

# Mitochondrial DNA Sequence From an Enigmatic Gorilla Population (*Gorilla gorilla uellensis*)

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**ABSTRACT** Although today gorillas are found in only two widely separate, discontinuous western and eastern African populations, rumors of the existence of an additional gorilla population in central Africa have inspired recent unsuccessful field expeditions in search of the “mystery ape” termed *Gorilla gorilla uellensis*. Such a gorilla population would have considerable conservation and scientific interest, and would presumably have descended from a population of gorillas that was thought to exist until the end of the 19th century on the Uele River in the current-day Democratic Republic of Congo. However, the sole evidence for the existence of these gorillas is three skulls and one mandible brought to the Royal Museum for

Central Africa (Tervuren, Belgium) in 1898. We determined a mitochondrial DNA sequence from one of these specimens and compared it to sequences from other gorillas. Contrary to expectations, the sequence obtained did not exhibit the phylogenetic distinctiveness typical of a representative of a peripheral isolated population. Rather, the results suggest a scenario in which the museum specimens did not originally derive from the northern Congo, but were brought from the area of current distribution of western gorillas to that location; the subsequent discovery and collection of the specimens there gave rise to the false inference of a local gorilla population. *Am J Phys Anthropol* 121:361–368, 2003. © 2003 Wiley-Liss, Inc.

Museum collections of animal specimens present an opportunity to assess not only the morphological but also the genetic variation of representatives of populations existing in the past. Comparison of results of genetic analysis of DNA from museum specimens with that from extant populations or species can clarify the systematics of extinct species (Higuchi et al., 1984; Thomas et al., 1989) and allow a direct assessment of changes in genetic diversity and dispersal over time (Thomas et al., 1990; Leonard et al., 2000; Hale et al., 2001). For two reasons, mitochondrial DNA (mtDNA) sequences are predominantly analyzed in studies examining “historical” DNA derived from museum specimens. First, mtDNA is present in a relatively high copy number in cells; therefore, the amplification of mtDNA is usually much easier to achieve than that of nuclear single-copy loci when samples containing degraded DNA are used (Greenwood et al., 1999). Second, portions of the noncoding control region of the mtDNA molecule possess high variability and have been extensively characterized in numerous taxa, and thus mtDNA represents an ideal locus to assess intraspecific genetic diversity. Within a species, peripheral or isolated populations may be expected to be particularly affected by such factors as restricted gene flow, population fragmentation, and isolation by distance. These factors are then typically reflected in an mtDNA gene tree in which deep phylogenetic splits correspond to populations in different parts of the species range (Avise, 2000). Even in the

absence of tree analysis, comparison of interindividual mtDNA genetic distances from such geographically distinct populations reveals greater differences among, as compared to within, populations. Although multiple highly variable nuclear-encoded microsatellite markers could in principle also be used in place of or in addition to mtDNA analysis, the lower copy number of nuclear DNA and the molecular characteristics of these markers make accurate results more difficult to achieve (Morin et al., 2001), while the lack of comparable information from living individuals would complicate interpretation of data from museum specimens.

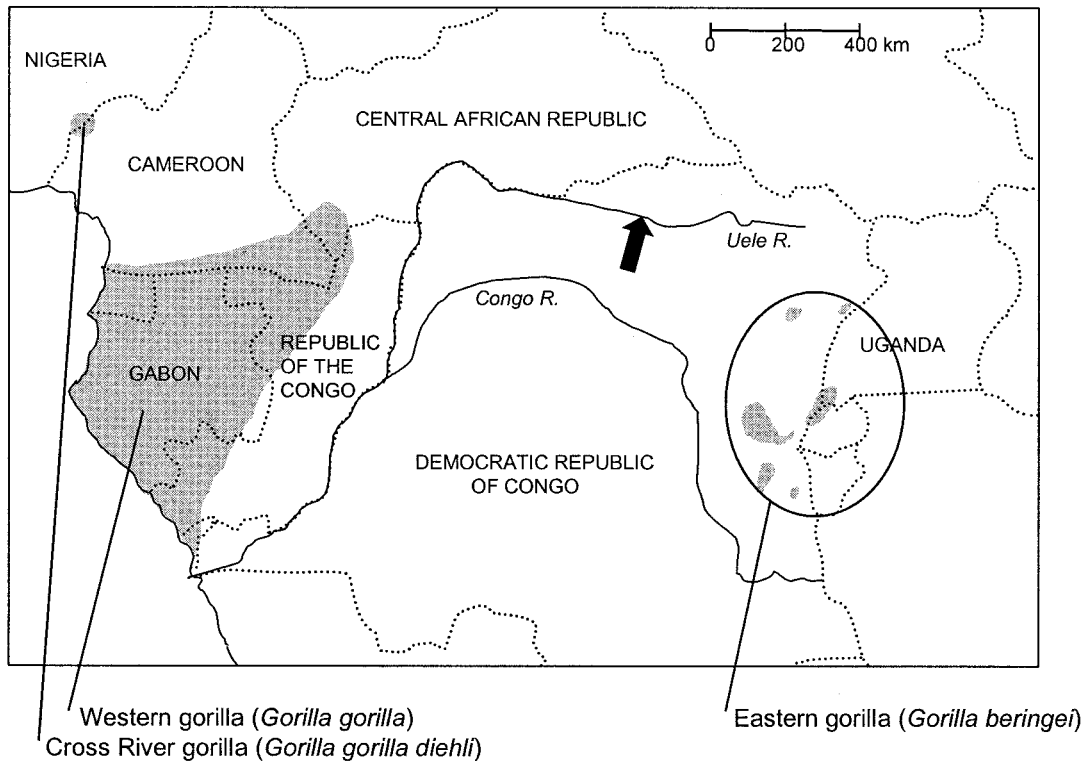
The classification of living gorillas is today in transition, and what has for decades been considered a single species is now classified as two geographically isolated species: the western gorilla (*Gorilla gorilla*) and the eastern gorilla (*Gorilla beringei*) (Fig. 1) (Groves, 2001). Subspecies have

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**Fig. 1.** Approximate current distributions of gorilla populations in Africa are indicated by shading. Arrow indicates origin of *G. g. uellensis* specimens near Bondo on the Uele River.

been described on the basis of assessments that consider morphological, geographic, or genetic distinctiveness as well as conservation concerns. Genetic analyses of mtDNA from gorillas, as well as other segments of mtDNA from gorillas, have produced phylogenetic trees consistent with current taxonomy in depicting a primary split between western and eastern gorilla mtDNA dated to some 2 million years ago, with a more recent divergence about 400,000 years ago between the two eastern subspecies (*G. b. beringei* and *G. b. graueri*; Ruvolo et al., 1994; Garner and Ryder, 1996; Jensen-Seaman and Kidd, 2001). Interestingly, these trees also show deep branches within the western gorilla clade that are suggestive of population structuring, although interpretation of the pattern is hampered by the fact that at present, only five reliable unique control-region sequences have been derived from individuals of approximately known geographic origin, with an additional nine sequences stemming from nine captive individuals of unknown origin. A further three reported western gorilla control-region sequences occupying some of the deepest divergences in a phylogenetic tree (Garner and Ryder, 1996) were convincingly shown to be artifactual, and most likely were inadvertently derived from nuclear insertions of mtDNA (numts) (Jensen-Seaman, 2000).

In eukaryotes, nuclear chromosomes appear to acquire fragments of mitochondrial genomes, which then evolve as nuclear pseudogenes at a slower rate than the original mtDNA counterpart (reviewed in

Bensasson et al., 2001). These numts are not equally prevalent in all animal species, and primates seem to have them in particularly high copy numbers. A recent analysis using the complete draft human genome found a total of 296 numts, with all parts of the mitochondrial genome represented, but with relatively fewer detectable insertions from the control region (Mourier et al., 2001). Cloning and sequencing of polymerase chain reaction (PCR) products and inference of the modes of evolution of the sequences obtained can serve to distinguish numts from genuine mtDNA sequences (Jensen-Seaman, 2000; Bensasson et al., 2001).

Comprehensive surveys of western gorilla genetic diversity are in progress (E.J. Wickings, personal communication), while particularly intense research interest has been focused in recent years on the geographically isolated, fragmented population of western gorillas, termed Cross River gorillas, living on the border of Nigeria and Cameroon (Fig. 1) (Oates, 2001). These gorillas were formerly considered to exhibit morphological variation consistent with that of western gorillas (*G. g. gorilla*) (Groves, 1971), but were recently awarded subspecies rank (*G. g. diehli*) on the basis of preliminary genetic analyses (Bergl et al., 2000), reassessments of morphology (Sarmiento and Oates, 2000; Stumpf et al., 2003), and conservation concerns (Groves, 2001). Other similarly isolated western gorilla populations are not known to currently exist, but in 1898 the Royal Museum for Central Africa registered the ac-

quisition of a collection of three gorilla skulls and a mandible originating near Bondo on the Uele River in the northern region of what is now known as the Democratic Republic of Congo, an isolated location more than 600 km distant from other gorilla populations (Fig. 1). These gorillas were termed *Gorilla gorilla uellensis* (note by Schouteden in Schwarz, 1927), and were reported at the time of collection to be exceedingly rare (Schouteden, 1930), but the remoteness and relatively undisturbed quality of the region has inspired recent unsuccessful searches for evidence of an existing remnant gorilla population (NPR Radio Expeditions, 2001). Here, we show that the mtDNA sequence from one of the Uele River specimens collected in 1898 falls within the genetic diversity of contemporary western gorillas, and we interpret this lack of phylogenetic distinctiveness as evidence against the belief that gorillas lived recently in that region.

### MATERIALS AND METHODS

The *G. g. uellensis* specimen used in this study was a P4 premolar from the lower mandible of specimen 103 in the catalog of the Royal Museum for Central Africa (Tervuren, Belgium). This specimen was chosen because, unlike the two type specimens 100 and 101, it does not have a “smoked” appearance (Coolidge, 1936), and exposure to excessive heat would not be expected to be favorable for the preservation of DNA. However, it was apparent, and has been noted in the literature (Groves, 1971), that this mandible perfectly matches the male type specimen 100, despite the assertion that it was collected at a different locality from a different individual (Schouteden, 1930). Furthermore, correspondence was found at the museum in which Matschie noted already in February 1905 that specimens 100 and 103 represented one individual. The mandible (103) had as original locality “Uelle” according to the correspondence, but the museum specimen register records the locality as “Mobele-Itimbiri.” The following was noted regarding the skull (100) in both the register and correspondence: “trouvé dans une hutte indigène près de Djabbir (Ht Uelle).” This information places the collection locality of the specimens in the region of Bondo in the northern Democratic Republic of Congo.

All work was done in a facility dedicated to the analysis of ancient DNA. DNA was extracted from approximately 40 mg of ground tooth root, using methods previously described (Vigilant et al., 2001). Amplifications were done on an MJ thermocycler with a 3 min activation step at 94°C, followed by 60 cycles of 93°C for 30 sec, 50–63°C for 1 min, 72°C for 45 sec, and a final incubation for 30 min at 72°C. The PCR mix contained 1.25 U AmpliTaq Gold (Perkin Elmer), 1 × AmpliTaq Gold buffer, and final concentrations of 250 μM for each dNTP, 250 pM for each primer, and 2.5 mM MgCl<sub>2</sub> in a total volume of 40 μl. PCR products were visualized on ethidium bromide-stained 2.8% agarose gels (NuSieve GTG). Amplifi-

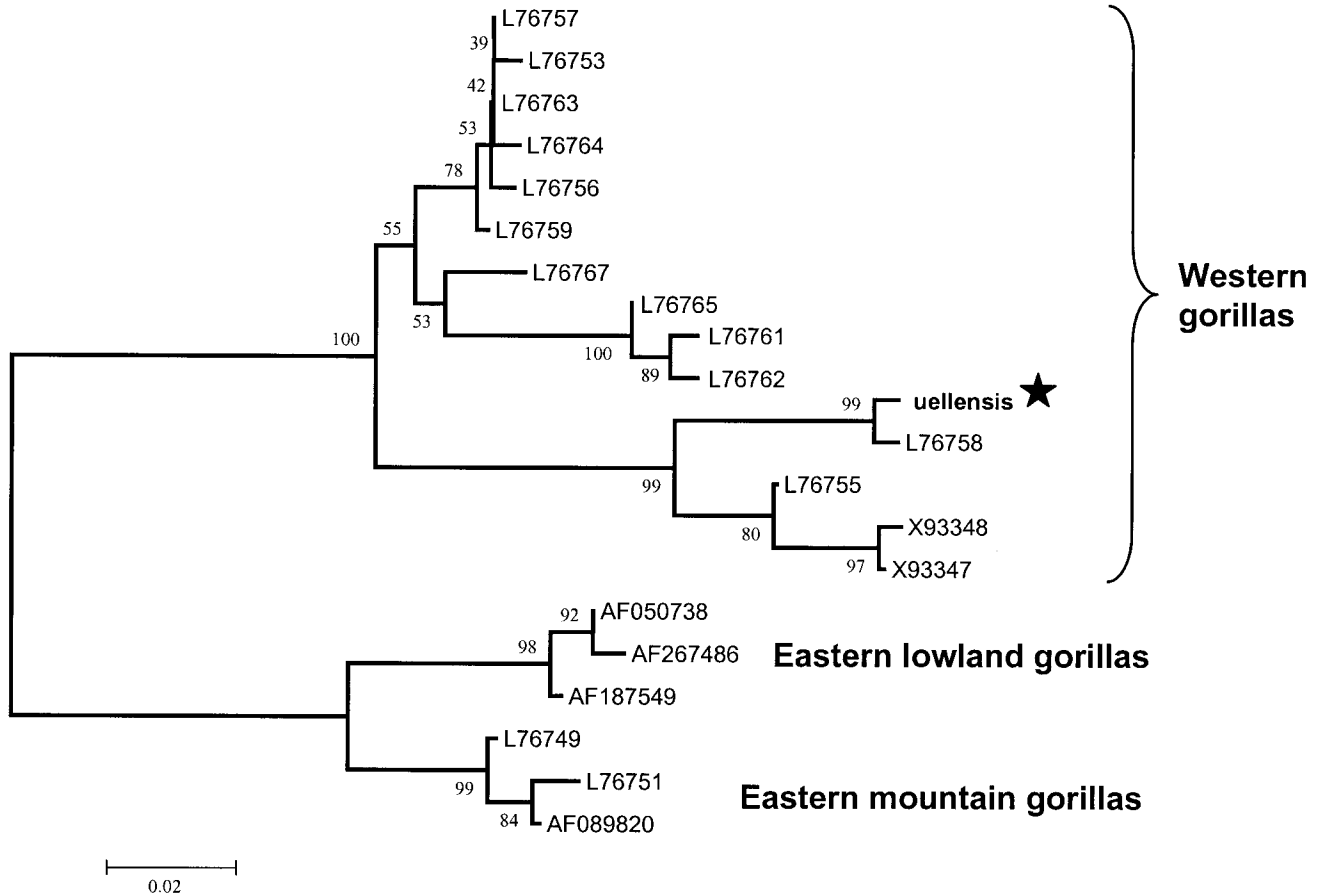
cation primers were designed to produce products of a total length between 90–140 bp, as the degraded condition of ancient or historical DNA prohibits longer amplifications (Hofreiter et al., 2001b). The following primers were used: first segment, L15996g (5'-CTC-CACCATCAGCACCCAAAGC-3') and H16082g (5'-AGGGATGCACGACATACATGATAA-3'); second segment, Bondo 3F (5'-AGTATTGGCTAACCCAT-CAAT-3') and WGR2 (5'-GGGGTTGTATGTGTT-ACAGGT-3'); third segment, Bondo 4F (5'-CGC-ACAGTACCACAAATGTCC-3') and Bondo1R (5'-CGGTAAATGACTTTATGTGTTATG-3'); fourth segment, Bondo 4F and WG R1 (5'-TAGTTGGTAT-TCCGTTGGG-3'); fifth segment, Bondo 5F (5'-GACCACCTGTAACACATACAGCC-3') and Bondo 3R (5'-GTGATTTAACTGAAATGCGCTATGT-3'); sixth segment, Bondo 6F (5'-CCACCCAATGGAAT-ACCAACC-3') and Bondo 3R; and seventh segment, WG F1 (5'-CATAACACATAAAGTCATTTATCG-3') and H16405 (5'-ATATTGATTTACGGAGGAT-3').

All products were cloned directly (without reamplification), using the TOPO TA cloning kit (Invitrogen). Each segment was cloned from a minimum of two separate PCRs, and for each amplification product at least three clones were sequenced. Colony PCR and sequencing were performed as previously described (Bradley and Vigilant, 2002). Positions that initially could not be unambiguously assigned because the clones from two separate PCRs showed consistent differences were resolved by sequencing clones of additional PCR products (Hofreiter et al., 2001a). Sequences were aligned by eye, using the program SeqEd (Applied Biosystems). The sequence obtained by alignment of these overlapping fragments was 334 bp long and begins at the position corresponding to 15997 in the human sequence (Anderson et al., 1981). The sequence from the *G. g. uellensis* specimen has been deposited in Genbank (accession number AJ422244). Phylogenetic analysis was done using the quartet-puzzling algorithm implemented in Tree-Puzzle (version 5.0) (Strimmer and von Haeseler, 1996) and the program package MEGA2 (version 2.1) (Kumar et al., 1993) for neighbor-joining and minimum-evolution tree analyses. Trees were calculated using the TN model for substitution (Tamura and Nei, 1993), assuming gamma-distributed rates with eight rate categories for puzzle trees, and with Kimura-two-parameter corrected distances for tree calculations done in MEGA2.

### RESULTS

Using seven overlapping PCR fragments, we reconstructed the sequence of the first hypervariable region of the mitochondrial control region for the *G. g. uellensis* specimen (Fig. 2). Clones from one amplification of the third segment, indicated in Figure 2, contained many substitutions that could not be verified by sequences from independent amplifications of the same or other overlapping segments. Hence, this set of clones may represent an amplifi-





**Fig. 3.** Phylogenetic tree, relating sequence of *G. g. uellensis* specimen to those previously reported from 14 western and 6 eastern gorillas. *G. g. uellensis* sequence is labelled “uellensis” and is denoted with a star; Genbank accession numbers are used to label other sequences. Numbers at branchpoints indicate % bootstrap support values, based on 1,000 replicates. The sequence alignment used is available from the authors upon request.

cation of a numt, and was not used in deriving the final consensus sequence. The consensus sequence derived for each segment amplified was subjected to a BLAST search (Altschul et al., 1990) in order to determine the most similar sequence in Genbank. With the exception of the anomalous set of clones mentioned above, for which the closest match was a reported numt generated from a gorilla, for each of the consensus segment sequences the closest match was a published gorilla mtDNA sequence (Genbank ID L76758). We were not able to derive an unambiguous sequence for a pyrimidine stretch in the middle part of the sequence. However, long runs of pyrimidines are notoriously difficult to sequence, as

**Fig. 2.** Sequence of first hypervariable region of mtDNA control region derived from *G. g. uellensis* specimen. At top is consensus sequence derived from comparison of sequences of individual clones shown below. At left, for labelling of individual clones, the letter denotes the segment; the first number, the PCR product used; and the second number, the individual clone. As mentioned in text, information from the segment denoted by a bracket with one star was not used in deriving the consensus sequence. The sequence in the bracket denoted by two stars corresponds to the pyrimidine-rich stretch that was omitted from the consensus sequence, shown below and used in the analyses.

DNA polymerases are prone to stuttering and errors in such regions. This region also exhibits considerable length variation in published gorilla sequences. Since length variation can arise from different mechanisms than base substitutions, neither the pyrimidine segment nor some positions exhibiting single-base gaps were used in the phylogenetic analysis.

A 244-bp alignment of sequences from *G. g. uellensis*, 14 western gorillas (*G. gorilla*), 3 eastern mountain gorillas (*G. b. beringei*), and 3 eastern lowland gorillas (*G. b. graueri*) was used to generate an unrooted neighbor-joining tree (Fig. 3). This tree supports the monophyly of eastern mountain gorillas with a 99% bootstrap value, that of eastern lowland gorillas with a 98% bootstrap value, and that of western gorillas with a 100% bootstrap value. The same tree topology, with slightly different bootstrap and support values, was obtained for minimum evolution and Puzzle trees, as well as for trees rooted with the human reference sequence (Anderson et al., 1981) as an outgroup. The *G. g. uellensis* sequence falls within western gorillas and occurs with a 99% bootstrap support value in a clade consisting of itself and the sequences from four captive individuals.

Inquiries were made concerning the geographic origins in Africa of the maternal ancestors of these four individuals. However, the relevant gorillas were imported to North America more than 40 years ago, and no specific locality information could be obtained for these, although some other gorillas imported at that time were reported to originate in Cameroon.

In addition to the tree analysis, we performed an analysis of pairwise genetic distances both within western gorilla mtDNA haplotypes and between those haplotypes and the *G. g. uellensis* sequence. The minimum and maximum pairwise distances within western gorillas are 0 and 28 differences, respectively, within the 244 bp analyzed, whereas there are 2 and 28 differences, respectively, between *G. g. uellensis* and western gorillas. Thus, both phylogenetic analysis and pairwise distance comparisons place the *G. g. uellensis* sequence clearly within the variation of western gorillas.

### DISCUSSION

The authenticity of the *G. g. uellensis* sequence presented here is supported by several lines of argument. First, negative controls to check for contamination by gorilla or other DNA of the extractions and amplifications were always processed along with the bone extract, and never yielded a PCR product. Second, the closest match by BLAST search for the consensus sequences used for each of the individual segments was gorilla mtDNA, with none showing a human sequence, the most common contaminating modern DNA (Hofreiter et al., 2001b). Third, PCR products were not directly sequenced, but rather each PCR product was cloned, and multiple clones were sequenced so that the inconsistent, irreproducible base substitutions characteristic of ancient DNA (Hofreiter et al., 2001a) could be examined. The strategy of sequencing multiple clones from multiple independent PCRs aids in the detection of inadvertent amplification of numts by revealing situations in which multiple classes of products for the same segment are obtained. For example, as mentioned above, we discounted the information obtained from one amplification of the third segment as the results were extremely divergent and not reproducible. The mechanisms favoring the amplification of numts rather than mtDNA are not completely clear, but likely involve the relative amounts of nuclear and mitochondrial DNA present as potential template molecules and the specificity of each individual primer (Kriings et al., 1997). The efficiency of amplifications from the *G. g. uellensis* extract was consistent with the presence of less than 10 copies of template mtDNA molecule per amplification attempt. Since mtDNA is typically an order of magnitude more prevalent than nuclear DNA, there was thus very little nuclear DNA present. The necessity of reconstructing the sequence from overlapping small fragments also reduced the chance that primers of higher specificity for nuclear rather than

mtDNA targets could have been inadvertently used for each and every fragment. Thus, multiple factors support the authenticity of the sequence of the first hypervariable region of the *G. g. uellensis* mtDNA control region.

The existence of an unrecognized gorilla population or subspecies would have considerable implications for gorilla systematics, and if still an extant population, gorilla conservation as well. Since their collection in 1898 and description early in the 20th century (Schouteden, in Schwarz, 1927; Schouteden, 1930), the gorilla specimens from the Bondo region have presented an enigma for primatologists. The area where they were discovered is about halfway between the range of the western and eastern lowland gorillas, suggesting that a population of gorillas at Bondo would represent a remnant of a formerly much larger continuous population.

The sole fact of the geographic isolation of the Bondo gorillas, as well as the suggested subspecies designation *G. g. uellensis*, imply a degree of isolation that would be reflected in a genetic monophyly of the Bondo gorilla relative to gorillas from other regions, as well as a greater genetic distance than that found in comparisons of sequences of individuals from a single continuous region. However, this was not found in the mtDNA analyses presented here. Rather, neither the tree analysis nor the analysis of pairwise sequence differences placed the sequence from *G. g. uellensis* outside the range of variation of contemporary western gorillas. Instead, the sequence falls within a clade containing four sequences from individuals of unknown origin, and has only two mutational differences distinguishing it from one of those individuals. There are two possible interpretations of these results. The first interpretation is that the relevant sequences L76758, L76755, X93347, and X93348 are derived from individuals also originating in the region of Bondo. We consider this unlikely, in view of the lack of evidence for gorillas existing within the past century in this region, but include this as a formal possibility. The second interpretation, which we favor, would be that the four mtDNA sequences of interest are derived from individuals originating within the currently recognized recent range of western gorillas.

As with the genetic analysis, morphological analyses of the three skulls and one lone mandible representing *G. g. uellensis* have failed to describe characters distinguishing them from specimens of western gorillas. In his monumental assessment of morphological variation within gorillas, Coolidge (1929) considered the male *G. g. uellensis* specimen to fall well within the variation exhibited by western gorillas, and more recent analyses concur with this finding (Groves, 1971, 2001). Coolidge (1929, p. 359) speculated that the gorilla specimens collected in the Bondo region had in fact originated hundreds of kilometers away in the eastern part of the range of western gorillas and were brought to Bondo by "Arab traders or wandering natives." He publicly

retreated from this suggestion in the face of assertions by Schouteden (1930) that information provided by the explorer Le Marinel clearly indicated that the specimens were collected in 1908 in the area of Bondo, with one of the specimens (102) actually being shot by a soldier known to Le Marinel (Coolidge, 1936). Coolidge (1936) either chose not to point out or did not actually know that these assertions could not be wholly accurate, since the samples were registered as having been received at the Royal Museum of Central Africa in 1898, 10 years before their supposed collection by Le Marinel. Besides these four specimens, no other evidence for gorilla populations in the region exists, as Coolidge (1936) also investigated in detail and discredited the three anecdotal reports and one picture supporting claims for gorillas existing between the bounds of longitudes 17° E and 28° E, the limits to the usual distribution of western and eastern gorillas. Recent expeditions have failed to find evidence of gorillas in the Bondo area (NPR Radio Expeditions, 2001), and all hair and fecal specimens collected on such surveys have been found to derive from chimpanzees (Gagneux et al., 2001; J. Eriksson and L. Vigilant, unpublished results).

In conclusion, the mtDNA sequence reconstructed from a more than 100-year-old specimen of *G. g. uellensis* closely resembles those obtained from living gorillas originating elsewhere within the recent range of western gorillas (*Gorilla gorilla*). This result supports a scenario in which the *G. g. uellensis* specimens did not originally stem from Bondo, but were transported in the past to Bondo from somewhere within the area of recent western gorilla distribution. This finding, in combination with 1) published morphological analyses that do not distinguish the *G. g. uellensis* specimens from western gorillas, 2) doubts about the reliability of the second-hand reports of the collector, and 3) the lack of any other evidence for gorillas in the region, suggests not only that the gorillas purportedly from Bondo should not be termed *G. g. uellensis*, but that no evidence remains to support the idea of a gorilla population living in recent times in this region.

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