

RESEARCH ARTICLE

Nuclear Integrations of Mitochondrial DNA in Gorillas

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Great ape systematics, particularly at the species level and below, is currently under debate, due in part to the recent influx of molecular data. The phylogenies of previously published mitochondrial control region (or D-loop) DNA sequences in gorillas show deep splits within West African gorillas (*Gorilla gorilla gorilla*), and very high levels of nucleotide diversity in this subspecies. Here we demonstrate that several previously reported D-loop haplotypes from West African gorillas are in all likelihood nuclear integrations of mitochondrial DNA. Revised estimates of the amount and pattern of mitochondrial DNA diversity in gorillas are provided, revealing two reciprocally monophyletic and highly divergent groups of gorillas, concurrent with their geographic distribution. *Am. J. Primatol.* 63:139–147, 2004. © 2004 Wiley-Liss, Inc.

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INTRODUCTION

A clear picture of the distribution of genetic variation in African apes is essential for understanding their evolutionary history, establishing conservation priorities, and examining our own evolution. Gorillas are found discontinuously in African tropical forests from the Atlantic coast to the western rift [Groves, 1971]. The absence of gorillas from the Congo basin effectively separates gorillas into eastern and western groups that are nearly 1,000 km apart. Currently, gorilla systematics is being revised, especially with respect to the exact number of

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species and subspecies involved, and what relationships they share [e.g., Groves, 1986, 1996, 2001; Ruvolo et al., 1994; Sarmiento & Butynski, 1996; Sarmiento et al., 1996; Sarmiento & Oates, 2000]. The notion of separating gorillas into two species (i.e., western gorillas (*G. gorilla*) and eastern gorillas (*G. beringei*)) has recently gained popularity, partly because of studies utilizing mitochondrial DNA data [Groves, 1996, 2001; Morell, 1994; Sarmiento & Butynski, 1996]. This two-species taxonomy is followed herein.

Previous studies that examined mitochondrial DNA (mtDNA) diversity in gorillas showed a deep split between western and eastern gorillas—as large or larger than the split separating chimpanzees (*Pan troglodytes*) from bonobos (*P. paniscus*) [Deinard, 1997; Garner & Ryder, 1996; Jensen-Seaman et al., 2003; Ruvolo et al., 1994]. However, a study in which Garner and Ryder [1996] examined the mtDNA control region, or “D-loop,” also revealed several very deep splits within western gorillas that were approximately as large as that between western and eastern gorillas, and called into question the simple division of the genus *Gorilla* into two main groups. That study included a phylogenetic analysis that suggested that the deepest split in western gorillas may have actually occurred before the split between western and eastern gorillas. A 50% majority rule consensus tree (see Fig. 2 in Garner and Ryder [1996]) failed to demonstrate monophyly of western gorillas. The most common branching (46% of trees) had two western gorilla D-loop haplotypes forming a clade that was the outgroup to a clade comprised of all the remaining eastern and western gorillas. Many authors who have advocated a two-species taxonomy for gorillas in recent years have cited the mtDNA data as evidence of an ancient split between the western and eastern groups, while ignoring the fact that some of the same data show that doing so could create a paraphyletic *G. gorilla*—at least with respect to the D-loop data of Garner and Ryder [1996].

It is imperative that if researchers are to use genetic data to make taxonomic decisions, establish conservation priorities, and infer evolutionary history, the data must be free from artifacts. One source of such artifacts, nuclear integrations of mitochondrial DNA (“numts”), are segments of the mitochondrial genome that have been inserted into the nuclear genome [reviewed in Zhang & Hewitt, 1996; Bensasson et al., 2001]. Numts may be a common occurrence in most eukaryotes, as they have been reported in vertebrates, invertebrates, yeast, and plants [Bensasson et al., 2001; Blanchard & Schmidt, 1995; Gellissen et al., 1983; Hadler et al., 1983; Louis & Haber, 1991; Tsuzuki et al., 1983]. Numts have been identified in many nonhuman primate species [Collura & Stewart, 1995; Mundy et al., 2000; van der Kuyl et al., 1995; Zischler et al., 1998]. Inadvertent PCR amplification of the nuclear copy can confound interpretations if it is assumed that the amplified product is the authentic mitochondrial product [Wallace et al., 1997; Zischler et al., 1995]. The nuclear copy may be preferentially amplified depending on the DNA extraction protocol [Herrnstadt et al., 1999] or tissue type [Greenwood & Pääbo, 1999] used. To determine whether any of the gorilla mtDNA D-loop sequences currently in GenBank are numts, and what effect this may have on our knowledge of gorilla systematics and phylogeography, we reexamined publicly available sequences and compared them with our own data.

MATERIALS AND METHODS

Samples of genomic DNA from eight western lowland gorillas (*G. g. gorilla*) (five of which were generously provided by Dr. O.A. Ryder, San Diego Zoo,

San Diego, CA) were used in a PCR amplification of the first hypervariable segment of the D-loop. The reactions contained standard 1X PCR buffer (1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris, pH 8.4) and the primers D-88 (5'-CTCTGTTCTTTCATGGGAAGC-3') and D-441 (5'-CGGGATATTGATTT-CACGGAGG-3'). The PCR cycle was as follows: 94°C for 4 min, followed by 30 cycles of 94°C for 30 sec, 61°C for 30 sec, and 72°C for 30 sec, followed by a final extension of 72°C for 10 min. The PCR products were purified and sequenced directly with the primers D-88 and D-441 with the use of the ABI Prism sequencing kit, and run on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA).

In addition, genomic DNA was extracted from an ~80-year-old tooth from one male eastern lowland gorilla (*G. b. graueri*) originally from near Lutunguru, Democratic Republic of the Congo (see Acknowledgments for details). We bisected the tooth at the crown line using a high-speed rotary tool equipped with a cutting attachment. Approximately one-fourth of the dried tooth pulp was removed and ground between sterilized stainless steel bolts under liquid nitrogen [Thomas & Moore, 1997]. We extracted the DNA from the powdered pulp using the DNeasy tissue kit (Qiagen, Valencia, CA). The D-loop was amplified with the primers ProFor2 (5'-CAGAGAAAAAGTCCTCGACTCCACC-3') and MidRev4 (5'-TAG-GAACCAGATGCCGATAACGT-3'). The PCR cycle was as follows: 94°C for 2 min, followed by 10 cycles of 94°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec; followed by 30 cycles of 89°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec; followed by a final extension of 72°C for 10 min. The reactions used the same buffer as above, but with the addition of 0.25µg/µl bovine serum albumin (BSA). The PCR products were purified and sequenced directly with the primers ProFor1 (5'-CTCCACCATCAGCACCCAAAGC-3') and MidRev2 (5'-TGCCGGA-TACAGTTGATTTTTAGC-3') as above. Since the resulting sequence appeared to be composed of multiple different sequences, the PCR was repeated and the products were cloned with the TOPO TA-cloning kit (Invitrogen, Carlsbad, CA). Transformation, growth, and isolation of bacterial colonies followed standard protocols [Sambrook et al., 1989]. Twelve individual colonies were picked with the assumption that the majority would possess the authentic mtDNA product. Plasmid DNA was purified (Qiagen miniprep kit), and sequenced with universal forward and reverse M13 primers.

All sequences were edited and aligned with the use of the Lasergene software package (DNASar, Madison, WI). All unique gorilla D-loop sequences available in GenBank at the time of this writing were included in the analyses. Haplotype phylogenies were constructed with PHYLIP [Felsenstein, 1993]. Gaps were ignored, and approximately 30 base pairs (bp) surrounding the polycytosine stretch in gorillas were deleted in order to facilitate alignment with the human reference sequence [Anderson et al., 1981], which was used as an outgroup. Sequence divergence estimates were calculated with the assistance of MEGA [Kumar et al., 1993]. The novel sequences are available as GenBank accession numbers AF240448-AF240458 and AF451954-AF451973.

RESULTS

The 12 clones derived from the eastern lowland gorilla tooth yielded 11 different sequences, abbreviated as LUT clones #1–12. LUT clones #3 and #5 gave identical sequences. In a neighbor-joining tree (Fig. 1), most of the cloned tooth sequences cluster among the three most divergent western lowland gorilla haplotypes (WL287, WL759, and WL495) reported by Garner and Ryder [1996],

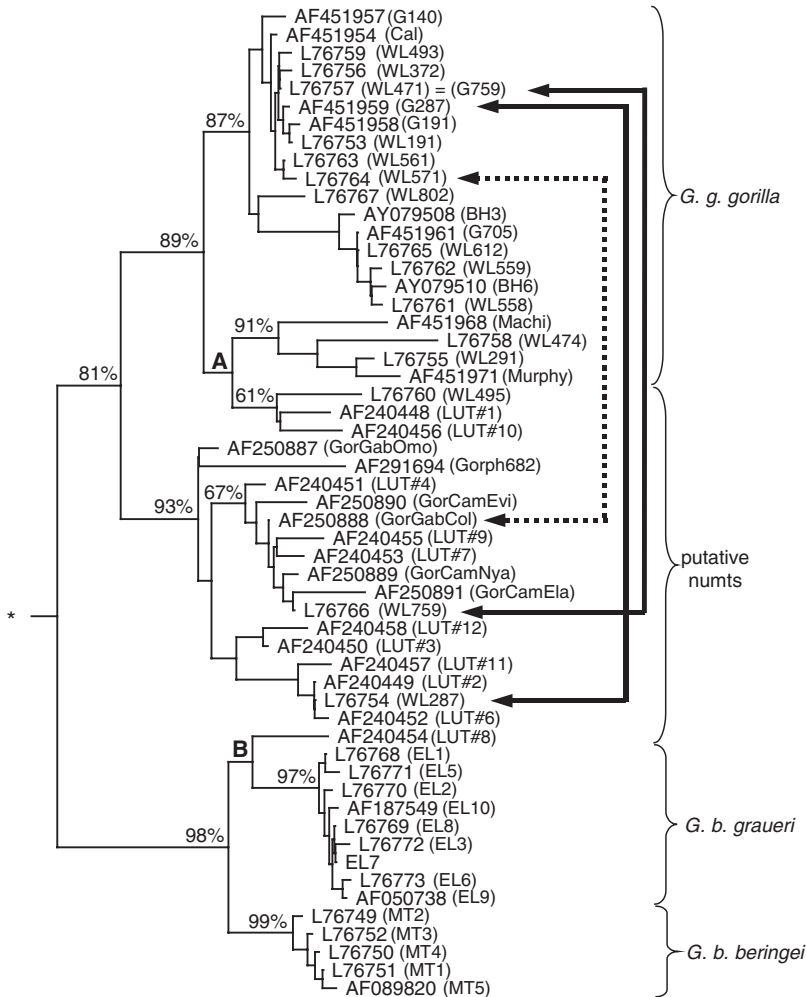


Fig. 1. Neighbor-joining tree of all previously reported gorilla mtDNA D-loop haplotypes, the 11 unique clones from the Lutunguru tooth that are believed to represent numts, and the sequences we obtained from genomic DNA, rooted with the human reference sequence [Anderson et al., 1981] indicated by an asterisk. GenBank accession numbers for all sequences are given, with sequence names in parentheses. Sequences beginning with “WL” are from western gorillas (taken from Garner and Ryder [1996]), those beginning with “Gor” are from western gorillas [Lacoste et al., 2001], and those beginning with “BH” are unpublished western gorilla sequences deposited in GenBank by M.I.J.-S. Eastern gorilla sequence names beginning with “MT” are from the Bwindi (Uganda) and Virunga (Rwanda, Uganda, and D. R. Congo) gorillas; those beginning with “EL” are from eastern lowland gorillas. MT and EL sequences are taken from Garner and Ryder [1996], Jensen-Seaman [2000], Jensen-Seaman and Kidd [2001], and Saltonstall et al. [1998]; and follow the simplified nomenclature for eastern gorilla D-loop haplotypes begun by Saltonstall et al. [1998]. Sequence EL7 was taken from Saltonstall et al. [1998], but is not available in GenBank. The sequence names G287 and G759 are western lowland gorilla sequences we obtained in the present study, using the DNA of the same individuals from which the respective haplotypes WL287 and WL759 originated (solid arrow). Sequence WL571 is from the same individual as sequence GorGabCol (dashed arrow). Bootstrap values (percentage of trees out of 10,000 supporting that node) are shown for major nodes only. Nodes A and B were supported in < 50% of bootstrapped trees. Genetic distances were estimated with the use of a two-parameter model [Kimura, 1980], with a transition/transversion ratio of 15. Other models and parameters did not change the tree in any appreciable way.

and among six western lowland gorilla sequences (GorGabOmo, Gorph862, GorCamEvi, GorGabCol, GorCamNya, and GorCamEla) reported by Lacoste et al. [2001]. LUT clone #9 differs from WL759 and GorGabCol by a single nucleotide substitution. LUT clone #2 differs from WL287 by one substitution and a 2-bp deletion. The sequences we obtained from the DNA of two western lowland gorilla individuals (G287 and G759) were very different than those reported by Garner and Ryder [1996], even though they were from the same individuals, as shown by the solid arrows in Fig. 1. In fact, the sequence we obtained from gorilla G759 was identical to that from gorilla WL471 [Garner & Ryder, 1996]. Furthermore, the sequence GorGabCol was obtained from Cola, a silverback male living at the Centre International de Recherches Medicales de Franceville in Gabon (V. Lacoste, personal communication). This same individual contributed the DNA for accession number L76764 [Garner & Ryder, 1996]. These two sequences from the same gorilla are very different (dashed arrow in Fig. 1), with the GorGabCol sequence clustering with several of the cloned *G. b. graueri* sequences.

DISCUSSION

The most likely explanation for these data is that nine of the previously reported western lowland gorilla haplotypes (WL287, WL759, WL495, Gorph682, GorCamEvi, GorGabCol, GorCamNya, GorCamEla, and GorGabOmo) are numts rather than authentic mitochondrial sequences. We obtained presumably authentic mtDNA D-loop haplotypes from two of the same individuals (G287 and G759) that yielded apparent numts in a previous study. Another individual, Cola, was analyzed independently by two other research groups. In this case, the phylogenetic position (Fig. 1) suggests that the Garner and Ryder [1996] sequence is authentic mtDNA, while the Lacoste et al. [2001] sequence is artifactual. Three individual gorilla samples each produced two different sequences in different laboratories, and therefore we can be confident that one of the sequences must be artifactual. We determined that six additional sequences are likely numts based on their position in the neighbor-joining tree, in that they cluster with several sequences cloned from a single eastern gorilla. Nonetheless, we cannot definitively rule out the possibility that these six sequences may actually be of authentic mtDNA origin. They could be true mtDNA haplotypes that are extremely similar to the mtDNA haplotypes that entered the nuclear genome to become numts. To obtain direct, unambiguous proof that all of the putative numts discussed here are of nuclear origin, one would have to clone them, and the flanking nuclear sequences, directly from the nuclear genome of a gorilla.

It is quite possible that several of the cloned sequences reported here that differ from one another by only one or two substitutions are from the same numt insertion, but differ because of polymerase error or autosomal allelic variation. They may also have resulted from independent integrations of similar mtDNA haplotypes into the nuclear genome, or from recent duplications of nuclear DNA. We believe that LUT clone #8 is derived from a recently translocated numt, and does not represent an authentic mitochondrial haplotype, since further attempts to amplify and sequence the D-loop from the Lutunguru tooth DNA extract yielded a sequence that appeared similar to haplotype EL6 but contained too many ambiguous nucleotides to be included in the analyses. Therefore, it is likely that the true authentic mitochondrial D-loop haplotype from the Lutunguru individual falls somewhere within the cluster of the 10 eastern lowland gorilla (EL) haplotypes. Amplification and sequencing of a portion of the mtDNA ND5

gene indicated that the Lutunguru individual is definitely an eastern lowland gorilla (data not shown).

It is remarkable that so many (nine of 23) of the western lowland gorilla mtDNA D-loop sequences previously deposited in GenBank appear to be nuclear pseudogenes. It is difficult to speculate why such a high rate of artifacts is seen in gorillas. Amplification of numts, as opposed to the authentic mtDNA, likely varies idiosyncratically depending on the laboratory methodologies used, including DNA extraction techniques, choice of tissue, and primer design [Greenwood & Pääbo, 1999; Herrnstadt et al., 1999]. Also, some species may simply possess more numts than others [Bensasson et al., 2001]. Recent analyses of the human genome sequence have revealed ~300–600 numts, several of which include the entire D-loop [Hazkani-Covo et al., 2003; Mourier et al., 2001; Tourmen et al., 2002; Woischnik & Moraes, 2002]. It is also unclear why so many numts were obtained from the eastern gorilla tooth, especially with the greater number of copies of mtDNA over nuclear DNA in most cells. Perhaps this individual's true mtDNA D-loop sequence contains a rare substitution at one of the primer binding sites, resulting in a bias toward nuclear DNA amplification.

It is important to reiterate the preliminary nature of these results. As stated above, for definitive proof of exactly which sequences are numts, they would have to be cloned (along with their flanking nuclear DNA) either from a genomic library or with the use of a PCR-based method, such as inverse PCR [Ochman et al., 1988]. In addition, a more exhaustive approach aimed at identifying as many numts in the gorilla genome as possible would allow for a more dense phylogenetic tree. This could be achieved, for example, through the use of degenerate PCR primers, followed by cloning and sequencing of hundreds of amplicons. Finally, the use of an old museum sample of uncertain provenance figured prominently in our results. Future studies using genomic DNA from known individuals will increase our confidence as to exactly which sequences are authentic mtDNA and which are not. Nonetheless, it is important to note that our conclusions are not entirely dependent on the eastern lowland gorilla tooth. Even without this sample, other independent lines of evidence point to the fact that multiple gorilla mtDNA sequences in GenBank are not authentic mtDNA.

After the nine putative numt sequences are removed, the maximum sequence divergence seen in western lowland gorillas (11.7%) is about one-third less than that (16.8%) reported by Garner and Ryder [1996]. This new estimate is not much greater than the maximum divergence seen within the West African chimpanzee (*P. t. verus*; 9.3%) or the Central African chimpanzee (*P. t. troglodytes*; 9.1%) according to Wise et al. [1997], or the maximum divergence between mountain gorillas (*G. b. beringei*) and eastern lowland gorillas (8.3%) [Jensen-Seaman & Kidd, 2001]. Garner and Ryder [1996] suggested that the deepest split between lineages within western gorillas was as great or greater than that between western and eastern gorillas. In fact, the trichotomy between eastern gorillas and the two deepest branches within western gorillas was not resolved in their 50% majority rule consensus tree. The most common resolution placed haplotypes WL287 and WL759 in a clade to the exclusion of all other western and eastern gorillas [Garner & Ryder, 1996]. When the nine previously published putative numt sequences are excluded from the analyses, all remaining western gorilla haplotypes cluster monophyletically in 99–100% of 1,000 bootstrapped neighbor-joining or maximum parsimony trees rooted with either a single human or chimpanzee sequence (trees not shown). Removing these numt sequences had no effect on the amount of mtDNA sequence divergence between western and eastern gorillas [Jensen-Seaman, 2000], which is about as large as that between

chimpanzees and bonobos, consistent with the suggestion that two species of gorillas should be recognized [Groves, 2001; Morell, 1994; Ruvolo et al., 1994; Sarmiento and Butynski, 1996]. Recognizing the previously published, highly divergent western lowland gorilla D-loop haplotypes as numts rather than authentic mtDNA strengthens the argument for a two-species taxonomy, in that it removes the previous reservation that doing so might create a paraphyletic *G. gorilla*.

It is essential to recognize that several of the published gorilla mtDNA D-loop sequences are actually nuclear pseudogenes, since investigators have used these mtDNA sequences to make statements regarding the evolutionary history, taxonomy, and conservation of the great apes [Garner & Ryder, 1996; Groves, 1996; Jensen-Seaman & Kidd, 2001; Jensen-Seaman et al., 2001; Ryder et al., 1999]. Furthermore, these sequences have been incorporated into larger studies examining the evolution of the African ape and human clade, despite explicit statements expressing confidence that all of the sequences were authentic and of mitochondrial origin [Gagneux et al., 1999]. We hope that the clarifications provided herein will help lead to a more complete understanding of the patterns of genetic diversity in these endangered great apes.

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