J Mol Evol (1995) 40:173–180

JOURNAL OF MOLECULAR EVOLUTION

Phylogeny of African Monkeys Based upon Mitochondrial 12S rRNA Sequences

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Received: 14 January 1994 / Accepted: 20 May 1994

Abstract. The suborder Anthropoidea of the primates has traditionally been divided in three superfamilies: the Hominoidea (apes and humans) and the Cercopithecoidea (Old World monkeys), together comprising the infraorder Catarrhini, and the Ceboidea (New World monkeys) belonging to the infraorder Platyrrhini.

We have sequenced an approximately 390-base-pair part of the mitochondrial 12S rRNA gene for 26 species of the major groups of African monkeys and apes and constructed an extensive phylogeny based upon DNA evidence. Not only is this phylogeny of great importance in classification of African guenons, but it also suggests rearrangements in traditional monkey taxonomy and evolution. Baboons and mandrills were found to be not directly related, while we could confirm that the known four superspecies of mangabeys do not form a monophyletic group, but should be separated into two genera, one clustering with baboons and the other with mandrills. Patas monkeys are clearly related to members of the genus *Cercopithecus* despite their divergence in build and habitat, while the talapoin falls outside the *Cercopithecus* clade (including the patas monkey).

Key words: African monkeys — mtDNA — 12S rRNA gene — Phylogeny

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The sequences reported in this paper have been deposited in the GenBank data base (accession nos. 35182—L35209)

Introduction

Among the Old World monkeys, the species living in Africa are clearly distinguishable from extant species in Asia. The large group of *Cercopithecus* monkeys is exclusively confined to Africa, and this is also true for the genera *Cercocebus* (mangabeys), *Papio* (baboons), *Mandrillus* (drills and mandrills), *Theropithecus* (gelada baboons), and *Colobus,* although other members of the subfamily Colobinae are present in Asia. The sacred baboon (*Papio hamadryas hamadryas*) is also found on the extreme southwestern tip of the Arabian peninsula. Two out of three species of great apes (chimpanzees and gorillas) find their habitat in Africa, the orangutan being the only Asian great ape. Two species of chimpanzee are known: the common chimpanzee (*Pan troglodytes*) and the pygmy chimpanzee or bonobo (*Pan paniscus*), while only one species of gorilla is known, subdivided into lowland (*Gorilla gorilla gorilla*) and mountain (*G. g. beringei*) gorillas. None of the lesser apes (genus *Hylobates,* gibbons and siamangs) are African. The majority of macaque species are located in Asia; only the Barbary macaque or Barbary "ape" (*Macaca sylvanus*) is still found in northern Africa.

Fossils from early Old World higher primates dating to the Oligocene (38 million years ago—mya) or possibly the Eocene (55 mya) have been found in the Fayum (Egypt). Primitive apes, dryomorphs and ramamorphs, appeared about 22–17 million years ago in Africa, suggesting that the ape–monkey split occurred at least 22 million years ago. Fossil records show that colobine monkeys appeared in the Upper Miocene (approx. 9 mya), while firm fossil evidence of Cercopithecines is found first in the Pliocene (Conroy 1990; Martin 1993).

At that time, *Theropithecus* species were widespread in Africa, of which only *Theropithecus gelada* remains today. The now-extinct *Theropithecus oswaldi* was probably replaced by *Papio* baboons in the Pleistocene (Eck and Jablonski 1984, and references therein). Upper molars and mandible fragments found in deposits 2.9–2.6 million years old, showing similarity to *C. aethiops, C. ascanius schmidti,* and talapoin monkeys were recovered from Omo Valley, Ethiopia, and Lake Turkana, Kenya (Leaky 1988). However, these fragmentary remains only indicate that by that time, guenons had evolved as a separate group. So colobine and cercopithecine monkeys probably split earlier (9–5 mya).

No strongly supported phylogeny based upon DNA evidence from all lineages of African monkeys and ages is currently available. Therefore we have sequenced, using PCR techniques, part of the mitochondrial (mt) 12S rRNA gene for every major group of Old World monkeys. Sequence variation between individuals belonging to one species was also studied. The mt 12S rRNA gene has been used successfully before to resolve phylogenetic questions (Ballard et al. 1992; Cooper et al. 1992; Janczewski et al. 1992; Meyer and Dolven 1992; Milinkovitch et al. 1993; Mindell et al. 1991; Miyamoto et al. 1990). As this gene codes for a ribosomal RNA molecule, more variation at the nucleotide level is allowed compared to a protein coding gene. However, some selective pressure will be acting upon the gene to maintain the correct secondary structure necessary for rRNA function. Indeed, extremely conserved sequence blocks are found in this gene, on which PCR primers have been based (Kocher et al. 1989). DNA phylogenies based upon other mitochondrial genes for a few Asian/African monkey lineages are known (Disotell et al. 1992; Hayasaka et al. 1988). Data were analyzed by neighbor-joining (Saitou and Nei 1987), maximum-parsimony (Swofford 1991), and maximum-likelihood methods (Felsenstein 1990), and for the maximum parsimony and neighbor-joining methods the robustness of the phylogenetic hypothesis was tested by bootstrapping (Felsenstein 1985).

Materials and Methods

Nonhuman Primate Samples. Samples from 26 species of the following African monkeys and apes were obtained (number [*n*] of individuals sequenced are indicated): *Pan troglodytes* (common chimpanzee, *n* = 12), *Pan paniscus* (pygmy chimpanzee, *n* = 1), *Gorilla gorilla gorilla* (lowland gorilla, *n* = 1), *Macaca sylvanus* (barbary macaque, *n* = 2), *Mandrillus sphinx* (mandrill, *n* = 10), *Papio cynocephalus anubis* (olive baboon, *n* = 6), *Papio cynocephalus cynocephalus* (yellow baboon, *n* = 2), *Papio hamadryas hamadryas* (hamadryas or sacred baboon, *n* = 8), *Papio cynocephalus ursinus* (chacma baboon, *n* = 2). *Colobus guereza* (Abyssinian black-and-white colobus, *n* = 6). *Cercocebus torquatus atys* (sooty mangabey, *n* = 2), *Cercocebus aterrimus* (black mangabey, *n* = 3), *Cercocebus galeritus* (Tana mangabey, *n* = 2), *Cercopithecus aethiops aethiops* (grivet, *n* = 1), *Cercopithecus aethiops pygerythrus* (vervet, *n* = 4), *Cercopithecus aethiops tantalus* (tantalus monkey, *n* = 2), *Cercopithecus aethiops sabaeus* (green monkey, *n* = 8), *Cercopithecus mitis* (Blue monkey, *n* = 2), *Cercopithecus patas* (patas monkey, *n* = 6), *Cercopithecus diana roloway* (diana monkey, *n* = 2), *Cercopithecus mona pogonias* (mona monkey, *n* = 2), *Cercopithecus ascanius* (redtail monkey, *n* = 3), *Cercopithecus nictitans* (spot-nosed guenon, *n* = 2), *Cercopithecus cephus* (moustached monkey, *n* = 2), *Cercopithecus neglectus* (De Brazza's guenon, *n* = 2), and *Miopithecus talapoin* (talapoin monkey, *n* = 1). Additionally, samples from two Asian monkey species were collected: *Macaca mulatta* (rhesus macaque, *n* = 1) and *Presbytis cristatus pyrrhus* (silvered leaf monkey or langur, *n* = 2).

The samples (serum/plasma/blood cells) were kindly provided by Jon Allan, Southwest Foundation for Biomedical Research, San Antonio, TX, USA (baboons, mandrills, vervets, and green monkeys); Michael Becker, University of Stellenbosch, Tygerberg, South Africa (chacma baboon, vervet); Vincent Hervé, Institut Pasteur, Bangui, Central African Republic (*Cercopithecus* species, *Colobus, Cercocebus* species); Vanessa Hirsch, NIH, Bethesda, MD, USA (*Cercopithecus* species); Michaela Müller, Institut Pasteur, Paris, France (tantalus monkeys, Barbary macaques); Martine Peeters, Institute for Tropical Medicine, Antwerp, Belgium (chimpanzees, mandrills, *Cercocebus* species); and Todd Disotell, University of New York, New York, NY, USA (pygmy chimpanzee). Further samples were provided by Peter Klaver, Artis Zoo, Amsterdam (gorilla, patas monkeys); Blijdorp Zoo, Rotterdam (Colobus, diana monkeys, leaf monkeys); CDI, Lelystad (*C. aterrimus*); TNO, Rijswijk (rhesus macaque); and RIVM, Bilthoven (talapoin monkey, *Cercopithecus* species)—all from The Netherlands.

DNA Extraction, Amplification, and Sequencing. Total DNA was extracted from serum, plasma, or blood cells by a procedure using silica and GuSCN (Boom et al. 1990). PCR amplifications were performed with primers L01091 and H01478 (Kocher et al. 1989) containing 5′ *Hin*dIII/*Not*I restriction sites, which amplify approximately 390 nt of the mitochondrial 12S rRNA gene. PCR products were purified on agarose gels and cloned into plasmid pDP14 (pSP73 from Promega, Inc., modified to contain a *Not*I restriction site). At least two clones from single individuals were sequenced in both directions using an Applied Biosystems 373A automated sequencer, following the manufacturer's protocols. For every species two to ten individuals were sequenced, except for *M. talapoin, P. paniscus, M. mulatta, C. a. aethiops,* and *G. gorilla gorilla,* in which cases only a single sample was available. Two monkeys, supposedly *C. a. aethiops,* showed hybrid (vervet) characteristics on closer inspection, so their DNA sequences were discarded.

Sequences have been deposited in Genbank (accession numbers 35182–35209).

Sequence Analysis. Alignment of the sequences was done using Clustal (Higgins and Sharp 1988) and corrected by hand. The phylogenetic analyses were done using the neighbor-joining method (Saitou and Nei 1987), as implemented in the MEGA package (Kumar et al. 1993). Additional analyses were done using the maximum-likelihood method (program DNAML in the PHYLIP package [Felsenstein 1990], abbreviation ML); and the maximum-parsimony analysis (Swofford 1991, abbreviation MP), as implemented in the DNAPARS program in the Phylip package). Because of the high number of transitions in the dataset (up to 30 times higher than the number of transversions when comparing sequences from *Papio cynocephalus ursinus* and *Cercopithecus mona pogonias*), distance matrices for the NJ method were made using Tamura's estimation method (Tamura 1992) which is able to take both different base frequencies and extreme transition/transversion ratios into account. For the ML method, initial estimates for transition/transversion rates were put at 2.0 and in a second run to 10.0, and base frequencies were estimated from the data; 100 bootstrap replicates were analyzed using the NJ and MP method.

A total of 183 clones derived from 82 individuals were sequenced

and compared. Sometimes PCR artifacts were encountered, amplified sequences that clearly not belonged to mtDNA 12S rRNA gene sequences. Variation in the other clones from a species did usually not exceed 1 nucleotide/clone/species. These differences were random and could easily have been generated by the Taq-polymerase used in the PCR reactions, which has an estimated level of nucleotide misincorporation of approximately 0.1–0.2%. No variation was observed between clones in some species.

Results and Discussion

An alignment of a 386–393-bp fragment of the mtDNA 12S rRNA gene for 26 species of African monkeys and apes and two Asian monkey species is shown in Fig. 1. Analyzing the obtained sequences by three different methods (neighbor-joining, maximum-likelihood, and maximum-parsimony) resulted in the phylogeny shown (Fig. 2). Clustering order in most cases was similar in the three methods. Differences when they occurred will be discussed below. The two different settings of the transition/transversion ratios parameters in the ML analyses (2 vs 10) yielded identical trees. Adding additional outgroups also did not affect tree topology.

Tree topology was highly reproducible in some parts of the tree, but not in others. Three different clusters can be clearly distinguished: apes, colobine monkeys, and cercopithecine monkeys, confirming traditional cladistic classification. The three ape species, *P. troglodytes* (common chimpanzee), *P. paniscus* (pygmy chimpanzee), and *G. gorilla gorilla* (lowland gorilla), are consistently clustered. As expected, the two chimpanzee species are closely related to each other. The colobine monkeys, represented by the African *C. guereza* and Asian *P. cristatus* sequences, were found to constitute a second cluster, being equally diverged from both apes and cercopithecine monkeys. This could indicate that this subfamily split early during monkey evolution. However, to obtain more insight in this cluster, more *Colobus* species should be sequenced. Most genera of colobine monkeys are at present found in Asia. Monkeys of the tribe *Papionini* (baboons, mandrills, mangabeys, and macaques) were found not to cluster monophyletically. Baboons (*Papio* species) and mandrills (*Mandrillus* species) form two separate clusters, indicating that referring to mandrills (*Mandrillus sphinx*) as *Papio sphinx* is not correct. The four superspecies of mangabeys (*Cercocebus*), of which we have sequenced three, are also not monophyletic, as was already proposed on immunologic (Cronin and Sarich 1979), protein electrophoretic (Lucotte 1979), and karyotypic (Dutrillaux et al. 1979) data. *C. aterrimus* (and probably also *C. albigena*) belong to the baboon cluster, while *C. torquatus* and *C. galeritus* are affiliated with mandrills. The clustering of *C. galeritus* with mandrills was also observed by Disotell et al. (1992), using mitochondrial COII gene sequences. Formerly, the *aterrimus/albigena* mangabeys were known as *Lophocebus* instead of *Cercocebus*. In line with our results, it might indeed be more correct to place them in the genus *Lophocebus*.

Comparing the obtained baboon sequences, a surprising outcome is that all 12S sequences are identical (alignment not shown), except for the South African chacma baboon (*P. c. ursinus*). This result is in contrast with common classification of baboons, in which a separate status is usually assigned to *P. hamadryas hamadryas* (sacred baboon), while the others are regarded as subspecies of *P. cynocephalus,* or alternatively all baboon types are considered to be distinct species (reviewed by Jolly and Brett 1973). The separate status of sacred baboon was found to be not appropriate using genetic distance markers (Williams-Blangero et al. 1990). In fact, these authors suggest that the five baboon types belong to a single polytypic species (*Papio hamadryas*). The observation is in line with the statement of Dutrillaux et al. (1982) that the chromosomes of different baboon species are identical. However, these authors did not examine the chacma baboon. An explanation could be that *Papio cynocephalus* species have recently experienced a bottleneck event, after which a small population is expanding again, or that speciation in baboons occurred recently and cannot yet be detected by 12S rRNA sequence variation.

The branch leading to the macaques (*Macaca sylvanus* and *Macaca mulatta*) originated relatively early during evolution of the Papionini. Phylogenetic placement of these monkeys was slightly controversial. The cluster of the two macaques was placed between the baboon and the mandrill cluster in both the maximum-likelihood and the maximum-parsimony analyses, while it was located outside these two clusters in the neighbor-joining analysis (Fig. 2). Our analyses confirmed that both macaques are taxonomically related, as was also observed in a study on Asian macaques (Hayasaka et al. 1988). The bootstrap value of the cluster representing the Papionini was low in both NJ and MP analyses, indicating low stability.

Cercopithecus monkeys (commonly named guenons) are an interesting group. Not only are there many species and over 70 subspecies, with chromosome numbers varying between $2N = 48$ and $2N = 72$ (Dutrillaux et al. 1980), but the whole group is exclusively confined to Africa. There have been attempts to classify guenons based on serum proteins (Ruvolo 1988). Also, vocal and morphological characteristics have been used for this purpose (Gautier 1988; Martin and MacLarnon 1988; and references therein). Figure 2 shows that the cluster *Cercopithecus* monkeys based upon 12S mtDNA sequences is not very stable. The bootstrap value for the whole group in the MP analysis was 55%. *C. neglectus* is an outlier in this group. Four subspecies of *C. aethiops* (African green monkeys) are currently recognized: *C. a. sabaeus* (green monkeys), *C. a. aethiops* (grivets), *C. a.*

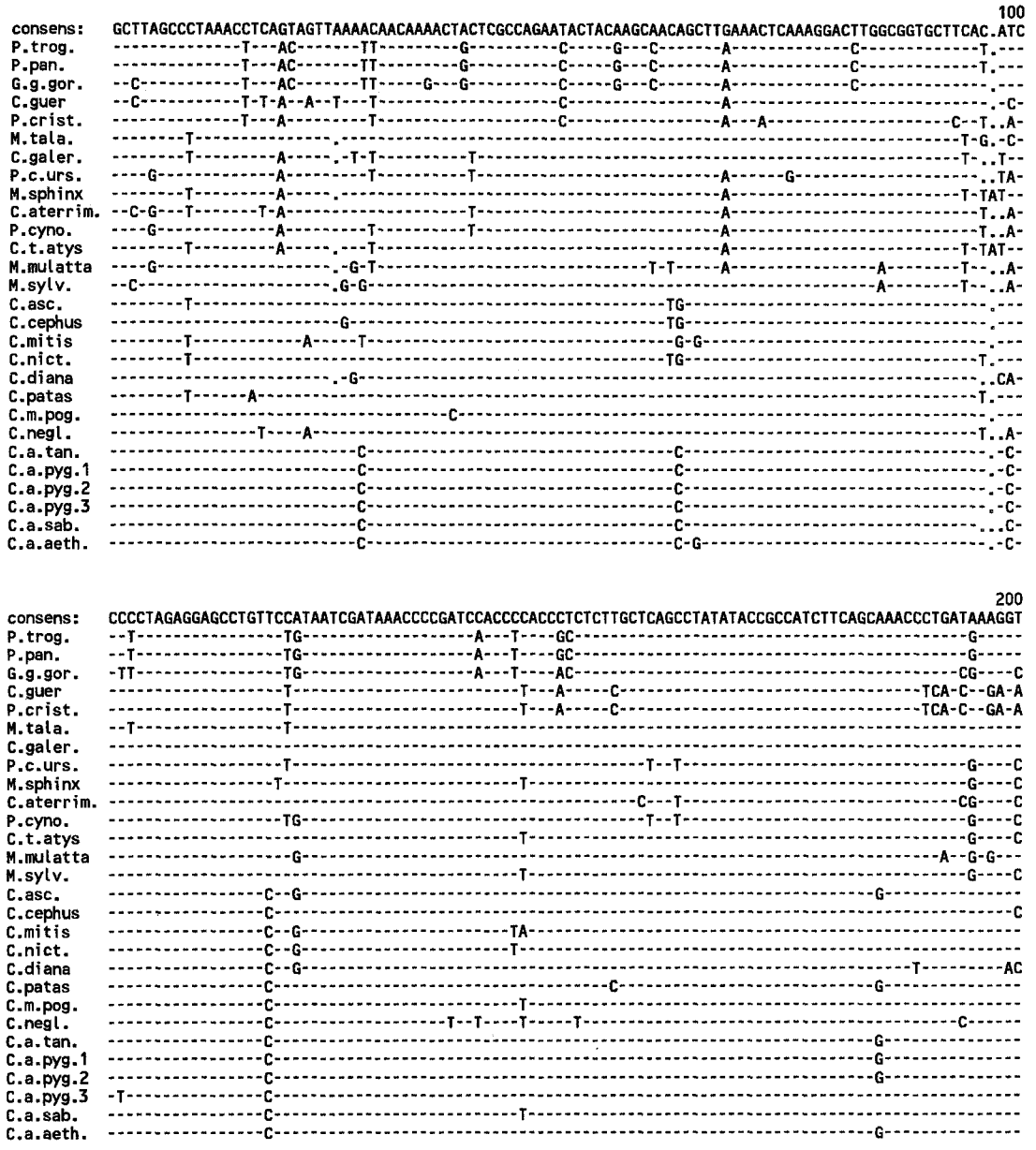

Fig. 1. Alignment of a 386–393-nt fragment (primers L01091/H01478, Kocher et al. 1989; exact size depending upon the particular species) of the mitochondrial 12S rRNA gene of 24 species of African primates and two species of Asian monkeys. A consensus derived from all sequences is shown in the *upper line*. Gaps introduced for optimal alignment are indicated by *dots*. Identical nucleotides are indicated by *dashes*. Sequences from three *Cercopithecus aethiops pygerythrus* (vervet) groups are shown. Geographic locations of the vervets sequenced were South Africa (number 1), unknown (number 2), and Tanzania (number 3). Tantalus monkeys were from the Central African Republic and *C. a. sabaeus* were from Senegal.

tantalus (tantalus monkeys), and *C. a. pygerythrus* (vervets). Placement of *C. a. pygerythrus* is not straightforward. In no analysis did vervet monkeys form clades according to the subspecies classification. The classifications obtained yielded low bootstrap values in both NJ and MP analysis. Materials from 16 monkeys belonging to the four proposed subspecies were obtained from different countries in Africa and were supplied by different laboratories and animal dealers. The analyses presented here indicate that phylogenetic placement of the monkey species is sometimes more dependent upon their geographic location rather than upon their morphological classification. In other words, based upon the mt 12S rRNA gene, *C. aethiops* can at this moment better be divided in distinct populations than in the four subspecies proposed earlier. Similar conclusions were drawn by Lucotte et al. (1982) based on serum proteins. *C. aethiops sabaeus* seems to be the most distinct subspecies. To test the hypothesis, more individuals of different subspecies (or populations) of these monkeys should be sequenced. *C. ascanius* and *C. cephus* were found to be closely related. Both species possess the same chromosomal karyotype (Dutrillaux et al. 1982). Also *C. nictitans* and *C. mitis* are reliably clustered. Two monkeys,

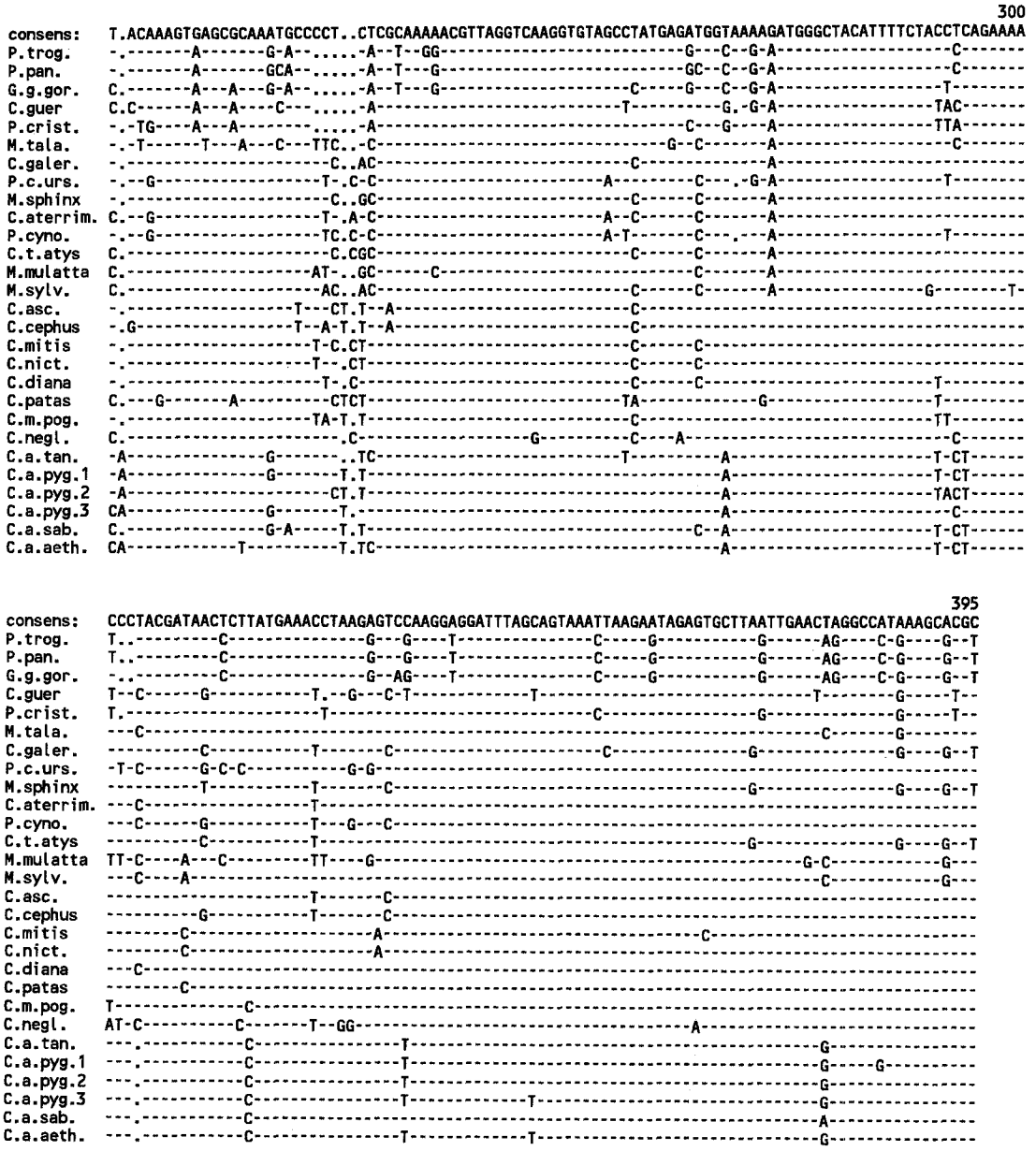

Fig. 1. Continued.

described to us as *C. mitis,* were in fact shown to contain *C. ascanius* mtDNA, indicating that they are probably interspecies hybrids, with *C. ascanius* monkeys in the female lineage. Fertile, wild hybrids between these species have been observed (Aldrich-Blake 1968). Although hybrids are easily distinguished morphologically, hybrid backcrosses are more difficult to separate visually. Patas monkeys are semiterrestrial, living in open wooded steppes and savannas and even rocky mountains, in contrast to the more arboreal forest guenons. They are large animals with long limbs and the capacity for fast running (Napier and Napier 1985). Because of this build and habitat, the patas monkey is generally excluded from the guenon group and placed in a separate genus (*Erythrocebus*). It was already noted that patas monkeys are closely related to *Cercopithecus* (Cronin and Sarich 1979) and our results suggest that they should be classified as *Cercopithecus patas* (Fig. 2).

A special problem is caused by the smallest of all African monkeys, the talapoin. Formerly assigned its own genus, *Miopithecus,* it is now commonly regarded as a subspecies of *Cercopithecus.* Our data, however, do not confirm the latter classification. The talapoin monkey could not be classified with any consistency in the phylogenetic analyses. Biologists have already noted that several morphological features, including sexual swellings in females (which are absent in guenons, but present in all *Papionini*), set it apart from other guenons (Hill 1972). An argument against placement of the talapoin in the Papionin tribe is its chromosome number of $2N = 54$ (Ponsà et al. 1980), while for the mentioned tribe the chromosome numbers are exceptionally stable at $2N =$

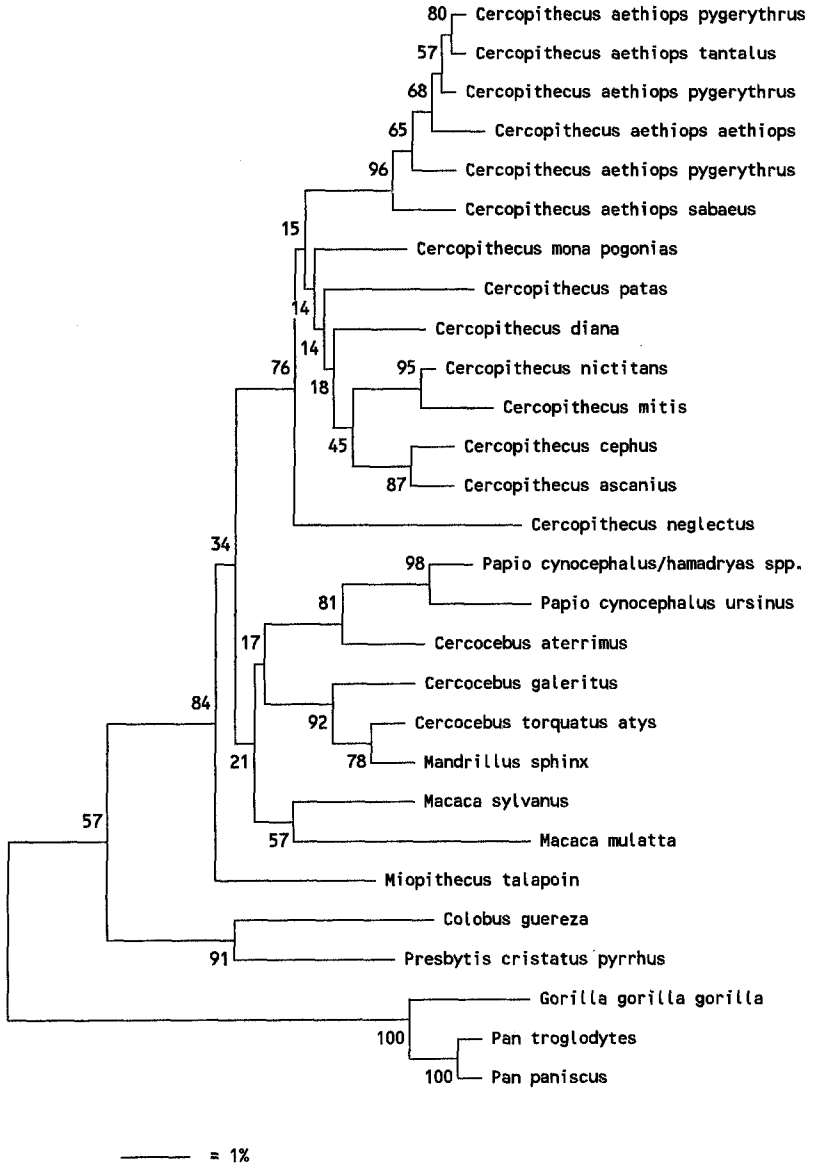

Fig. 2. Neighbor-joining tree based upon a 386–393-nt fragment of the mitochondrial 12S rRNA gene of 24 species of African primates and two species of Asian monkeys. As sequences derived for groups of vervet (*C. a. pygerythrus*) monkeys acquired from different locations differed substantially, several were included in the analysis. The African apes (chimpanzees and gorilla) were designated outgroups in this analysis. Bootstrap values (node reproduction frequencies out of 100 trees) are represented from bootstrap analysis.

42. To obtain a more reliable clustering of the talapoin, more genes should be sequenced, as no other *Miopithecus* species is known. Generally, phylogenetic trees are better resolved by adding more taxa than by adding more sequence data per species (Hillis and Huelsenbeck 1992).

To compare our data with other DNA evidence, a few options are available. For several African primate species, nucleotide sequences have been published for the nuclear CD4 gene (Fomsgaard et al. 1992). The CD4 molecule, a T-cell surface glycoprotein, acts as the receptor for immunodeficiency viruses. Also, phylogenies based on nucleotide sequences from simian immunodeficiency virus (SIV) isolates have been generated (Müller et al. 1993; Hirsch et al. 1993). Applying the same phylogenetic analyses to CD4 gene sequences resulted in phylogenies with the same topology as our mtDNA-based trees. CD4 sequences were compared for *C. aethiops sabaeus, C. aethiops aethiops, C. aethiops pygerythrus, C. patas, C. torquatus atys, P. troglodytes, M. mulatta,* and *H. sapiens* (human) (Fig. 3). Chimpanzees and humans were consistently clustered, and *Cercopithecus* species formed another separate cluster. In our mtDNA analyses, humans also cluster with the great apes (result not shown). Although based on fewer species, genomic CD4 sequences analyzed by our methods form clusters comparable to mitochondrial sequences. This result enhances the confidence in our phylogeny.

Simian immunodeficiency viruses have been isolated from many wild-caught African monkey species. Several of these natural strains have been sequenced (Müller et al. 1993; Hirsch et al. 1993; and references therein), and phylogenetic analyses have been performed. Viruses from the four proposed subspecies of *C. aethiops* (Allan et al. 1991) cluster according to the subspecies hypothesis in an NJ analysis (Müller et al. 1993). Subsequent mtDNA analysis (this paper) shows that the *C. aethiops* host cannot always be distinguished accordingly. An explanation could be that the mt 12S rRNA gene sequences

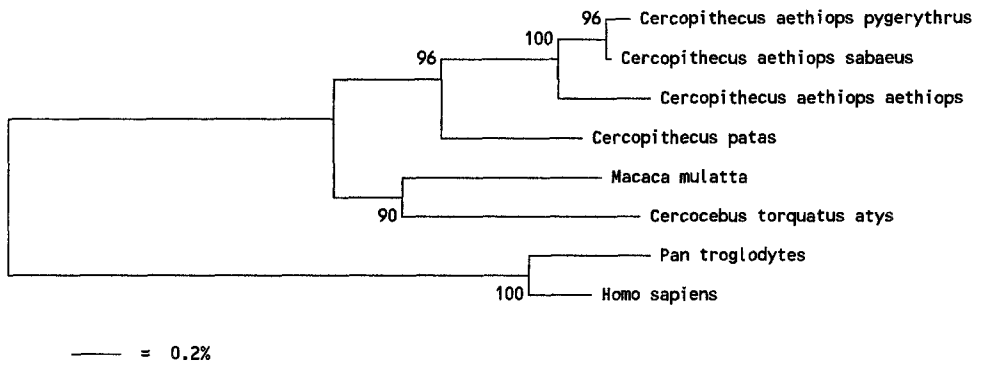

Fig. 3. Phylogenetic tree obtained from ML and NJ analysis of CD4 sequences (Fomsgaard et al. 1992). Both analyses yielded trees with the same topology. Bootstrap values as in Fig. 2.

of the subspecies are not enough diverged yet to be informative for phylogenetic analysis. Viruses belonging to the lentiviral group of retroviruses, like SIV, have a high mutation rate, thus being able to adapt to the species faster than mitochondrial DNA, making it possible that African green monkeys were infected with SIV before speciation. However, virus strains isolated from mandrills showed them to be highly divergent from other SIV strains, including those from sooty mangabeys (Tsujimoto et al. 1989), while in our phylogeny mandrills and sooty mangabeys are confidentially clustered. This could indicate that in the case of the virus, interspecies transmissions could have occurred after the mangabey\mandrill split. So, a tree based upon a nuclear gene (CD4) is comparable to the mtDNA phylogeny presented here, but the mtDNA phylogeny is not comparable to a virus (SIV)-based tree.

The phylogenetic hypothesis proposed on the basis of mitochondrial DNA only partially supports the traditional classification of African monkeys which is based on geographical distribution, morphological characters, social life, and habitats. A different classification is required in some cases when the robustness of the genetic phylogeny reported is accepted.

Acknowledgments. We thank Koen Brouwer (National Foundation for Research in Zoological Gardens, The Netherlands) for advice and Todd Disotell for stimulating suggestions concerning the phylogenetic analyses.

This study is funded in part by The Institute of Virus Evolution and the Environment.

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