sequencing, Kilger *et al*. 1997). Multiple clones from each of the three amplifications were sequenced using M13 universal primers, resulting in a total of at least six complete, full-length sequences per amplification for a total of 18–36 sequences per wild individual. For the analyses in which primer pairs or sample types were compared, at least

12 complete sequences were obtained for each of three amplifications.

Sequences were aligned by eye using BIOEDIT v5.0.9 (Hall 1999) and, as a conservative measure against irreproducible results that might result from contamination or *in vitro* recombination, were considered authentic only if they were observed in two or more independent PCRs from a single individual. Some of the clones exhibited one to three sporadic substitutions over the total analysed length of 217 bp, as would be expected because of occasional misincorporations of nucleotides by the *Taq* polymerase (Kobayashi *et al*. 1999), and the identity of any such position was determined using the consensus of at least three sequences.

Since the primers used are expected to amplify exon 2 sequences from multiple DRB loci, subsequent attribution of sequences to particular loci was performed by phylogenetic analysis, using the neighbour-joining method, of the entire 217 bp obtained using primers GH46 and GH50 (Saitou & Nei 1987) using MEGA 2.1 (Kumar *et al*. 2001) with Kimura two-parameter distances (Kimura 1980). Included in the phylogenetic analyses were previously published gorilla DRB alleles available from the ImMunoGeneTics database (Robinson *et al*. 2003). Additional analyses were performed including chimpanzee and human alleles representing the different loci and lineages. Following convention, alleles that grouped together in the tree analyses were assumed to be from homologous loci (Kenter *et al*. 1993). After sequences were assigned to loci, it was possible to use available information concerning the family relationships among the individuals studied to identify haplotypes, which we define as a fixed combination of DRB exon 2 alleles on a single chromosome. MEGA was also used to estimate the number of synonymous nucleotide substitutions per synonymous site (K_s) and the number of nonsynonymous changes per nonsynonymous site (K_n) using the modified Nei and Gojobori method (Nei & Kumar 2000), which assumes rate differences in transitional and transversional changes. These calculations were performed independently for the nucleotides within and outside the antigen-binding-region (Brown *et al*. 1993), and a Z-test (Nei & Kumar 2000) was used to test for positive selection.

Microsatellite analysis

All individuals characterized for MHC variation in this study, as well as additional individuals from both populations, were genotyped at nine microsatellite loci that had been first characterized in humans. These loci had previously been tested on chimpanzees and gorillas and those found to be highly variable within gorillas were used in this study (D1s550, D2s1326, D4s1627, D5s1470, D7s2204, D7s817, D10s1432, D16s2624, vWF; Clifford *et al*. 1999; Bradley *et al*. 2000). Each allele scored at a heterozygous locus was observed from two or more independent amplifications, while a genotype at a locus was considered homozygous only after scoring of multiple amplifications as required by the amount of template DNA present in the PCR and by the rates of allelic dropout observed using gorilla DNA (Morin *et al*. 2001). In practice, nearly all homozygous genotypes were unambiguously observed a minimum of seven times because the estimated amounts of DNA present in the PCRs rarely exceeded 100 pg, the level that would be needed to allow fewer repetitions. Any genotypes for which the criteria could not be met, for example in cases of poor amplification success or insufficient sample material, were considered unknown. Only individuals with complete genotypes at six or more loci were included in the analyses. GENEPOP v3.4 (Raymond & Rousset 1995) was used to check for departure from Hardy–Weinberg equilibrium. The alternative hypotheses of heterozygote deficiency and excess were considered by using the *U*-test (Rousset & Raymond 1995) and a Markov chain method was used to calculate exact *P*-values (Guo & Thompson 1992). This software was also used to test the populations for linkage disequilibrium between all pairs of loci.

We calculated observed (H_O) and expected (H_E) heterozygosities for all loci, as well as individual heterozygosity (H_I), which is the proportion of heterozygous loci per individual (Slate *et al*. 2000). We did not correct individual heterozygosity values for missing data (Coltman *et al*. 1999) for two reasons. First, the pattern of missing data does not noticeably differ between populations and thus is not expected to affect comparisons at the population level. Second, differences in the allelic composition of the loci in the two populations mean that there is no common basis for standardization. The three different types of heterozygosity estimates and the average number of alleles per locus were compared between the populations and were analysed for statistical significance using a *t*-test to assess relative levels of inbreeding.

Family relationships among the subset of gorillas analysed for MHC variation were determined as previously described (Vigilant *et al*. 2001; Bradley *et al*. 2004). In brief, the sharing of at least one allele at every microsatellite locus was confirmed for individuals known to be, or suspected of being, mother–offspring. Within each of the two population samples, all genotyped adult males were considered as possible sires and nonsires were excluded by mismatches at two or more loci, leaving in each case a single male as the assigned father. The requirement of a minimum of two mismatches is a conservative measure to guard against inadvertent exclusion of the true father by a single mismatch possibly resulting from a mutation at these rapidly evolving loci (Ellegren 2000; Mackiewicz *et al*. 2002).

Results

DRB methods assessment

A number of comparative tests were conducted to evaluate the validity of the data obtained using DNA from faecal samples for MHC analysis of gorillas. For one test we used DNA obtained from blood samples of two captive gorillas to compare directly the results obtained using three different primer sets. Using the primers recently recommended for use in humans, GH46 and 2DRBAMP-B (Hurley *et al*. 2001), a total of four and seven sequences were detected for the two individuals, respectively. The second set of primers, TU68 and TU139 (Kupfermann *et al*. 1992), produced only two of those four and four of those seven sequences, and no additional ones. The last primer set, GH46 and GH50 (Scharf *et al*. 1988), produced all four of the sequences from the first individual, and six of the seven sequences previously observed from the second individual, and no additional sequences. The single sequence not detected using GH46 and GH50 was attributed using phylogenetic analysis to the DRB6 locus, which is known to be nonfunctional (Kasahara *et al*. 1992; the alleles detected here are identical to those in their cluster A). These results suggest that the first and third sets of primers work equally well at amplifying functional DRB loci, and because it had not yet been established at the start of this study that GH46 and 2DRBAMP-B were useful in nonhuman primates, we used GH46 and GH50, which were previously used in studies of gorillas (Gyllensten *et al*. 1991a), for all the analyses of wild individuals.

A further test used the primers GH46 and GH50, and compared the results just described to those obtained from DNAs extracted from faeces from the same two captive individuals. For each gorilla, results obtained using faecal DNA were identical to those obtained using blood DNA. There was no significant difference in the frequency of missing alleles in individual amplifications [homozygous individual (three alleles): all six PCRs contained the three alleles; heterozygous individual (six alleles): blood sample produced four, five and six alleles per PCR, faecal sample produced four, five and five alleles per PCR], but nonetheless the issue of missing sequences (allelic 'dropout') was further investigated in the following analysis.

Since extrapolating from results using samples from captives to samples from the wild requires the assumption that all samples perform similarly, we performed another analysis in which we retrospectively assessed the relationship between the proportion of alleles detected using faecal DNA from wild individuals and the number of sequences evaluated. We first identified 16 faecal samples from 16 different wild individuals for which the maximum possible number of DRB alleles, six, had been observed. We chose to analyse individuals possessing six alleles because we presumed that using the maximal number of loci (three) would produce the greatest chances of observing allelic dropout. We then plotted the number of alleles found as a function of the number of cloned sequences analysed from single amplifications (Fig. 1). This revealed that in this worst-case scenario, in which six different alleles were present in an individual, analysis of at least 10 sequences from each of two amplifications, or eight sequences from each of three amplifications, was necessary to obtain all alleles with confidence ($P = 0.029$ and $P = 0.036$, respectively).

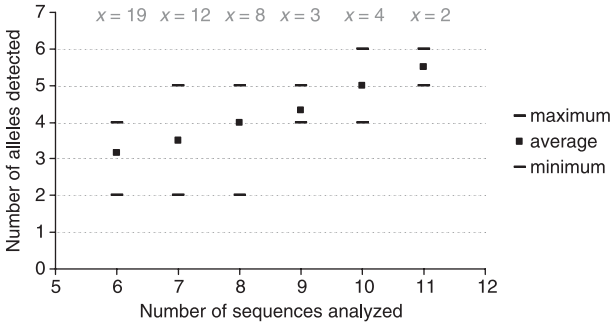

Fig. 1 The probability of detecting alleles in relation to number of sequences analysed for a single PCR. For the 16 individuals for which the maximum number of alleles, six, was expected, we compared for each of the three PCRs the number of alleles detected in relation to how many sequences had been analysed. The numbers across the top indicate the total number of independent PCRs analysed for each category.

In the study of DRB variation in wild gorillas, we used a minimum of six sequences from each amplification for a overall minimum of 18 sequences. In not all cases did we exceed 24 sequences, the minimum needed according to the worst case scenario described above, because in many cases we were analysing known parent–offspring pairs. This is relevant because of the nature of variation at DRB. Because multiple loci are closely linked and the pattern of variants on a single chromosome defines a haplotype, in cases in which a parent or offspring of an individual in question is already characterized, it is possible to infer the necessary haplotype of the relative. In our analysis, no mutational differences were observed in the alleles shared between parent and offspring. In the limited number of cases (11/72 haplotypes) in which only two of three alleles of a haplotype were detected in the individual being analysed, the remaining allele was inferred from the matching haplotype that was completely characterized in the relative. The number of replications performed suggests that the frequency with which a haplotype would have been entirely missed is less than 1%, even for cases in which two haplotypes differed by one allele.

DRB sequence analysis and locus assignment

The 17 wild western gorillas and 19 wild mountain gorillas analysed yielded a total of 656 putative DRB exon 2

sequences. Of these, 61 sequences (9.3%) were unusual because they were always only observed in a small number of sequences from a single amplification and appeared to be a combination of other sequences reproducibly found in the individual analysed.

The 595 remaining sequences were each observed from a minimum of two independent amplifications from a single individual and represented a total of 21 different DRB exon 2 sequences, seven of which have been previously described (Gyllensten *et al*. 1991a; Kasahara *et al*. 1992; Kupfermann *et al*. 1992; Kenter *et al*. 1993). The 14 novel sequences described here and a total of 37 published sequences (Robinson *et al*. 2003) of this segment of the gorilla DRB exon 2 that have been attributed to loci were used in the phylogenetic analysis. We excluded three published sequences (Gogo1*0306, Gogo5*0201, Gogo5*0401) that are probably products of *in vitro* recombination. This occurrence of artificial sequences while typing the MHC-DRB has been both described and demonstrated experimentally by others (Fattorini *et al*. 2000; Longeri *et al*. 2002). The three sequences were excluded for the following reasons: (i) it was not clear if these sequences were observed more than once, (ii) the sequences could be explained as mosaics of other alleles detected in the same respective individuals, and (iii) they would otherwise derive from a locus duplication that has not been detected in other individuals. However, we were conservative and did not exclude two published sequences (Gogo1*0201 and GogoW*1001), even though their unusual phylogenetic affiliations (Fig. 2) suggested that they were recombinants, because we lacked information on other alleles present in the relevant individuals. In the tree analysis, the 14 novel sequences fell within six clusters (DRB1*10, DRB1*03, DRB3, DRB5, DRBW and DRB6) of sequences from known loci (Fig. 2). The distribution of alleles in these clusters was the same when chimpanzee and human alleles are included (results not shown), consistent with previous studies showing high conservation across primates (Klein 1989; Kupfermann *et al*. 1992). The new sequences were given names consistent with the locus assignment suggested by the tree analysis and following consultation with the nonhuman primate group at the international ImMunoGeneTics database (GenBank accession numbers: AJ619785–AJ619803). Even though current taxonomy describes two species of gorillas, since some of the sequences are identical in both taxa we labelled all sequences 'Gogo-DRB' (Klein *et al*. 1990), while indicating in the database entries the species in which they had been detected. Inclusion of the 61 irreproducible sequences in a phylogenetic analysis resulted in odd placements outside the clusters, as would be expected given the recombinant appearance of these sequences.

The inclusion of parent–offspring pairs in the sampling allowed us to infer the combinations of loci occurring on a single chromosome (linkage groups) by following the principle that no two alleles at one locus can occupy one chromosome (Fig. 3). The linkage groups I and III have been previously described from western gorillas (Kasahara *et al*. 1992; Kenter *et al*. 1993), and were observed in our study in both gorilla species, while linkage group II was detected only in western gorillas. We included the DRB6 allele 6*0204 in linkage groups I and II, but prefaced it with a question mark. This is because although the allele was not consistently detected, it was observed in four wild individuals and the DRB6 locus was found in the haplotype of the captive individuals and included in earlier descriptions of gorilla linkage groups (Kasahara *et al*. 1992; Kenter *et al*. 1993). Furthermore, the comparative tests of primers suggested that the primers used in this study tend to miss the nonfunctional DRB6 locus, which is probably the result of evident mismatches in the primer annealing sites (Bergström *et al*. 1999).

Multiple haplotypes, which are defined as particular combinations of alleles on a single chromosome, were found within linkage groups I and III. The total number of haplotypes and their frequency distribution did not differ between the two populations (Table 1). The haplotypes found in the two captive individuals were also compatible with the described linkage groups (Fig. 3).

As in humans (Bergström *et al*. 1999), in gorillas the DRB1 locus contains the most alleles, although we found haplotypes that differed only in alleles at other loci (Fig. 3). Interestingly, the two most similar haplotypes were found in the two different gorilla populations and differed by a single substitution at a single allele (Fig. 3, linkage group III). Considering all loci, the alleles differed at an average of 26.2 of the 217 sequenced positions (range 1–44).

Test for selection at functional DRB loci

A total of 41 sequences of the 217-bp segment of exon 2 were analysed for evidence of selection. Alleles from the nonfunctional locus DRB6 were excluded, as were the three sequences (Gogo1*0306, Gogo5*0201, Gogo5*0401) mentioned earlier.

Amino acid substitutions were found to be disproportionately located in codons that have been identified as antigen-binding sites (Brown *et al*. 1993). An average of 12.9 nucleotide substitutions occurred between sequences at these 42 positions, while only 9.6 changes occurred in the remaining 175 positions (31% vs. 6%; $t = -40.3$; $P < 0.001$). Significantly more of the changes at the antigen-binding sites were nonsynonymous than synonymous (ratio of 1.6, $P = 0.02$), which is consistent with the proposed maintenance of high variation by diversifying selection.

Comparison of variation at DRB between western and mountain gorillas

Within each gorilla population sample a total of 13 different alleles was detected (Table 1). These represented four and

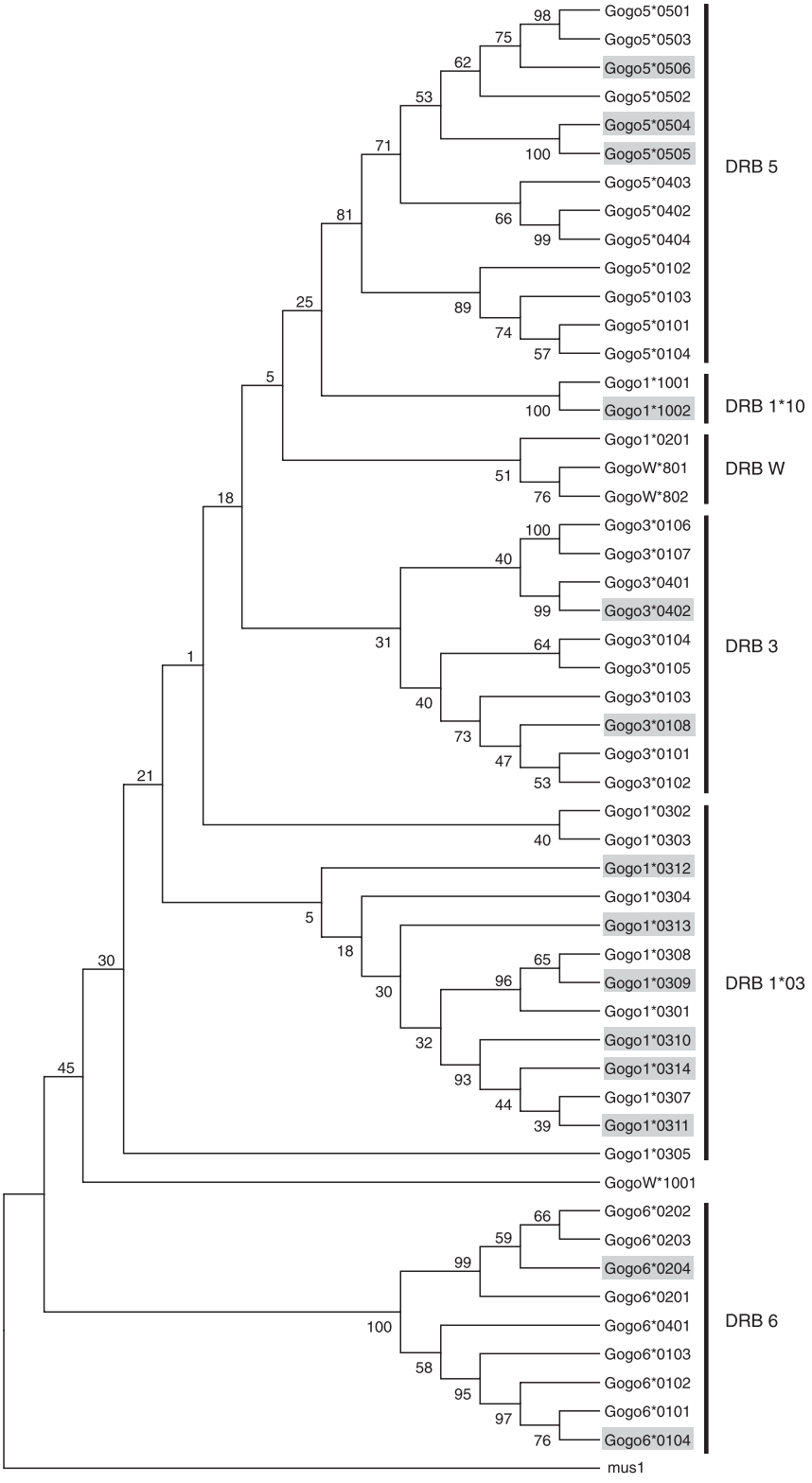

Fig. 2 Phylogenetic tree reconstructed using the 217 bp of exon 2 of 51 gorilla DRB sequences and a mouse Eβ1 sequence as an outgroup. The tree, constructed using the neighbour-joining method with Kimura two-parameter distances, reveals six clusters as indicated. Numbers at branch-points represent bootstrap support values. DRB1*03 and DRB1*10 are on different linkage groups and are therefore expected to have diverged, as is apparent. Grey shading indicates sequences reported in this study.

six haplotypes from 17 western and 19 mountain gorillas, respectively. However, they included 10 mother–offspring pairs and nine father–offspring pairs. This reduces the total number of chromosomes sampled to 27 and 26 for western and mountain gorillas, respectively. A similar average number of 4.6 and 5.0 alleles per individual was detected for western and mountain gorillas, respectively ($t = 0.79$, $P = 0.43$). The observed heterozygosity was similarly high

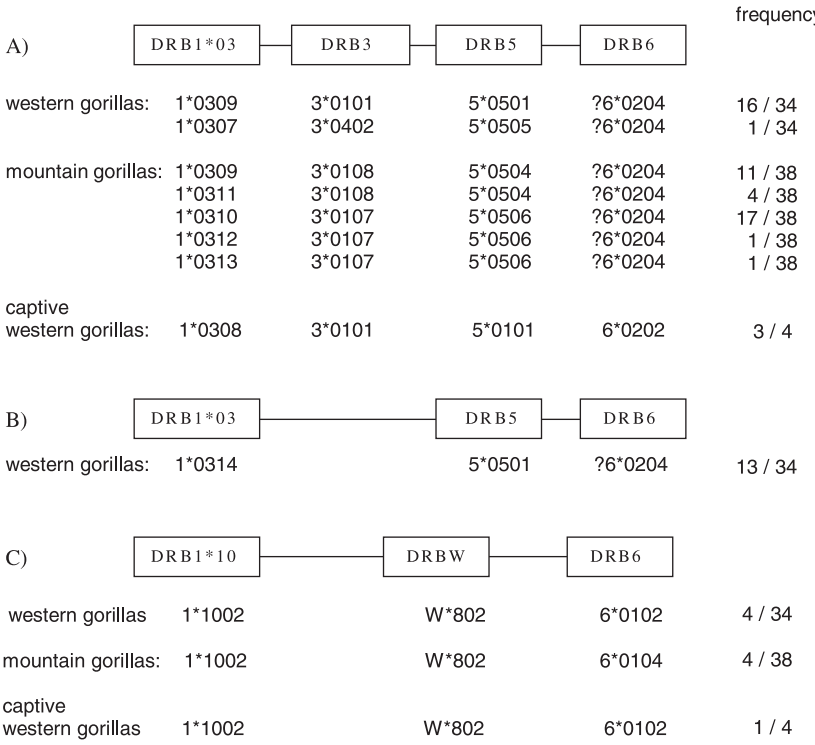

Fig. 3 (A), (B) and (C) represent the three linkage groups, the particular loci comprising a linkage group are shown in boxes and the alleles are listed below each linkage group. The allele DRB6*0204 was detected only once for each of four individuals representing both gorilla species and because the published sequences analysing a longer part of the DRB show several mismatches in the primer annealing site (Bergström *et al*. 1999), we assumed that the allele was present on all haplotypes (indicated with a question mark). The observed chromosomal frequencies of the different haplotypes are indicated.

Table 1 Variation in gorillas at the exon 2 of the MHC-DRB loci

			Number of alleles					
	N	*h*	DRB1	DRB3	DRB5	DRBW	DRB6	Total
All gorillas	54	21	17	10	15	3	9	54
Western*	35	15	13	9	13	3	8	46
Western (this study)	17	4	4	2	3	1	3	13
Mountain	19	6	6	2	2	1	2	13
Shared between the species		0	2	1	0	1	1	5

N, sample size; *h*, number of characterized haplotypes.
*Data from this study and the ImMunoGeneTics database (Robinson *et al*. 2003).

in both samples, with 13 of the 17 western (average H_O = 0.76) and 14 of the 19 mountain gorillas (average H_O = 0.74) exhibiting two different DRB haplotypes ($t = -0.19$, $P = 0.85$).

Comparison of microsatellite variation between western and mountain gorillas

A total of 66 western and 69 mountain gorillas were typed at six or more of the nine microsatellite loci and the genotypes were on average 87 and 90% complete for western and mountain gorillas, respectively. The complete microsatellite data sets are available from the authors upon request. Deviation from Hardy–Weinberg equilibrium was observed in western gorillas, with two loci showing a heterozygote excess for the western gorillas after Bonferroni correction. In western gorillas we also found four cases of significant linkage disequilibrium between single pairs of loci, but in a nonlinear manner. These results were probably caused by sampling related individuals because when we did the same calculations using only a single generation (defined as individuals that have produced offspring, a total of 14 males and 15 females), no deviation from Hardy–Weinberg or linkage equilibrium was found.

Observed and expected heterozygosities and the average number of alleles were calculated for western and mountain gorillas using both the complete data sets and

Table 2 Comparison of measures of microsatellite variation in regional samples of gorillas

	N	*A*	H_O	H_E	H_I
Western					
all	66	6.71[a]	0.80	0.73	0.80[c]
subset	17	5.22[a]	0.84[b]	0.75	0.85[d]
Mountain					
all	69	5.89	0.67	0.66	0.69[c]
subset	19	5.00	0.66[b]	0.68	0.67[d]

N, sample size; 'subset' includes the individuals also typed at the MHC DRB; *A*, average number of alleles; H_O, H_E, H_I, observed, expected and individual heterozygosities, respectively.
[a,b]comparison significant at $P < 0.05$ and [c,d]comparison significant at $P < 0.001$ between values that share the same superscript letter.

reduced data sets, which included only the individuals that were analysed for DRB variation. This allowed us to determine to what extent the smaller set of individuals characterized for MHC variation were representative of microsatellite variation in the larger sample. Comparison between the complete data sets revealed that although the diversity measures were greater in the western than in the mountain gorilla sample, the differences were not significant (Table 2; H_O, $t = 1.77$, $P = 0.10$; H_E, $t = 1.44$, $P = 0.17$; number of alleles, $t = 1.15$, $P = 0.27$, respectively). The average individual heterozygosity for western gorillas was 0.80, as compared to 0.69 for mountain gorillas, and this difference was significant ($t = 4.04$, $P < 0.001$). Thus, using this set of microsatellite markers, our sample of western gorillas had higher estimates of diversity than the sample of mountain gorillas, but the difference was small and only statistically significant when a substantial number of comparisons were made by comparing the average heterozygosities across individuals rather than just across loci.

Direct comparison using just the two sets of individuals sequenced at DRB also revealed reduced microsatellite variation of mountain gorillas relative to western gorillas (Table 2). Although the average number of alleles was similar, the average observed and individual heterozygosities of western gorillas were significantly higher (H_O, $t = -2.39$, $P = 0.029$; H_I, $t = 4.82$, $P < 0.001$).

When we compared the results of the reduced data set to those of the complete data set *within* each population, we found for the western gorillas a significantly lower number of alleles per locus (5.22 vs. 6.71, $t = 2.25$, $P = 0.04$). In contrast, the subsample of mountain gorillas typed at DRB contained a high proportion of the average number of alleles (5.00 vs. 5.89 alleles per locus, $t = 1.51$, $P = 0.15$).

In summary, the results suggest that western and mountain gorillas exhibit similar levels of variation at these microsatellite loci, but a higher amount of genetic uniformity was observed within the mountain gorillas, as indicated by the significantly lower levels of individual heterozygosity. In addition, the subsample of western gorillas analysed for MHC variation slightly under-represents the microsatellite variation seen in the complete western gorilla sample.

Discussion

As anticipated, the use of low concentration DNA templates extracted from noninvasive samples made the acquisition of data on both MHC and microsatellite variation challenging. The majority of the PCRs contained less than 100 pg of template DNA, and allelic dropout, the amplification of only one of two alleles at a heterozygous locus, was observed. In the case of the microsatellite analysis, dropout rates were similar to those observed previously and while extensive replication of genotyping served to ensure accuracy of results (Morin *et al*. 2001; Bradley *et al*. 2004), it was not possible to genotype all individuals completely because of amplification failures and finite sample materials. Our examination of the relationship between the number of MHC sequences analysed and frequency of undetected alleles suggests that researchers relying upon the use of low amounts should include analysis of many sequences from multiple amplifications. This is consistent both with standard practice in microsatellite genotyping, and with recommendations for MHC analysis of humans, where even though comparison with the wealth of already accumulated data facilitates typing, several (three for allele-level typing) independent PCRs per sample are recommended for full resolution (Hurley *et al*. 2001). As dropout was expected to be present in this study, we included analysis of related individuals, and so were able to arrive at substantiated conclusions regarding MHC variation in each of the individuals examined.

Nearly 10% of the individual MHC-DRB sequences examined were only observed once and always appeared to be a combination of other sequences that were repeatedly found in the analysed individual. These can be explained as instances of jumping PCR, a phenomenon in which the polymerase switches between template molecules during the PCR, resulting in the production of a molecule that is a hybrid between two or more different sequences. Although such template switching was noted in early descriptions of PCR applications (Saiki *et al*. 1988), it had been thought to primarily affect analyses of DNA containing polymerase-blocking lesions, such as DNA extracted from ancient samples (Pääbo *et al*. 1990). However, researchers have more recently reported the occurrence of jumping PCR under a variety of experimental conditions, particularly when an excessive number of cycles are used in the PCR (Judo *et al*. 1998). Concern regarding the low amount of template present in the PCRs led us to use 40 cycles in the amplifications of the MHC-DRB loci, and the proportion of apparent recombinants we observed was similar to that described

from the use of damaged DNA from forensic samples (Fattorini *et al*. 2000). We suggest that the occurrence of such artefacts could be reduced by using the minimum number of cycles necessary to produce a visible product (Zylstra *et al*. 1998; Thompson *et al*. 2002), and if necessary by conducting a second 're-amplification' so that additional cycles can be performed in a new reaction mixture that does not have depleted primers (Meyerhans *et al*. 1990). It is worth noting that criteria used by other researchers to evaluate MHC locus sequences might not have excluded recombinants from consideration as real alleles (Gyllensten *et al*. 1991b; Kenter *et al*. 1993; Go *et al*. 2002).

Including the results reported here, a total of 21 haplotypes at the MHC-DRB loci have been described from 54 gorillas. For the western gorilla species, even though this study doubled the number of individuals analysed from 18 to 35, we added only four to the 11 previously described haplotypes (Table 1). This may be an effect of regional sampling, with our study of one population including related individuals while previous studies analysed captive animals of various, unknown origins. By comparison, a sample of 35 unrelated captive western chimpanzees of unknown origin exhibit 16 haplotypes (Kenter *et al*. 1992), and 100 humans from an isolated traditional society show 13 haplotypes (Titus-Trachtenberg *et al*. 1994), while a total of 430 haplotypes have been described to date from humans (Robinson *et al*. 2003). The linkage groups, as well as some of the alleles, are identical between the two species of gorilla. This is consistent with the reported *trans*-species mode of evolution of the MHC (Klein 1989), in which selection acts to conserve alleles for long periods of time, in this case for more than the 2 million years suggested for the split between the two gorilla species (Jensen-Seaman *et al*. 2003). As expected from results in many taxa (Wenink *et al*. 1998; Hambuch & Lacey 2002; Hedrick *et al*. 2002; Richman *et al*. 2003) signs of positive selection were detected at the antigen binding sites of the DRB, although this does not indicate whether selection is still ongoing (Garrigan & Hedrick 2003).

Levels of variation in our comparative regional samples of the two gorilla species at both the selected and the neutral loci were similar and concordant, as has been reported in other species that have not undergone substantial demographic changes (Boyce *et al*. 1997; Hedrick *et al*. 2001; Landry & Bernatchez 2001). Loss of variation at MHC loci has been observed in wild mammal populations after severe bottleneck events, such as a reduction to fewer than 20–30 breeding individuals (Hedrick *et al*. 1999, 2000; Hoelzel *et al*. 1999; van der Walt *et al*. 2001; Smulders *et al*. 2003), as well as in isolated island populations (Seddon & Baverstock 1999). Bottlenecks leave temporary traces, such as altered frequencies of alleles, upon the pattern of microsatellite variation present in subsequent generations (Luikart *et al*. 1998), but detection of such events is difficult in the absence of sampling from periods prior to the suspected bottleneck event or in the case of a gradual decline in population size (Luikart & Cornuet 1999; Larson *et al*. 2002; Maudet *et al*. 2002; Beaumont 2003). Our results suggest that genetic drift in the small population of mountain gorillas has not been severe enough to reduce genetic variation greatly at either the neutral microsatellite loci or the selected MHC locus. The significantly higher individual homozygosity in mountain gorillas can be attributed to the smaller average number of alleles per locus. This lower level of genetic variation is expected in a population of reduced size, but the results taken as a whole do not indicate a major difference in population-level genetic variation between the mountain and western gorillas.

The determination of the influences of genetic variation upon the social behaviour of wild animals is a topic of great interest, and our results suggest that a project using similar methods aimed at investigating the effects of immune system variation upon, for example, mate choice in gorillas could be feasible. One published study examined parentage of almost 600 semi-free-ranging macaques and found evidence for greater reproductive success of males, but not females, heterozygous at the DQB MHC locus although no mating preference associated with DQB genotype was detected (Sauermann *et al*. 2001). Unfortunately, few studies of wild primates would be able to amass samples from sufficient individuals for a powerful study, and the redundancy of the MHC system with its many different functional loci means that the choice of target loci for investigation is not obvious. A recent study in sticklebacks revealed that the interaction between ecological factors and genotypes is complex (Wegner *et al*. 2003), as is the way mate choice might work to enhance genetic variation (Roberts & Gosling 2003).

Finally, very few data exist from other genetic loci with which to compare western and mountain gorillas. Analysis of sequence variation at eight nuclear loci in a limited number of western and eastern gorillas found higher sequence divergence between the two chimpanzee species (chimpanzee and bonobo) than between the two gorilla species (Jensen-Seaman *et al*. 2003). In contrast, a study of sequence variation at a noncoding locus on the X-chromosome did not distinguish western and eastern gorillas (Kaessmann *et al*. 2001). In a study limited to western gorillas, analysis of sequence variation at 50 noncoding nuclear loci in 15 individuals revealed slightly higher nucleotide diversity than found in a world-wide sample of 30 humans or a sampling of 17 chimpanzees (Yu *et al*. 2004). One possible explanation might be greater population substructuring within gorillas as suggested by an excess of intermediate frequency variants, suggesting that further analyses of individuals of known origin, at multiple nuclear loci, are needed to understand the geographical distribution of genetic variation in wild-living gorillas.

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D. Lukas conducted the analyses of MHC variation in wild gorillas whilst he was a visiting student from the University of Münster and the results were the basis of his diploma thesis supervised by N. Michiels. The microsatellite analysis of the western gorillas of Mondika comprised part of B. Bradley's PhD dissertation research on the molecular ecology of western gorillas. A. M. Nsubuga is also using microsatellite data to complete PhD dissertation research on the genetic social structure of the Bwindi mountain gorillas. D. Doran and M. M. Robbins are field researchers interested in the behavioural ecology of gorillas and other primates and in the use of genetic techniques to produce data relevant to the understanding of social behaviour. L. Vigilant is interested in using genetic analyses in combination with observational data to address topics such as the distribution of paternity or patterns of relatedness among individuals in primate social groups and the implications for the evolution of social behaviour.
