Molecular Ecology (2001) **10**, 2241–2247

Mitochondrial DNA variation and biogeography of eastern gorillas

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Abstract

Mitochondrial DNA variation in 109 individuals from four populations of wild living gorillas in East Africa was ascertained by sequencing the first hypervariable segment of the control region, or 'D-loop', amplified from noninvasively collected hair and faeces. D-loop haplotypes from eastern gorillas fell into two distinct clades, each with low levels of genetic diversity; most observed haplotypes within each clade differing by only one or two mutations. Both clades show evidence of population bottlenecks in the recent past, perhaps concomitant with the tropical forest reduction and fragmentation brought on by global cooling and drying associated with the last glacial maximum.

Keywords: *beringei*, D-loop, *graueri*, hominid, hominoid, phylogeography

Received 10 January 2001; revision received 7 June 2001; accepted 7 June 2001

Introduction

The climatic history of Africa during the last 1 Myr has been one of change, with warm wet times alternating with cool dry periods (Hamilton 1975; Livingstone 1975; Jolly *et al*. 1997). The lowered temperatures and increased aridity during the Pleistocene glaciations reduced and fragmented tropical forests into island refugia (Livingstone 1967, 1982; Grubb 1982; Haffer 1982; Kingdon 1989). As large-bodied, forest-dwelling primates, the history of gorillas (*Gorilla gorilla*) is expected to be closely tied to the history of the African forests, with the fragmentation of forests potentially fragmenting gorilla populations. Also, as gorillas live at reasonably uniform densities across sites and across habitats (Yamagiwa 1999), population sizes are predicted to decrease in proportion to the decrease in the size of the forests, which may have been reduced to only 5–25% of the present during the last glacial maximum at ≈ 18 000 years ago (Livingstone 1967, 1975; van Zinderen Bakker & Coetzee 1972; Moeyersons & Roche 1982; Chapman 1983).

Gorilla populations are found in equatorial Africa in two widely separated regions: West Africa and East / Central Africa, separated by nearly 1000 km. The eastern gorilla populations (Fig. 1) are usually assigned to the subspecies *G. g. beringei* and *G. g. graueri*, although increasingly they are being referred to as the separate species *G. beringei* (Groves 1996, 2001; Sarmiento & Butynski 1996). The mountain gorillas of the Virungas are the type population of *G. g. beringei*, and today number around 300 individuals (Harcourt 1995). The gorillas of the Bwindi Impenetrable National Park, located ≈ 40 km to the north of the Virungas also number ≈ 300 individuals (McNeilage *et al*. 1998), and have traditionally been placed in *beringei*, although Sarmiento *et al*. (1996) suggest that these gorillas are not the same subspecies as the Virunga gorillas based on morphological data. The isolated population of gorillas found on Mt. Tshiaberimu, numbering only ≈ 16 – 18 individuals (Butynski & Sarmiento 1995), has been placed by Groves (1970) in *graueri*, though later noting they 'tend towards *beringei* in some respects' (p. 168, Groves & Stott 1979). Recently Sarmiento & Butynski (1996) suggested reviving the subspecies *rex-pygmaeorum*, originally proposed by Schwarz (1927), for the Tshiaberimu gorillas. The Kahuzi-Biega populations, usually considered *graueri*, currently account for well over half of all gorillas in East Africa (Hall *et al*. 1998). Schouteden (1947, referenced in Groves & Stott 1979) favoured including the Kahuzi-Biega populations in *rex-pygmaeorum*, restricting the subspecies *graueri* to the type population from the Itombwe Massif west of Lake Tanganyika.

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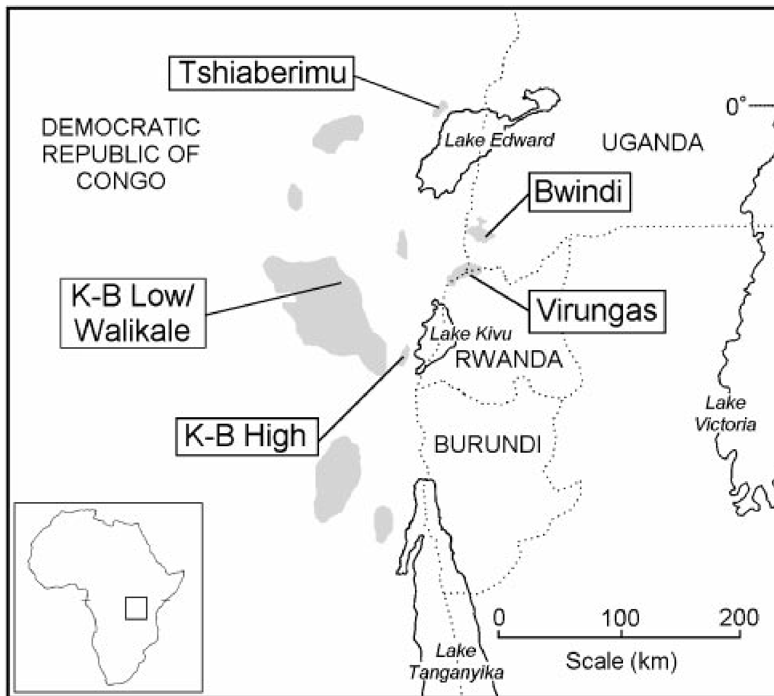

Fig. 1 Locations of populations of gorillas in East Africa sampled in this study. Shaded areas indicate extant populations of gorillas. 'K-B High' and 'K-B Low' refer to the highland and lowland sectors of the Kahuzi-Biega National Park, D. R. Congo. Distributions of populations are taken from Hall *et al*. (1998) and Omari *et al*. (1999).

The maternally inherited mitochondrial DNA (mtDNA) control region, or 'D-loop', is one of the most rapidly evolving, highly polymorphic genetic markers in most mammals examined to date (Avise 2000). Here we examine the patterns of D-loop DNA sequence diversity in several populations of eastern gorillas in order to infer relationships between populations, genetic structure within populations, and past changes in population size. These results are examined in the light of climatic and vegetation changes in Pleistocene Africa.

Materials and methods

Hair samples from vacated night nests were collected from (Fig. 1): Mt. Tshiaberimu, D. R. Congo ($n = 15$); Virunga Volcanoes National Park, Rwanda ($n = 17$); Bwindi Impenetrable National Park, Uganda ($n = 48$). Additional hair samples were obtained from captive gorillas (Houston Zoological Gardens, $n = 1$; Royal Zoological Gardens at Antwerp, $n = 2$). Two of these captive gorillas, 'M'kubwa' (Houston) and 'Mukisi' (Antwerp), were captured in the 1950s in the Walikale region, D. R. Congo, probably from in or near the northern part of the lowland sector of the Kahuzi-Biega National Park. Fecal samples were collected from nests from the highland sector of the Kahuzi-Biega National Park, D. R. Congo ($n = 29$), and stored in 100% ethanol until extraction. The Tshiaberimu samples are from two different identified social groups, and the samples from highland Kahuzi-Biega and the Virungas are from habituated social groups, making it unlikely that the same individuals were repeatedly sampled.

Using a slight modification of a protocol described by Walsh *et al*. (1991), ≈ 5 mm of the root portion of individual hairs was cut directly into 0.5 mL screw-top microcentrifuge tubes, using a fresh disposable scalpel blade and disposable tweezers. To each tube 250 μL of 5% Chelex resin (Bio-Rad, Hercules, CA, USA), 0.039 M DTT, and 0.2 μg / μL proteinase K was added. Tubes were then vortexed briefly, incubated 4–12 h at 55 °C while shaking, vortexed, boiled for 8 min, vortexed, centrifuged for 1 min, and stored at 4 °C. Fecal material with a wet volume of ≈ 300 μL was added to a 1.5-mL screw-top microcentrifuge tube and desiccated in a vacuum centrifuge. To this, 1 mL of 5% Chelex resin was added. Tubes were vortexed thoroughly, boiled 10 min, vortexed, and centrifuged. Proteinase K was added to a final concentration of 0.4 μg/μL, and the tubes were incubated 4–12 h at 55 °C while shaking, vortexed, boiled for 8 min, vortexed, and centrifuged for 1 min. An aliquot of 100 μL of the supernatant was purified with silica-based mini-columns (Qiagen, Valencia, CA, USA) using an elution volume of 50 μL.

For most hair and fecal samples, 10 or 2 μL of the supernatant, respectively, were used as template in a 100 μL polymerase chain reaction (PCR), using the primers ProFor2 (5′-CAGAGAAAAAGTCCTCGACTCCACC-3′) and MidRev4 (5′-TAGGAACCAGATGCCGGATACAGT-3′), with the PCR cycle: 94 °C for 2 min; followed by 10 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s; followed by 30 cycles of 89 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s; followed by 72 °C for 10 min. The reactions contained 1× PCR buffer (Perkin-Elmer), 1.5 mM $MgCl_2$, 200 μM dNTPs,

200 μM each primer and 0.25 μg/μL BSA. This PCR yields products of ≈ 485 bp in gorillas. Alternatively, some samples were amplified in 25 μL reactions with the above primers, followed by re-amplification of an aliquot of this first PCR with the primers ProFor1 (5′-CTCCACCATCAG-CACCCAAAGC-3′) and MidRev2 (5′-TGCCGGATA-CAGTTGATTTTTAGC-3′) in a 100-μL reaction to increase yield for sequencing.

PCR products were purified (Qiagen PCR Purification kit), and sequenced with ProFor1 and MidRev2 using the ABI Prism dye terminator sequencing kit and run on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA, USA). Most individuals were only sequenced with the forward primer. Any individuals with a novel haplotype or ambiguous base were then sequenced with the reverse primer. Sequences were edited and aligned with the LASERGENE software package (DNAStar, Madison, WI, USA). Novel haplotypes have been deposited in GenBank (Accession nos AF089820, AF050738 and AF187549).

To estimate the mutation rate for the region analysed (bases 16041–16391 of the human reference sequence; Anderson *et al*. 1981), an alignment was constructed using published haplotypes from 18 humans (Vigilant *et al*. 1991), 18 West African chimpanzees (*Pan troglodytes verus*) (Deinard & Kidd 2000), 18 Central African chimpanzees (*P. t. troglodytes*) (Morin *et al*. 1994), 18 East African chimpanzees (*P. t. schweinfurthii*) (Goldberg & Ruvolo 1997), 20 bonobos (*P. paniscus*) (Gagneux *et al*. 1999; Deinard & Kidd 2000) and 15 western lowland gorillas (*Gorilla gorilla gorilla*) (Garner & Ryder 1996), along with the 15 eastern gorilla haplotypes that are the focus this study. As gorillas possess a large deletion in the centre of the first hypervariable region, only the two domains flanking it (16041–16165 and 16268–16391 of the human reference sequence) were used in interspecific analyses, so the derived mutation rate was based only on nucleotides homologous in all species. Within *Gorilla*, the entire region was analysed, including a polycytosine stretch, for a total of between 271 and 278 bases. Gaps were included in the analyses, and conservatively weighted equal to transitions, in order to use all available information as is important when the number of observed substitutions is small (Giribet & Wheeler 1999; Simmons & Ochoterena 2000).

Phylogenetic analyses were performed with the help of PHYLIP v3.57c (Felsenstein 1993). Estimates of sequence divergence and transition/transversion ratios were calculated with MEGA v1.02 (Kumar *et al*. 1993). Nucleotide diversity, analyses of molecular variance (AMOVA; Excoffier *et al*. 1992), exact tests of population differentiation (Raymond & Rousset 1995), and pairwise mismatch distribution parameters were estimated with ARLEQUIN, Versions 1.0 and 2.000 (Schneider *et al*. 1997, 2000). All mismatch distributions used one copy of each unique haplotype, in order to avoid artefacts based on the possibility that the same individuals were sampled repeatedly, or that mother–offspring pairs were included in the analyses.

Results

Only 10 different haplotypes were observed in the 112 individuals sequenced, seven of which have been reported previously (Garner & Ryder 1996; Saltonstall *et al*. 1998). Combined with available published sequences, the resulting 15 unique eastern gorilla haplotypes fall into two distinct clades (Fig. 2). One includes all of the individuals from Bwindi and the Virungas. The other includes the individuals from Tshiaberimu, the two populations from Kahuzi-Biega, and the captive individuals. Because these clades correspond to the subspecies *Gorilla gorilla graueri* and *G. g. beringei* defined morphologically by Groves & Stott (1979), or *G. beringei graueri* and *G. b. beringei* if the eastern gorillas are considered a distinct species, we use the subspecies names *graueri* and *beringei* hereafter to refer to these two eastern gorilla clades. No haplotypes were shared between *graueri* and *beringei*, nor were any haplotypes seen previously in western lowland gorillas (Garner & Ryder 1996) found in any of the eastern gorillas.

By calculating the mean number of observed transversions between all pairs of *Homo* and *Pan* haplotypes and assuming these genera diverged 5–7 Ma, we estimated the transversion mutation rate to be 5.97×10^{-9} to 8.36×10^{-9} transversions per site per year. The transition to transversion ratio has been estimated from large numbers of human and chimpanzee sequences to be ≈ 25 (Excoffier & Yang 1999), which is similar to the value of ≈ 19 estimated from the 15 eastern gorilla haplotypes discussed here, but using far fewer substitutions. We therefore estimated the actual rate of transitions to be 25 times the rate of transversions, or 1.49×10^{-7} to 2.09×10^{-7} transitions per site per year. For dating events in eastern gorillas we used only transitions as the number of transversions is small (one transversion between *graueri* and *beringei*, and no transversions within either clade). The mean number of transitions per site between all pairwise comparisons between *graueri* and *beringei* is 0.0660, placing the split between these gorilla mtDNA clades at 316 000–443 000 years ago, but with an unknown, probably large, confidence interval.

The mean sequence divergence between unique haplotypes within *graueri and beringei* is 0.899 and 0.794%, with maxima of 1.81 and 1.44%, respectively. Nucleotide diversity (π), estimated from all available individuals, is 0.660 and 0.580% in *graueri* and *beringei*, respectively. These values of π are an order of magnitude smaller than in western lowland gorillas (*G. g. gorilla*; $n = 20$; $\pi = 5.19\%$), estimated herein from previously published sequences (Garner & Ryder 1996), but excluding three haplotypes shown elsewhere to be nuclear pseudogenes by cloning and sequencing multiple highly divergent PCR products from a single

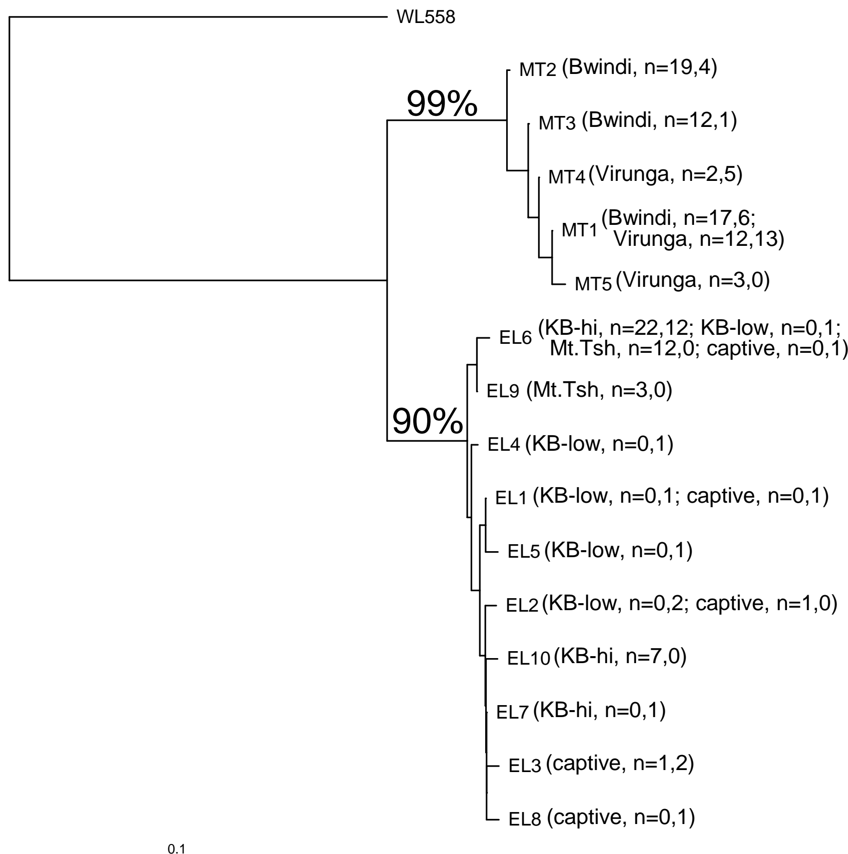

Fig. 2 Neighbour-joining tree of all eastern gorilla D-loop haplotypes, rooted with a single western lowland gorilla (WL558). The simplified sequential haplotype nomenclature of *graueri* (EL) follows that of Saltonstall *et al*. (1998), and is extended to *beringei* (MT), where haplotypes MT1, MT2, MT3 and MT4 are identical to haplotypes MT074, MT045, MT504 and MT374, respectively, described by Garner & Ryder (1996). Locations where each haplotype was found are given in parentheses, followed by the number of individuals with that haplotype in this study (first number) and two previous studies combined (second number) (Garner & Ryder 1996; Saltonstall *et al*. 1998). Distances between haplotypes were estimated using the Kimura 2-parameter model, with a transition / transversion ratio of 25. Bootstrap values based on 10 000 bootstrapped trees are given on the two main branches of eastern gorilla haplotypes.

individual gorilla (Jensen-Seaman 2000; M. I. Jensen-Seaman *et al*. unpublished data), and about one-fourth as large as that estimated from published human sequences ($n = 188$; $\pi = 2.47\%$; Vigilant *et al*. 1991).

No population is reciprocally monophyletic, with all populations sharing at least one haplotype with another population within each subspecies. However, some population structure exists within each subspecies. Results from the AMOVAS demonstrate that 36.2% of the genetic variation within *graueri* and 27.5% of the variation within *beringei* is partitioned among populations, with the remainder within populations. All pairwise population comparisons revealed significant F_{ST} values ($P < 0.001$; permutation method as implemented in the ARLEQUIN package), except the comparison between Mt. Tshiaberimu and the highland population of Kahuzi-Biega ($P = 0.060$). Exact tests of population differentiation (Raymond & Rousset 1995) indicate that all populations differ significantly ($P < 0.01$).

The shallow star-like phylogeny of haplotypes within each eastern gorilla clade (Fig. 2), as well as the unimodal distribution of pairwise mismatches (Fig. 3) suggest a recent population expansion (Slatkin & Hudson 1991; Rogers & Harpending 1992). Values of the mutational parameter τ under the model of Rogers (1995) were estimated to be 2.49 and 2.20 for *graueri* and *beringei*, respectively. Similar estimates

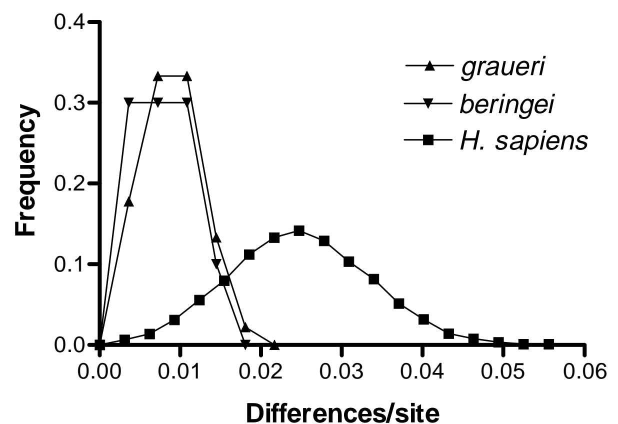

Fig. 3 Mismatch distributions between unique haplotypes seen in eastern gorillas, with humans as a comparison. Human distribution is generated from data in Vigilant *et al*. (1991), and also uses only unique haplotypes. The observed numbers of mismatches for each pairwise comparison were converted to differences per site based on the length of the sequence compared.

of τ (2.65 and 2.38) were obtained using a generalized nonlinear least-squares method (Rogers & Harpending 1992; Schneider & Excoffier 1999). Using the mutation rate derived above and the conversion from τ to time (t) with

the formula $t = \tau/2\,\mu l$, where μ is the mutation rate in units of mutations / site / year and l is the length of the sequence, the time of population expansions in *graueri* and *beringei* are estimated to have occurred around 25 100 and 22 100 years ago, respectively (ranges: 21 500–30 200 and 19 000–26 700, respectively, based on the range of mutation rates), using Rogers' (1995) moment estimator of τ. Slightly earlier dates of 26 700 (22 900–32 100) and 24 000 (20 600–28 800) years ago for *graueri* and *beringei*, respectively, are obtained using the least-squares estimate of τ (Schneider & Excoffier 1999). As these ranges only reflect the uncertainty in the human–chimpanzee calibration point of the mutation rate estimate, they are not a true confidence interval for the time of population expansion.

Discussion

Mitochondrial D-loop haplotypes of East African gorillas fall into two strongly supported clades, corresponding to the morphologically and geographically defined subspecies *Gorilla gorilla beringei* and *Gorilla gorilla graueri*. These two clades meet the major requirement of reciprocal monophyly of mtDNA haplotypes for consideration as evolutionary significant units (ESUs), as proposed by Moritz (1994). No other populations or group of populations meet this requirement, although it must be remembered that several eastern gorilla populations were not sampled, including those of the Itombwe Massif, west of Lake Tanganyika, the type population of *graueri*. The dating of the split between *beringei* and *graueri* mtDNA lineages at ≈ 380 000 years ago is similar to that of Ruvolo (1996), who estimated a date of ≈ 400 000 years ago from the mtDNA COII gene, and is in general agreement with Groves & Stott (1979), who suggested that the dispersal of gorillas from west to east across the Albertine rift was probably much earlier than 100 000 years ago, based on volcanic data. These early dates imply that the splitting of the ancestral population of eastern gorillas into the two present clades was not due to the forest fragmentation concurrent with the most recent glacial maximum, but may have coincided with an earlier Pleistocene arid phase or a vicariance event associated with tectonic or volcanic activity along the rift.

Within each of these subspecies all populations shared haplotypes with others, indicative of recent gene flow. However, there is evidence of significant population differentiation in haplotype frequencies, suggesting that these populations were not entirely panmictic in the recent past. In *graueri*, the most frequent D-loop haplotype in Tshiaberimu was also the most frequent in the highland sector of Kahuzi-Biega, suggesting substantial gene flow or a common female ancestry from Tshiaberimu, the northernmost population of eastern gorillas, to at least as far south as Kahuzi-Biega, perhaps including most or all of the current *graueri* range. Taxonomically, the Tshiaberimu gorillas would seem to have to be placed in *graueri*, consistent with Groves & Stott (1979). Alternatively, if the subspecies *rex-pygmaeorum* is resurrected for the Tshiaberimu gorillas, our data indicate that it should also contain the populations from Kahuzi-Biega, therefore restricting *graueri* to the type population from the Itombwe massif, in agreement with Schouteden (1947, referenced in Groves & Stott 1979), but in disagreement with Groves & Stott (1979). As with the previous study by Garner & Ryder (1996), we find a close relationship between the gorillas from Bwindi and Virunga, suggesting that these populations too were in recent reproductive contact. This genetic similarity is intriguing given their differences in anatomy (Sarmiento *et al*. 1996); perhaps these populations have undergone rapid morphological divergence, assuming that mtDNA is representative of the rest of the genome. Unfortunately, the molecular data cannot provide a good estimate of the time since these populations have had reproductive contact because so few mutations have occurred, but it may be relevant that the whole of the mitochondrial genetic diversity of the Bwindi / Virunga clade would appear to be about one-fourth as old as the diversity in modern humans.

The shallow star-like phylogeny and unimodal mismatch distributions in both *graueri* and *beringei* indicate a recent population expansion, or bottleneck followed by a return to the original population size (Slatkin & Hudson 1991; Rogers & Harpending 1992). These inferred demographic events appear to have occurred simultaneously in both subspecies, suggesting that a common extrinsic factor such as climatic change is likely responsible. The modal numbers of mismatches in these gorilla subspecies are about one-third that of humans, and correspondingly these eastern gorilla subspecies appear to have experienced a population bottleneck much more recently than have humans. The last 100 000 years have seen great climatic fluctuations, with the last glacial maximum at around 20 000 years ago (Livingstone 1975; Brooks & Robertshaw 1990; Hamilton & Taylor 1991). The estimated population expansions in *graueri* and *beringei* beginning at around 20 000 years ago are consistent with population bottlenecks in these subspecies concurrent with the substantial reduction in the size of tropical forests during the last glacial maximum, with all of their existing mtDNA diversity originating in an expansion during the current postglacial. Examination of multiple codispersed African mammals may reveal if these patterns are typical for tropical forest-dwelling species, or if gorillas are unusually susceptible to climatic and vegetational changes because of their large body size and restricted diet (Watts 1984).

There is tremendous uncertainty in the estimated mutation rate; different methods for estimating the mutation rate in the hominoid D-loop have produced disparate results (Howell *et al*. 1996; Parsons *et al*. 1997; Pesole *et al*.

1999). So, although our estimated date of a population bottleneck in eastern gorillas is near the time of the last glacial maximum and consistent with a population decrease concomitant with the associated reduction in tropical forests, the inability to precisely estimate a mutation rate for the D-loop precludes the practice of definitively linking postulated demographic changes to specific environmental changes. The stochastic aspects of mutation and sampling further increase this uncertainty. Finally, we note that the splitting of mtDNA lineages does not necessarily correspond to, but could predate, the separation of the populations.

Acknowledgements

We are indebted to the many people who generously gave their time and effort to collect and donate hair and fecal samples from wild-living populations of gorillas collected during the course of their field work: Dr Esteban Sarmiento (American Museum of Natural History, New York); Dr Thomas Butynski (Zoo Atlanta); Dr Martha Robbins (Institute for Evolutionary Anthropology, Leipzig); Dr Michele Goldsmith (Tufts University, Boston); Dr Andrew Plumptre and Dr Alastair McNeilage (Wildlife Conservation Society, New York); Mbake Sivha (Gesellschaft für technische Zusammensarbeit); and Dr Angela Meder (Berggorilla & Regenwald Direkthilfe, Muelheim). We also thank the Houston Zoological Gardens and the Royal Antwerp Zoo for providing hair samples of their captive gorillas, which proved invaluable to this study. DNA sequences were kindly made available prior to publication by Dr Amos Deinard (University of California-Davis). Helpful comments on earlier drafts of this manuscript were kindly offered by two anonymous reviewers, as well as by Amos Deinard, Tony Goldberg, Richard Lawler, Martha Robbins, Kristin Saltonstall, Esteban Sarmiento, and David Watts. This work was supported by the National Science Foundation (SBR-9900100) to MIJ-S.

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This work is part of a larger study examining the evolutionary genetics of gorillas and their closest relatives, humans and chimpanzees, using mitochondrial and nuclear DNA sequence haplotypes conducted by Michael Jensen-Seaman while a graduate student in the lab of Kenneth Kidd, who for many years has been actively studying the population genetics of humans and nonhuman primates. Jensen-Seaman is currently a postdoc studying comparative genomics in the Human and Molecular Genetics Center at the Medical College of Wisconsin.