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REPLY

Reply: Facts, faeces and setting standards for the study of MHC genes using noninvasive samples

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In a commentary on our recent article (Lukas *et al*. 2004), Knapp (2005) highlights challenges facing studies of the MHC loci. As researchers who have been active in developing and promoting adherence to rigorous guidelines in studies using ancient DNA (Pääbo *et al*. 2004), mitochondrial DNA (Thalmann *et al*. 2004) and microsatellite typing (Morin *et al*. 2001; Bradley & Vigilant 2002), we agree that coherent standards are needed to obtain reliable results. Contrary to the implications by Knapp, in our study of MHC variation in wild gorillas, we employed even more stringent standards than she discusses, and summarize these briefly here.

We suggest that any study of MHC locus diversity in little-studied taxa, and particularly (but not exclusively) those employing low-quality DNA sources such as noninvasively collected samples, should employ a strategy as follows. First, results from different sets of primers should be compared in order to estimate the probability that alleles may have been missed because of mutations in primer annealing sites (Kwok *et al*. 1990; Hurley *et al*. 2001). Second, in order to identify changes arising out of errors in the polymerase chain reaction (PCR), for each individual the sequences of a minimum of two clones, each derived from an independent reaction, should be compared. Although not specified by Knapp, the use of two independent amplification products is important because while the sequencing of multiple clones from a single PCR may reveal erroneous-base changes in single clones (Kobayashi *et al*. 1999), sequences that arise from nucleotide misincorporations or artefactural recombination in PCRs that start from few or single molecules can only be detected by screening more than one reaction (e.g. Fattorini *et al*. 2000; Nino-Vasquez *et al*. 2000). Parenthetically, while Knapp suggests that recombinant products originate from heteroduplex mismatch repair during cloning (Longeri *et al*. 2002), a substantial body of work strongly implicates artificial recombination occurring during the PCR as a likely source of such artefacts (Meyerhans *et al*. 1990; Huang & Jeang 1994; Odelberg *et al*. 1995; Bradley & Hillis 1997; Judo *et al*. 1998; Zylstra *et al*. 1998; Thompson *et al*. 2002). Third, particularly if low-concentration DNA sources such as faeces are employed, quantification of the amount of amplifiable DNA in the sample using a 5′ exonuclease PCR is strongly recommended (Morin *et al*. 2001). As we and others have previously described, the use of low-concentration DNAs in the PCR can result in the stochastic amplification of only one of two alleles at a heterozygote locus and quantification of the DNA allows the establishment of standards for adequate replication (Taberlet *et al*. 1996; Morin *et al*. 2001; Creel *et al*. 2003; Lukas *et al*. 2004).

After determination of confirmed alleles and assignment to the particular loci using phylogenetic analyses (Klein *et al*. 1990), the next step is haplotype construction. As mentioned by Knapp, the most stringent and reliable method is analysis of parents and their respective offspring, and this should represent a fourth criterion for these studies. Contrary to the statement by Knapp, in our study we characterized seven offspring with both parents (some with multiple offspring). The haplotypes we constructed based on these three-way comparisons were consistent with results from an earlier study in which overlapping cosmids were sequenced using high-quality DNA from the blood of a single captive gorilla (Kasahara *et al*. 1992; note that their cosmid clones for cluster A end within an additional locus, which they did not consider in their further analysis). In addition, we agree with Knapp that the haplotypes of an individual should not be inferred by using haplotypes of unrelated individuals because the high mutation rate means that haplotypes can easily differ at any of several loci. As we clearly pointed out, we inferred that single alleles of a particular haplotype were not detected in a few individuals in our study based on the facts that the respective alleles had all been found in the parent or offspring necessarily sharing this haplotype and that we never observed mutations between parents and offspring. As Knapp mentions, new results need to be interpreted in the context of previous publications, and we would add the caveat that the reliability of previous studies must also be evaluated. Prior to our study, data available on haplotypes in gorillas were quite limited, and authors had even made

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cautionary remarks highlighting possible shortcomings of their approach (e.g. in Kupfermann *et al*. 1992: 'the *DRB5* may not have been amplified by chance'). In sum, the numerous safeguards used in our study provide confidence that the overall results and specifically the haplotypes detected in our study represent a consistent addition to the previously limited data.

In conclusion, we concur with Knapp that it is still a major challenge to determine MHC haplotypes from large numbers of individuals from the wild. However, using standards such as those outlined in our study and here, it is possible to generate reliable data that will further our understanding of the functional role of the MHC in natural populations.

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