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Phylogeny and Evolution of Selected Primates as Determined by Sequences of the ε-Globin Locus and 5′ Flanking Regions

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We studied phylogenetic relationships of 39 primate species using sequences of the ε-globin gene. For 13 species, we also included flanking sequences 5′ of this locus. Parsimony analyses support the association of tarsiers with the anthropoids. Our analysis of New World monkeys supports the model in which the callitrichines form a clade with Aotus, Cebus, *and* Saimiri, *with* Cebus *and* Saimiri *being sister taxa. However, analysis of the 5′ flanking sequences did not support grouping the atelines with* Callicebus *and the pitheciins. Our data support the classification of platyrrhines into three families, Cebidae (consisting of* Cebus, Saimiri, Aotus, *and the callitrichines; Atelidae—the atelines; and Pitheciidae—*Callicebus *and the pithiciins. The strepsirhines form well-defined lemuroid and lorisoid clades, with the cheirogaleids (dwarf and mouse lemurs) and* Daubentonia *(aye-aye) in the lemuroids, and the aye-aye being the most anciently derived. These results support the hypothesis that nonhuman primates of Madagascar descended from a single lineage. Local molecular clock calculations indicate that the divergence of lemuroid and lorisoid lineages, and the earliest diversification of lemuroids, occurred during the Eocene. The divergence of major lorisoid lineages was probably considerably more recent, possibly near the Miocene–Oligocene boundary.*

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Within hominoids some estimated dates differ somewhat from those found with more extensive noncoding sequences in the β-globin cluster.

KEY WORDS: ceboids; strepsirhines; primate phylogeny; ε-globin gene; molecular evolution; molecular clock.

INTRODUCTION

Several recent researchers (Schneider *et al.*, 1993, 1996; Harada *et al.*, 1995; Porter *et al.*, 1995) have used globin DNA sequences to establish relationships among primates. Their molecular studies support many of the relationships indicated by more traditional morphological studies, but in some cases, they have supported different phylogenies (Shoshani *et al.*, 1996). Primate taxa that receive strong support from a variety of molecular and morphological data sets include Hominoidea (apes and human), Cercopithecoidea (Old World monkeys), Catarrhini (hominoids and Old World monkeys), Platyrrhini (ceboids or New World monkeys), Anthropoidea (catarrhines and platyrrhines), and Strepsirhini (lemurs and lorises). However, the relationships within and among them are not entirely resolved. For example, relationships among the platyrrhines and among the strepsirhines are in dispute, because various molecular and morphological data sets produce conflicting results.

The systematic status of the tarsiers (Tarsioidea) has been especially controversial (Martin, 1990; Shoshani *et al.*, 1996). Traditional classifications group them with the strepsirhines in the suborder Prosimii, with Anthropoidea comprising the other primate suborder (Simpson, 1945; Schwartz, 1986; Fleagle, 1988), or have them as a sister group to all other living primates (Gingerich, 1973, 1975). Other classifications divide the primates into a suborder Haplorhini, consisting of tarsiers and anthropoids, with the lemuroids and lorisoids being placed in the suborder Strepsirhini (Nowak, 1991). Martin (1990) reviewed the morphological evidence linking tarsiers and anthropoids.

Schwartz (1986) considers the tarsiers to be closely related to the lorisoids, and recognizes tarsiers, lemuroids, and lorisoids as members of the Prosimii. A study of sequences of αA-crystallin (Jaworski, 1995) supports this arrangement. Hayasaka *et al.* (1988) also found some support for the prosimian clade, though they regard their result, based on mtDNA sequences, to be inconclusive. Other molecular studies (Bailey *et al.*, 1992a, Porter *et al.*, 1995; Adkins *et al.*, 1996) place tarsiers with the anthropoids.

Major systematic issues within the strepsirhines involve the cheirogaleids (dwarf and mouse lemurs) and the aye-aye (*Daubentonia madagascariensis*). Both are endemic to Madagascar, but their relationships

with other nonhuman Malagasy primates are arguable (Martin, 1990). Traditional studies have included all Malagasy species, including the aye-aye and the cheirogaleids, in a lemuroid clade and all mainland African and Asian strepsirhines in a lorisoid clade (Simpson, 1945). This classification is based on the hypothesis that all nonhuman primates of Madagascar are descended from a single lineage that traversed the Mozambique Channel into Madagascar. However, some researchers have questioned the monophyly of Malagasy strepsirhines, and have suggested instead that the ancestors of the aye-aye or the cheirogaleids or both colonized Madagascar independently from other primates. Various ecological, behavioral, and morphological similarities have led some workers (Charles-Dominique and Martin, 1970; Szalay and Katz, 1973; Groves, 1974; Schwartz and Tattersal, 1985) to propose that cheirogaleids are more closely related to the lorisoids of the mainland than to the lemuroids of Madagascar. Contrarily, other studies of morphological and molecular data (Yoder, 1994, 1996; Porter *et al.*, 1995), generally support monophyly of Malagasy strepsirhines.

Some morphological (Groves, 1989) and molecular (Adkins and Honeycutt, 1994) evidence suggests that *Daubentonia* is the sister group to the remaining strepsirhines. This hypothesis implies at least two migrations of nonhuman primates to Madagascar—the first being the ancestor of *Daubentonia*, and the second, the lineage leading to the more typical lemuroids.

Schneider *et al.* (1993, 1996), Harada *et al.* (1995), and Porter *et al.* (1995) used the ε-globin gene sequences to resolve relationships among the New World primates. The results of these molecular studies not only confirm some of the relationships proposed by morphological studies (Rosenberger, 1984; Ford, 1986; Kay, 1990), but also provide evidence for relationships that are in consistent with previous morphological results. Harada *et al.* (1995), Schneider *et al.* (1996), and Barroso *et al.* (1997) also used IRBP (interphotoreceptor retinoid binding protein) gene intron 1 sequences to resolve relationships among these primates and produced results similar in most respects to those from ε-globin gene sequences.

The New World monkey ε-globin gene sequences studied by Porter *et al.* (1995) were the same as those analyzed by Schneider *et al.* (1993). In addition, Porter *et al.* (1995) analyzed ε-globin sequences from *Nycticebus*, *Microcebus*, *Cheirogaleus*, *Propithecus*, and *Daubentonia*, as well as sequences of an upstream flanking region for eight of the species. We expanded this ε-globin data set by adding additional species, and by including additional upstream sequences for some species. These additional data provide resolution for some relationships that were left unresolved or were poorly supported in the previous study.

The ε-globin gene is part of the β-globin gene cluster, the genes of which arose from a series of tandem gene duplications (Goodman *et al.*, 1984). This cluster in primates consists of the ε-, γ-, ψη-, δ-, and β-globin genes (Fig. 1). The 5′ genes (ε and γ) are expressed earlier in ontogeny than the 3′ genes (δ and β). The ε gene is the most 5′ gene, and is embyonically expressed. The γ-globin gene is also embryonically- expressed in strepsirhine primates (Tagle *et al.*, 1988), but it was tandemly duplicated and became fetally expressed in anthropoids (Fitch *et al.*, 1991; Hayasaka *et al.*, 1992). In some ceboids, one of the γ-globin genes is inactivated, or in the case of *Aotus*, the two loci are combined into a hybrid gene (Meireles *et al.*, 1995; Chiu *et al.*, 1996). The ψη-globin locus is a pseudogene. The δ- and β-globin genes are active in adults.

The coding sequence of the ε-globin gene (Fig. 1) is distributed among three exons, which are 92, 223, and 129 bp in length. Exons 1 and 2 are separated by a short intron, of approximately 125 bp. Exons 2 and 3 are separated by a much more extensive intron which is approximately 850–900 bp in most species. The sequences that we analyzed include all three exons as well as the intervening introns. For some species, we also sequenced and analyzed the region 5′ of exon 1.

The purposes of this study were to use sequences associated with the ε-globin gene to provide information on the overall history of primate evolution. We selected species for study to address (1) the relationship of tarsiers to the anthropoid and strepsirhine lineages, (2) the relationships

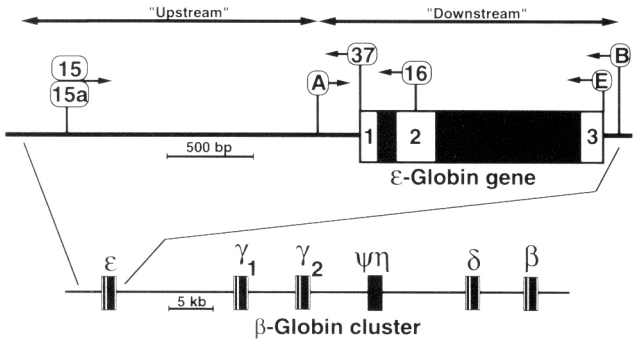


Fig. 1. Diagrammatic representation of the ε-globin gene, showing its location in the β-globin cluster. The cluster illustrated is based on *Homo*, but other primate species differ in the intergenic distances and in the nature of the γ-globin gene(s). Exon coding sequences are white, and the exons of the ε-globin gene are numbered 1, 2, and 3. The annealing sites of PCR primers are shown above the illustration of the ε-globin gene. The upstream and downstream regions are defined as used in our analysis.

among the genera of New World primates, and (3) the monophyly of Malagasy primates and the systematic placement of the aye-aye and the cheirogaleids.

Phylogenies based on molecular data should produce more reliable conclusions as larger amounts of sequence are included, and also as more species are examined. This study amplifies previous work in both of these dimensions and provides strong support for certain clades, as determined by bootstrap and strength of grouping measures.

We also use the aligned sequences to examine rates of molecular evolution in various clades and to estimate divergence times via a molecular clock.

MATERIALS AND METHODS

Species Examined

Sources of previously published sequences are listed in Table I. Data newly determined in this study include sequences of the ε-globin gene proper from one ape—the siamang (*Hylobates syndactylus*)—and a lorisoid: the potto (*Perodicticus potto*). Newly determined data also include 5′ flanking sequences from five species of ceboid primates, including common marmoset (*Callithrix jacchus*), squirrel monkey (*Saimiri sciureus*), night monkey (*Aotus azarae*), bald uakari (*Cacajao calvus*), white-bellied spider monkey (*Ateles belzebuth*), and a lemuroid, Verreaux's sifaka (*Propithecus verreauxi*).

All of the species examined for data in the upstream flanking region had been previously sequenced in the ε-globin gene proper. Combined with orthologous sequences previously published, sequence data for the full span of the ε-globin gene proper are now available for 39 species of primates, 1 species of goat, and 1 species of rabbit. One of the 39 primate species (*Saimiri sciureus*) is represented by individuals from two localities representing different subspecies. The upstream region has been sequenced in 13 species of primates as well as 1 species of rabbit (Table I). We used previously published sequences from the domestic goat (*Capra hircus*) (Shapiro *et al.*, 1983) and rabbit (*Oryctolagus cuniculus*) (Hardison, 1983) as outgroups.

PCR Amplification

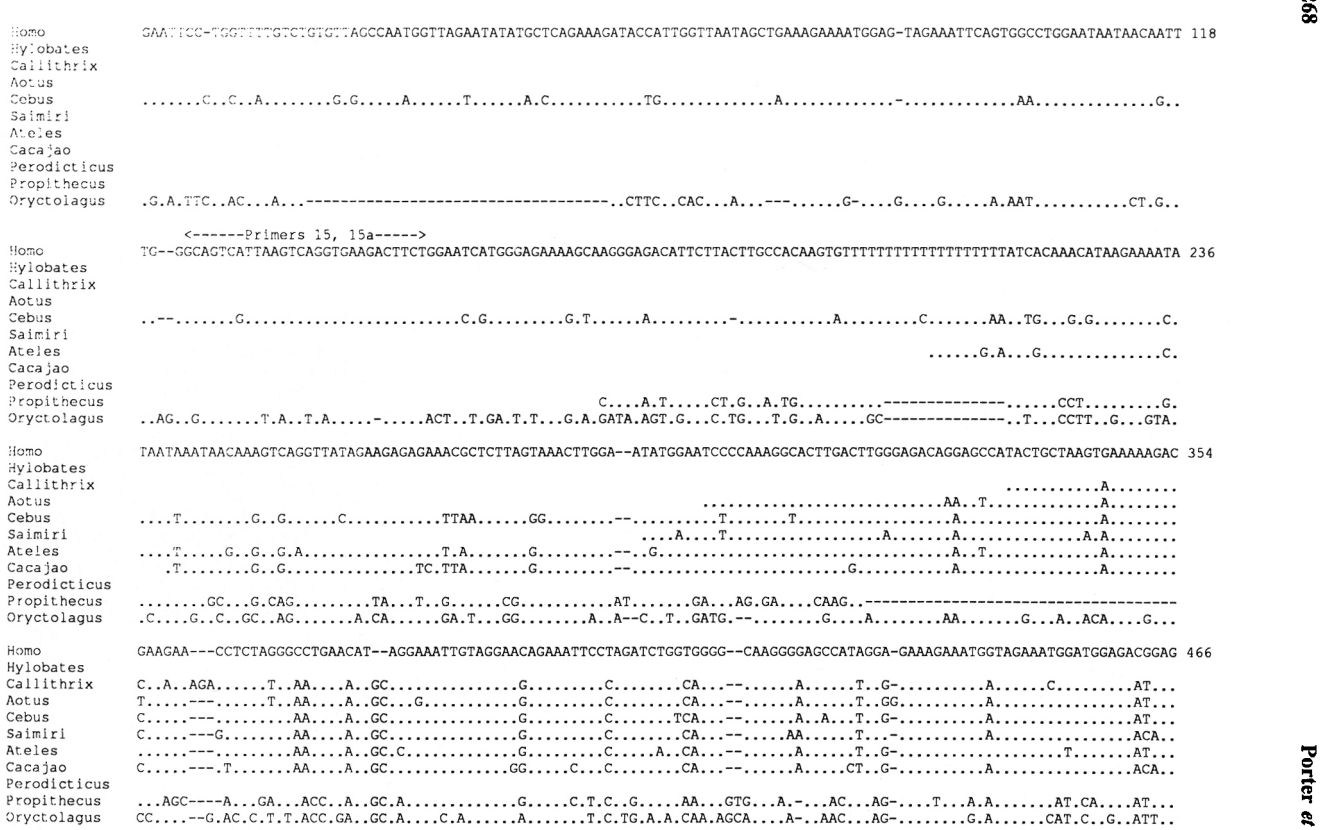
We amplified sequences of interest from genomic DNA via the polymerase chain reaction (PCR), using the protocol described by Porter *et*

Table I. Species Examined and Sources of Data[a]

Taxon	Common name	Source
Primates		
Catarrhini		
*Homo sapiens**	human	Collins and Weisman, 1984
Pan paniscus	bonobo	Bailey *et al.*, 1992a
Pan troglodytes	chimpanzee	Bailey *et al.*, 1992a
Gorilla gorilla	gorilla	Bailey *et al.*, 1992a
*Pongo pygmaeus**	orangutan	Koop *et al.*, 1986
*Hylobates lar**	white-handed gibbon	Bailey *et al.*, 1992a
Hylobates syndactylus	siamang	U64616
Macaca mulatta	rhesus macaque	Bailey *et al.*, 1992a
Platyrrhini		
Cebulella pygmaea	pygmy marmoset	Schneider *et al.*, 1993
*Callithrix jacchus**	common marmoset	Schneider *et al.*, 1993, U64615
Callimico goeldii	Goeldi's monkey	Schneider *et al.*, 1993
Leontopithecus rosalia	golden lion tamarin	Schneider *et al.*, 1993
Saguinus midas	golden-handed tamarin	Schneider *et al.*, 1993
*Aotus azarae**	red-necked night monkey	Schneider *et al.*, 1993, U64612
*Cebus albifrons**	white-fronted capuchin	Bailey *et al.*, 1992a
Cebus kaapori	Kaapor capuchin	Harada *et al.*, 1995
Cebus nigrivittatus	weeper capuchin	Harada *et al.*, 1995
Saimiri bolivensis	Bolivian squirrel monkey	Harada *et al.*, 1995
Saimiri sciureus	common squirrel monkey (Brazil)	Harada *et al.*, 1995
*Saimiri sciureus**	common squirrel monkey (Peru)	Schneider *et al.*, 1993, U64619
Alouatta belzebul	red-handed howler	Schneider *et al.*, 1993
Alouatta seniculus	red howler	Schneider *et al.*, 1993
Ateles geoffroyi	black-handed spider monkey	Schneider *et al.*, 1993
*Ateles belzebuth**	white-bellied spider monkey	Schneider *et al.*, 1993, U64613
Lagothrix lagothricha	woolly monkey	Schneider *et al.*, 1993

Brachyteles arachnoides	woolly spider monkey	Schneider *et al.*, 1993
Pithecia irrorata	bald-faced saki	Schneider *et al.*, 1993
*Cacajao calvus**	bald uakari	Schneider *et al.*, 1993, U64614
Chiropotes satanas	bearded saki	Schneider *et al.*, 1993
Callicebus torquatus	collared titi monkey	Schneider *et al.*, 1993
Callicebus moloch	dusky titi monkey	Schneider *et al.*, 1993
Tarsioidea		
Tarsius syrichta	Philippine tarsier	Koop *et al.*, 1989
Strepsirhini		
Perodicticus potto	potto	U64617
Nycticebus coucang	slow loris	Porter *et al.*, 1995
*Galago crassicaudatus**	thick-tailed greater bush baby	Tagle *et al.*, 1988
*Propithecus verreauxi**	Verreaux's sifaka	Porter *et al.*, 1995 U64618
Lemur fulvus	brown lemur	Harris *et al.*, 1986
Cheirogaleus medius	fat-tailed dwarf lemur	Porter *et al.*, 1995
*Microcebus murinus**	gray mouse lemur	Porter *et al.*, 1995
*Daubentonia madagascarinsis**	aye-aye	Porter *et al.*, 1995
Artiodactyla		
Capra hircus	domestic goat	Shapiro *et al.*, 1983
Lagomorpha		
*Oryctolagus cuniculus**	domestic rabbit	Hardison, 1983

[a]Each species analyzed in the study is listed, along with the published source of sequence data. For sequences newly determined in this study, the GenBank accession number is listed. For species with both a published source and an accession number, the accession number is for the upstream 5′ flanking sequences, and the cited publication gives the downstream sequences. Species marked with an asterisk include upstream data.


Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus
<------Primers 15, 15a----->
Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus
Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus
Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus

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                 /Ins
Homo          GCAGAGAAGGAGGAACTGGGCTGGGGCAATGAGATTATGGTGATGTAA-GGGACTTTTAT-AGAATTAA-CAATGCTGGAATTTGTGGAACTCTGCTTCTATTATTCCCCAATCATTAC 583
Hylobates
Callithrix    C...--...A...............A............A........-....T....C.-...T....-.......A.........C............G.....T............A
Aotus         C...GA.G.A...-..........A......................-.........C.AG.......A.......A.....T..C.................T.............
Cebus         C...--.G.A...............T...........C.........-..C.....C.-......G.-................C.................T..T.....GC...
Saimiri       C...--...A.....T........................A......-.........C.-.........-...C............C.................T..TA.........
Ateles        C...--...A.........C.....A.....................-.........C.-.........-....................C...A.............T.............
Cacajao       C...--...C.......A.......A..G.......CA...C.....-......G..C.-.........-.....C.........C.................T............
Perodicticus
Propithecus   C...--...C...........A....TC.CT...C...A........GG..A......C.-.........-...A........G..-.C..........TC.........TT......C.A
Oryctolagus   C...--.TAT.A.....A.TTAA..AAT.CT....A...A.T.....GT..A.TG...C.-G.......-A..G..........C..C.GT...-....C......T.T.TG.....C.A

Homo          TTCTGTCACATTGATAGTTAAATAATTTCTGTGAATT-TATTCCTTGATTCTAAAA-------TATGAGGATAATGACAATGGTATTA-TAAGGGCAGATTAAGT-GATATAGCATAAGC 693
Hylobates
Callithrix    C...............G...T.....C........C..-..................AA-----A.....A..............A....-....A...........C-A.....C....G.T
Aotus         ..........C..............C........C..-...........C......AA-----A......................-..................C-A.....C....G..
Cebus         ..........C..............C........C..-..................-------A......................-........C......C-AG....C....G..
Saimiri       ....A.....C..............C.......GC..-..................AAAAAAAA.......................-....T.T.AC.....C-AG....C..C.G..
Ateles        ...CA.....C..............C........C..-...A.......A......A------A.......................-..................C-A.....C....G.T
Cacajao       ....C.....C..............C........C..-..................AA-----.......................-...T............C-A.....T....GA.
Perodicticus
Propithecus   ........TC.C..C.....C.C..C.........CC.-.G......T.........-------A.CA.......AC.....TA.....C..G.T.....----..-.....T.T.C.TAG
Oryctolagus   C.T.T..CTT....C.A...T....C.........CC.C.C..T...T....C.TTC---------C.T.........C....T....G.C.C..TA....GC.-..-..A...A..A.G.G

Homo          AATATTCTTCAGGCACATGGATCGAATTG---AATACACTGTAAATCCCAACTTCCAGTTTCAGCTCTACCAAGTAAAGAGCTAGCAAGTCATCAAAATGGGGACATACAGAAAAAAAAA 810
Hylobates
Callithrix    G.........G..................---G.......A.......................A.....G......................C....A.T....-A.A..........
Aotus         T............................---........................T.A.....GT.......................C..CA.......TA.A..........
Cebus         G.....T......................---....A...A...............T...C....T.A.....G.......A...............C..CA.......-..A..........
Saimiri       G............................---.......AA......................A.....G....C.................C..CA.A.....-A.A.........
Ateles        G..........................C..---..........................A.....G......................C...........-..A..........
Cacajao       ............................---........A......................A..A..G..........G.........G.C..C.......TA.A..........
Perodicticus
Propithecus   ...........T........G.G.....---...G..-.A.....G..G....GG......T....C.A..C........................C..ACA.......-.GA...C..---
Oryctolagus   .....CT....T.A....CA..--....CACT......-.A.....GTGG.T..A.......TGT...C.A..T.C.....AT....C.........C.TCA...GGC.T..A...C..---

Homo          A--------------------GGACAC--TAGAGGAAT-------------------------------------AATATACCCTGACTCCTAGCCTGATTAATATATCGATT-CACTTT 870
Hylobates
Callithrix    .AAGGAAAC------------.A....--.........TTTAAGTCTCTGCTATCTGTGGCTACA---AACACTAA.......................ATA.G......T....-......
Aotus         .AAAAAAAA------------.T....--.........TTGAAGTCTCCGCTATCTGTGGCTACA---AACACTAA....C.T..................A...G......T....-......
Cebus         .AAAAAAAAAAAAAAAAAAA......AC.........TTGAAGTCTTTGCTATCTGTGGCTACA---AACACTAA....C.T........T.......A...G......T....-......
Saimiri       .AA--------------------.GGG.AC.........TTGAAGTCTCTGCTATCTGTGGCTACA---AACACTAA.-..CCT..................A...GC..C..T....-......
Ateles        .AAAAAAAAAA----GAGGG......--.........TTGAAGTCTCTGCT--CTCTGGCTACA---AACACTAA.......................A..........T....-......
Cacajao       .AA------------------...GC.--.........TTGAAGTCTCTGCTATCTGTGGCTACA---AACACTAA..C...................A....G.....T....-G..C..
Perodicticus
Propithecus   --------------------..G.GT--..A......TTGAAGCCTCTGGCATGGGTGGCTACAGCAAACACTAA.-.T..A.....T......T...............A...-TG.C.G
Oryctolagus   --------------------..G...-T....A....TTGAA-CCTCTACCCTG-GTGACTACAGCACACACTA---------------------------G.C.A...A...-A....A
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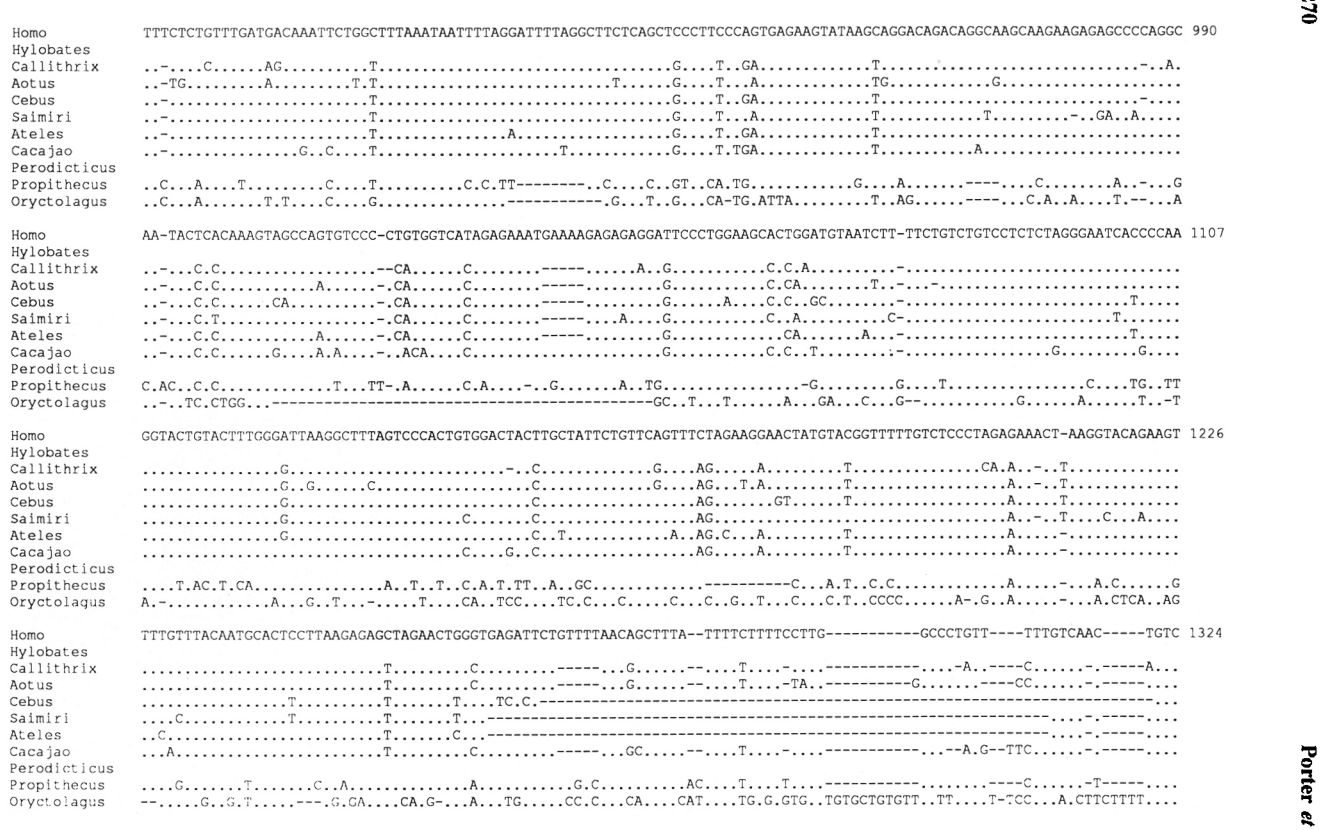
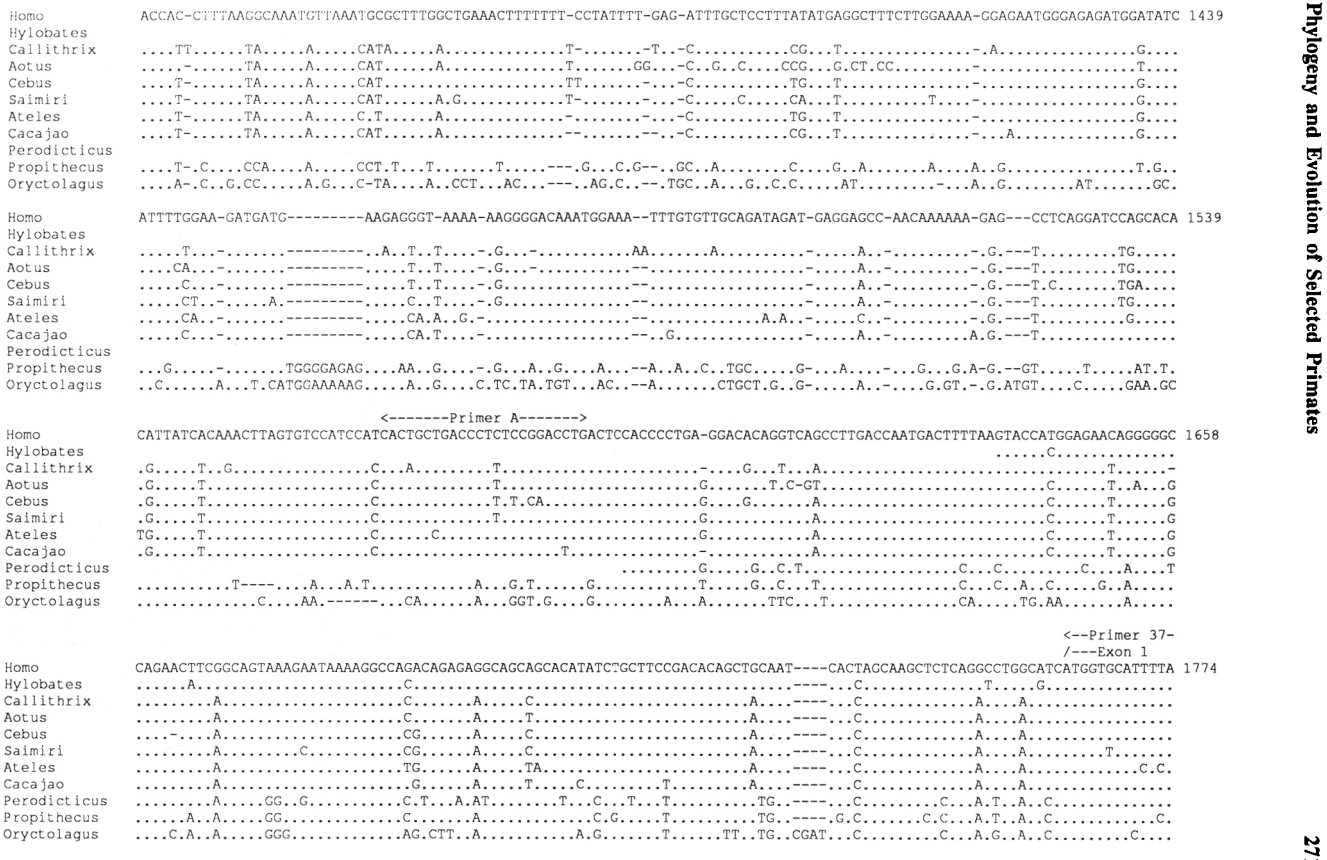
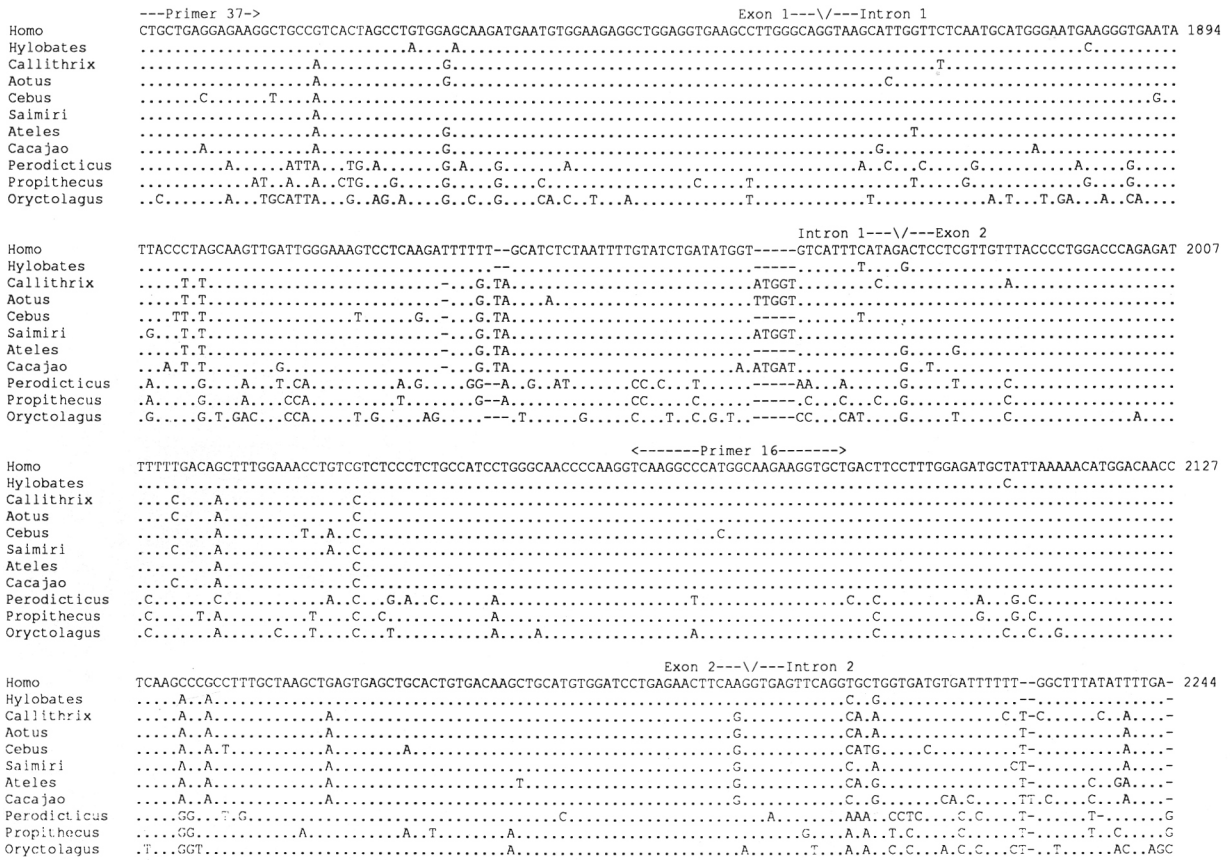

Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus
TTTCTCTGTTTGATGACAAATTCTGGCTTTAAATAATTTTAGGATTTTAGGCTTCTCAGCTCCCTTCCCAGTGAGAAGTATAAGCAGGACAGACAGGCAAGCAAGAAGAGAGCCCCAGGC 990
AA-TACTCACAAAGTAGCCAGTGTCCC-CTGTGGTCATAGAGAAATGAAAAGAGAGAGGATTCCCTGGAAGCACTGGATGTAATCTT-TTCTGTCTGTCCTCTCTAGGGAATCACCCCAA 1107
GGTACTGTACTTTGGGATTAAGGCTTTAGTCCCACTGTGGACTACTTGCTATTCTGTTCAGTTTCTAGAAGGAACTATGTACGGTTTTTGTCTCCCTAGAGAAACT-AAGGTACAGAAGT 1226
TTTGTTTACAATGCACTCCTTAAGAGAGCTAGAACTGGGTGAGATTCTGTTTTAACAGCTTTA--TTTTCTTTTCCTTG----------GCCCTGTT----TTTGTCAAC-----TGTC 1324


Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus
<-------Primer A------->
<--Primer 37-
/---Exon 1


---Primer 37->
Exon 1---\/---Intron 1
Homo
Hylobates
Callithrix
Aotus
Cebus
Saimiri
Ateles
Cacajao
Perodicticus
Propithecus
Oryctolagus
Intron 1---\/---Exon 2
<-------Primer 16------->
Exon 2---\/---Intron 2

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Homo          -CATTAATTGAAGCTCATAATCTTATTGGAAAGACCAACAAAGATCTCAGAAATCATGGGTCGAGCTTGATGTTAGAACAGCAGACTTCTAGTGAGC-ATAACCAAAACTTACATGATTC 2362
Hylobates     -.............T.C........................................A.........T.........................-............A..T......
Callithrix    -.........CA..G.G.C.....C......C.T.......................T.......T....................G..-........GG..A..T......
Aotus         -TC.......G...G.A...A..........C.TG......................T.......T....................G..-........GG..A..T......
Cebus         -.........G...G.G..............C.T...T...........G....AT...T.A......T....................G-.-..G.....GG..A..T......
Saimiri       -.........G...G.G..............C.T...............G.........T.......T....................G..-........GG..A..TA.....
Ateles        -.........G...G.G..............C.T..................CA....T.......T....................G..-T.......GG..A..T......
Cacajao       -......C.A.G...G.G.............C.TG......................T.......T......G.............G..-........GG..A..T......
Perodicticus  A..A....A......T.G.C.A.G...A.C.G..T...GG....CT.......A.G.A....C......G.C......ACA....-...C....G..G.....GG.GC.CA..T......
Propithecus   A..A....G......T.G.C.A.A...A......T.....................-----AC......G.......GGA........C....G..-..G.....G..CA.TT......
Oryctolagus   A..A....G.G.TA.A.G.C.A.G......G...T..........T......G....A.A.ATGTT...T.......------..TC..C.A..GT.-..C.A...G..CA.TT.A....

Homo          AGAACTAGTGACAGTAAAGGACTAC--------------TAACAGCCTGAATTGGCTTAA--CTTTTCAGGAAA-TCTTGCCA-GAACTTGATG-TGTTTATCCCAGAGAATTGTATTAT 2463
Hylobates     .................G.......--------------.....................--............-........-..........-.........TG.........G....
Callithrix    ...G...............G....--------------..G..................--............-........-..........-.........TG......CA......
Aotus         ...G...............G....--------------..G..................--............-........-..........-.........TG......CA......
Cebus         ...G..............AG....--------------.GG.........GA......--............-......A.-..........-.........TG......CA......
Saimiri       ...G...............G...GATTGGCTAAACATT--------..............--............-....T...-..........-.........TG......CA......
Ateles        ...G........--......G....--------------..G..................--.......A....-........-..........-.........TG......C.......
Cacajao       ...G.......G........G....--------------..G...A..............--T...........-........-......A...-.........TG......C.......
Perodicticus  ....TG..G.......G..ACA...--------------......G.......TT....CCA...........TA.T...G..-......G....-GC.......T.......AA......
Propithecus   ...G...A.............GA...--------------C.G...G.......TA.A...AA-...........-.T...T..-..-..G....-.T.......TG......AAC.....
Oryctolagus   ....A....A.-G.....A.G...T--------------......T..A.------------T.......A...---------------....-.........TG-......AA.G....

                                                                                      /Alu
Homo          AGAATTGTAGACTTGTGAAAGAAGAA-TGAAATTTGGCTTTTGGTAGA-TGAAAGTCCATTTCAAGGAAATAGAAATGCC-----TTATTTTATGTGGGTCATGAT----AATTGAGGTT 2572
Hylobates     ..........................-.....................-...............................-----.....................----.......T...
Callithrix    .A...............C...GC...-.....-----...........A.-....C.TG......................-----.......-.A............----..C.......
Aotus         .A...............C...G....-.....-----...........-.A....C.TG......................-----.......-.A............----..C.......
Cebus         .A...............C...G....-.....-----...........-......C.TG......................-----.......-.A............----..C.......
Saimiri       .A...............C...G....-.....-----...........-......C.TG......................-----.......-.A.....C......----..C.......
Ateles        .A...............C...G....-................A....-......C.TG......................-----.......---A...........----..C.A.....
Cacajao       .A...............C...G....-.....................-.....CC.TG......................-----.......-.........G....----..CC......
Perodicticus  ....GA.C....C.C...C..AG....A..C...........G.A....-CC-.G.C.TG.C.........GG.C....CT.----T.....CC.........T..GG----.C.CC-A.G.
Propithecus   .T...AAC......C..C...G.T..A.TC.......A..........-....G.C.TG.C....A...GG.C....T..-----...C....C.C.....C....----...CT.A...
Oryctolagus   T.........CT....AC...G.A..A.AG......T..G..A.....-....G.C.T..C....T...GG.........AATGC-..........A.TTT.TC....GATG..-CT...AC

Homo          TAG-AAGA-GATTTTTGCAAAAAAAA------------------T-AAAAG----ATTTG-CTCAAAGAAAAATAAGACACATTTTCTAAAATATGTTAAATTTCCCATCAGTATTGTG 2665
Hylobates     ...A...C-......G..........AAAAAAAAAAAACAAAAC-.-.....----....T-...........................................T............
Callithrix    ...G....-...G....A.G.....------------------T.-...CT----....T-...................A.............CA....................
Aotus         ...G....-...G....A.......------------------T.G.....----....T-.......G...........A.............A.....................
Cebus         ...G....-...G....A.......------------------T.-.....----....T-...................A.............A...............A.....
Saimiri       C..G....-...G....A.......------------------T.-...G.----T...T-...................A.............A.........T.......C....
Ateles        ...G....-...G....A......-------------------T.-.....----....T-..................TA.............GA....................
Cacajao       ...G....-..AG....A.......------------------T.-.....----....T-...................A.............GA....................
Perodicticus  ...G..A.-T.-....TTTG..C.-------------------T.TTG...ACAT....TT..GG..C.......CAGA.A...C....G....CA..-----CT.GC....-------
Propithecus   ...A..A.-..-.....GTG.G...------------------T.T-....ATAG....T-...............CA...A...C.................T.TG..........
Oryctolagus   .T.ATTAGG..CGA..TATGCG.G.--------------------------------.CT-..TG..A.......CA...A.......G...A..A.......CT.--...........
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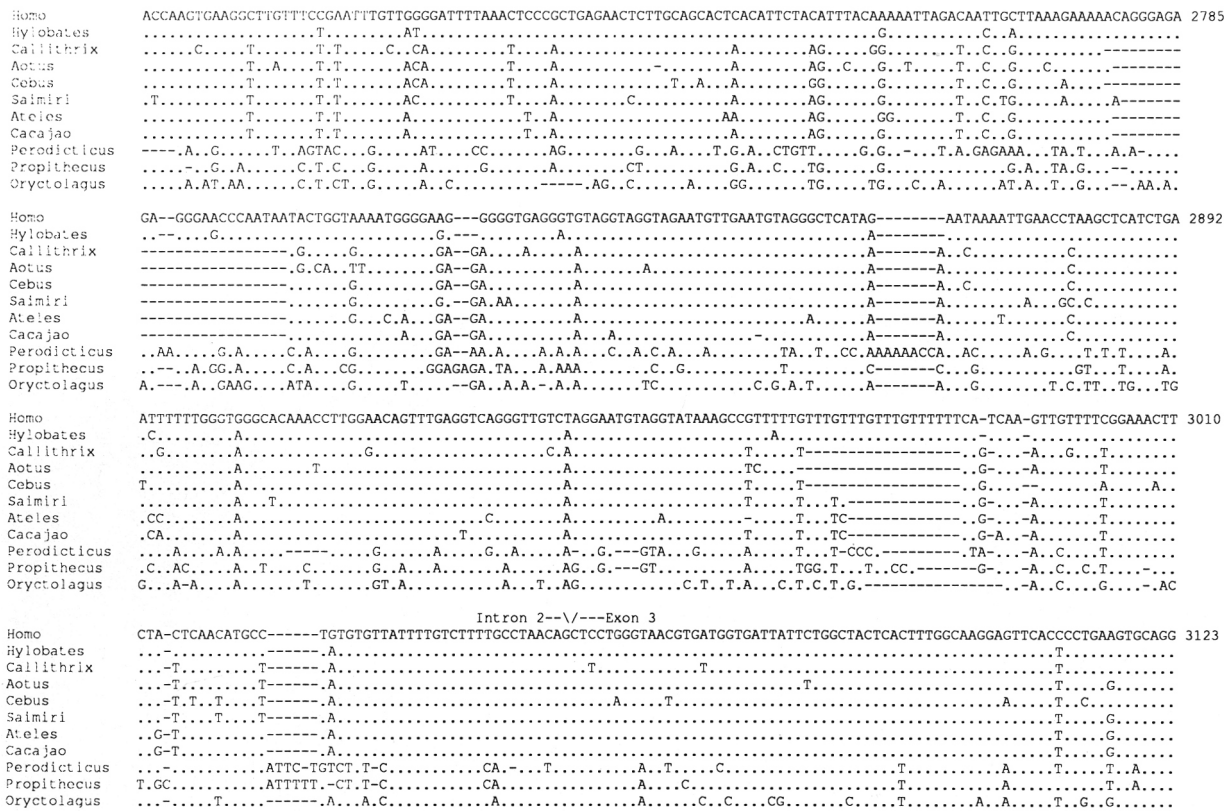
Homo ACCAAGTGAAGGCTTGTTTCCGAATTTGTTGGGGATTTTAAACTCCCGCTGAGAACTCTTGCAGCACTCACATTCTACATTTACAAAAATTAGACAATTGCTTAAAGAAAAACAGGGAGA 2785
Hylobates .....T.....AT.....G.....C..A.....
Callithrix .....C.....T.....T.T.....C..CA.....T....A.....A.....AG.....GG.....T..C..G.....---------
Aotus .....T..A....T.T.....ACA.....T....A.....-.....A.....AG..C...G..T.....T..C..G...C.....--------
Cebus .....T.....T.T.....ACA.....T....A.....T..A...A.....GG.....G.....T..C..G.....A....---------
Saimiri .T.....T.....T.T.....AC.....T....A.....C.....A.....AG.....G.....T..C.TG.....A.....A-------
Ateles .....T.....T.T.....A.....T..A.....AA.....AG.....GG.....T..C..G.....--------
Cacajao .....T.....T.T.....A.....T..A.....A.....AG.....G.....T..C..G.....--------
Perodicticus ----.A..G.....T..AGTAC...G.....AT....CC.....AG.....G...A....T.G.A..CTGTT.....G.G..-...T.A.GAGAAA...TA.T...A.A-....
Propithecus .....-..G..A.....C.T.C...G.....A.....G.....A.....CT.....G.A..C...TG.....G.....G.A..TA.G...--.....
Oryctolagus .....A.AT.AA.....C.T.CT..G.....A..C.....-----.AG..C.....A....GG.....TG.....TG...C..A.....AT.A..T..G...--.AA.A.
Homo GA--GGGAACCCAATAATACTGGTAAAATGGGGAAG---GGGGTGAGGGTGTAGGTAGGTAGAATGTTGAATGTAGGGCTCATAG--------AATAAAATTGAACCTAAGCTCATCTGA 2892
Hylobates ..--....G.....G.---.....A.....A--------.....
Callithrix ----------.G.....G.....GA--GA...A.....A.....A-------A..C.....C.....
Aotus ----------.G.CA..TT.....GA--GA.....A.....A.....A-------A.....C.....
Cebus ----------.....G.....GA--GA.....A.....A-------A..C.....C.....
Saimiri ----------.....G.....G.--GA.AA.....A.....A-------A.....A...GC.C.....
Ateles ----------.....G...C.A...GA--GA.....A.....A.....A-------A.....T.....C.....
Cacajao ----------.....A...GA--GA.....A...A.....-.....A-------A.....C.....
Perodicticus ..AA.....G.A.....C.A....G.....GA--AA.A....A.A.A...C..A.C.A...A.....TA..T..CC.AAAAAACCA..AC.....A.G....T.T.T....A.
Propithecus ..--..A.GG.A.....C.A...CG.....GGAGAGA.TA...A.AAA.....C..G.....T.....C-------C...G.....GT...T....A.
Oryctolagus A.---.A..GAAG....ATA....G.....T.....--GA..A.A.-.A.A.....TC.....C.G.A.T.....A-------A...G.....T.C.TT..TG...TG
Homo ATTTTTTGGGTGGGCACAAACCTTGGAACAGTTTGAGGTCAGGGTTGTCTAGGAATGTAGGTATAAAGCCGTTTTTGTTTGTTTGTTTTTTTCA-TCAA-GTTGTTTTCGGAAACTT 3010
Hylobates .C.....A.....A.....A.....-.....-.....
Callithrix ..G.....A.....G.....C.A.....T.....T----------..G-...-A...G...T.....
Aotus .....A.....T.....A.....TC....----------..G-...-A.....T.....
Cebus T.....A.....A.....T.....T----------..G....--.....A.....A..
Saimiri .....A...T.....A.....T.....T...T.----------..G-...-A.....T.....
Ateles .CC.....A.....C.....A.....-.....T...TC----------..G-...-A.....T.....
Cacajao .CA.....A.....T.....A.....T.....T...TC----------..G-A..-A.....T.....
Perodicticus ....A....A.A.....-----.....G.....A.....G..A.....A-..G.---GTA...G.....A.....T...T-CCC.---------.TA-...-A..C....T.....
Propithecus .C..AC.....A..T....C.....G..A...A.....A.....AG..G.---GT.....A.....TGG.T...T..CC.-------G-...-A..C..C.T....-...
Oryctolagus G...A-A....A.....T.....GT.A.....A...T..AG.....C.T..T.A...C.T.C.T.G.----------..-A..C....G....-.AC
Intron 2--\/---Exon 3
Homo CTA-CTCAACATGCC------TGTGTGTTATTTTGTCTTTGCCTAACAGCTCCTGGGTAACGTGATGGTGATTATTCTGGCTACTCACTTTGGCAAGGAGTTCACCCCTGAAGTGCAGG 3123
Hylobates ...-.....------.A.....T.....
Callithrix ...-T.....T------.A.....T.....T.....T.....
Aotus ...-T.....T------.A.....T.....T.....G.....
Cebus ...-T.T..T....T------.A.....A.....T.....A.....T..C.....
Saimiri ...-T....T....T------.A.....T.....G.....
Ateles ..G-T.....------.A.....T.....G.....
Cacajao ..G-T.....------.A.....T.....G.....
Perodicticus ...-.....ATTC-TGTCT.T-C.....CA.-...T.....A..T.....C.....T.....A.....T.....T..A....
Propithecus T.GC.....ATTTTT.-CT.T-C.....CA.....A....C.....T.....A.....T..A....
Oryctolagus ...-.....T.....------.A...A.C.....A.....A.....C..C....CG.....C.....T.....A..A.....T..G..G.....

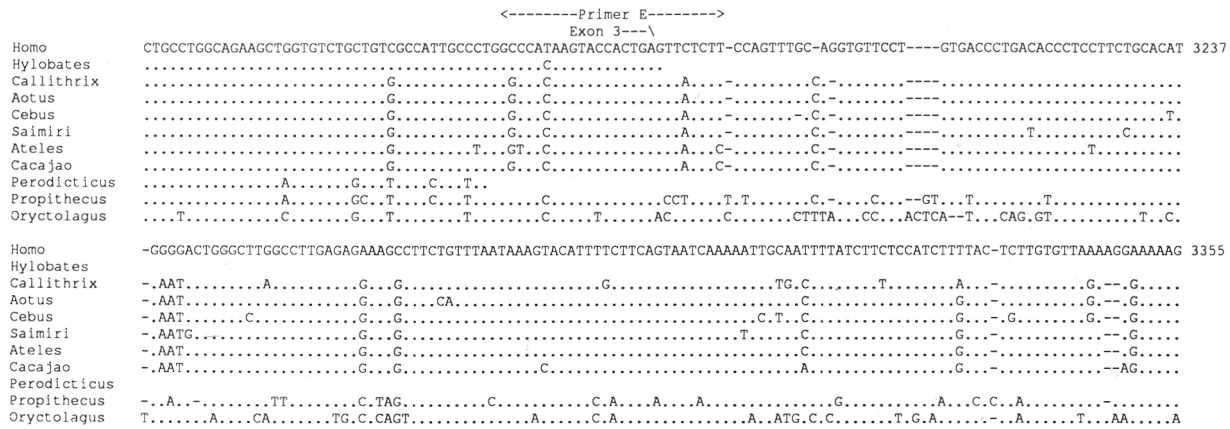

Fig. 2. Alignment of ε-globin and upstream flanking regions for eight primate species for which we report new sequence data. Sequences of human, capuchin, and rabbit are also included for reference with previously published data. The sequences shown are from *Homo sapiens*, *Hylobates syndactylus*, *Callithrix jacchus*, *Aotus azarae*, *Cebus albifrons*, *Saimiri sciureus*, *Ateles belzebuth*, *Cacajao calvus*, *Perodicticus potto*, *Propithecus verreauxi*, and *Oryctolagus cuniculus*. Blanks indicate missing data, dots represent nucleotides identical to human ones, and hyphens represent deletions. Annealing sites of PCR primers are indicated above the alignment, along with indications of the boundaries of protein coding regions of the three exons. Primer B anneals just beyond the 3′-most sequences in the alignment. Two long insertions were deleted from the figure. Ins indicates the location of a 298-bp insertion in the 5′ flanking sequence of *Homo*. A corresponding insertion also occurs in the genomes of *Pongo* and *Hylobates. lar.* Alu indicates the location of a 124-bp insertion of an Alu sequence in intron 2 of *Saimiri* (Schneider *et al.*, 1993). Numbers indicate cumulative number of base pairs in human, disregarding insertions not shown and deletions.

al. (1995). Annealing sites of PCR primers are shown in Figs. 1 and 2. We used primers A and B of Bailey *et al.* (1992) and E of Porter *et al.* (1995) to amplify the ε-globin gene proper. These primers span a region of approximately 2 kb from just upstream of exon 1 (primer A) to either the 3′ end of exon 3 (primer E) or just 3′ of the end of exon 3 (primer B). In addition to primers 15 and 16 of Porter *et al.* (1995), we made use of newly designed primers 15a (5′ GCA GTC ATT AAG TCA GGT GAA GAC TTC 3′) and 37 (5′ CTT CTC CTC AGC AGT AAA ATG CAC CAT 3′) to amplify the upstream sequences (Figs. 1 and 2). In most species, primers 15 and 15a anneal to a sequence approximately 1.6–1.9 kb upstream of the beginning of exon 1. Primer 16 anneals to a region within exon 2, and primer 37 is complementary to the extreme 5′ end of exon 1 (Figs. 1 and 2). The PCR product which includes the "upstream" (5′ flanking region) sequences overlaps by several hundred base pairs with the product which includes the "downstream" (ε-globin gene proper) region (Fig. 1).

We used one of the following two PCR thermal profiles as required to produce positive results: 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, and 3 min of 65°C, with a final extension of 10 min at 65°C; or 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, 20 sec at 55°C, 20 sec at 60°C, 20 sec at 65°C, and 1 min at 70°C, with a final extension of 10 min at 70°C.

Approximately 3.1–3.6 kb of continuous sequence was determined in the sample species in both the upstream and the downstream regions. After insertion of gaps to facilitate sequence alignment among the species, the data set extends over 4.3 kb, much of which is due to long inserts in the genomes of goat and squirrel monkeys.

Sequencing

We sequenced PCR products from most species directly by automated methods after they were purified with QIAquick PCR purification columns (QIAGEN). If PCR produced more than one size class of fragment, we isolated the desired fragment from an agarose gel via the QIAEX II gel extraction kit of QIAGEN. We cloned the amplified PCR product of *P. potto* into a Promega pGEM-T plasmid vector before sequencing. Using helper phage, we prepared single-stranded DNA from selected clones. We performed automated sequencing on either an ABI sequencer, Model 373A, or a Pharmacia A.L.F. sequencer. We prepared sequencing reactions for samples analyzed on the ABI sequencer with the ABI PRISM Dye terminator cycle sequencing kit, per the manufacturer's recommended protocols. We prepared the sequences analyzed on the Pharmacia se-

quencer with fluroescent-labeled primers via the Thermo Sequenase fluorescent-labeled primer cycle sequencing kit from Amersham and the protocol recommended for one-dye primer labeling. We subjected the Amersham sequencing reactions to the following thermal cycles: 30 cycles of 20 sec at 95°C, 20 sec at 60°C, and 1 min at 75°C, followed by 20 cycles of 20 sec at 95°C, and 1 min at 75°C. We sequenced the cloned potto gene by automated methods as described above and also by standard procedures using the Sequenase (Version 1.0) kit of Amersham, and long polyacrylamide gels (Slightom *et al.*, 1991). We examined at least three clones and determined consensus sequences via the ESEE sequence editor (Cabot and Beckenbach, 1989), protected mode version, 1995.

Sequence Alignment

We performed initial sequence alignment with the Clustal V program (Higgins *et al.*, 1992), using a fixed gap penalty of 35 and a floating gap penalty of 3 to lengthen each gap by 1 base pair. These parameters, compared to a range of others, yielded the best initial alignment by the parsimony criterion. Nevertheless, in some cases, the alignment produced by these parameters could still be improved by inspection. In doing so using the sequence editor, the resulting alignment to produced a maximum-parsimony tree shorter than the unmodified alignment. The final alignment varied slightly from the alignment used by Porter *et al.* (1995).

Phylogenetic Analysis

We generated maximum-parsimony (MP) trees from aligned nucleotide sequences using the program DNAPARS of the PHYLIP package (Felsenstein, 1989). We treated all insertions and deletions, regardless of length, as a single nucleotide substitution. To determine confidence in the various nodes of the MP trees, we determined bootstrap percentages SEQBOOT, DNAPARS, and CONSENSE of PHYLIP. These percentages reflect the number of replications in which a particular node was supported in an analysis of randomly sampled portions of the data. As an additional measure of confidence, we determined the strength of grouping values by calculating the shortest tree not compatible with each interior node of the MP tree, via PAUP (Swofford, 1993). This procedure calculates the minimum additional length that must be added to the MP tree(s) to disrupt the monophyly of each clade in the MP tree. This strength of grouping value is identical to that presented by Porter *et al.* (1995) using other programs.

To determine the effect of including data from additional species, or of adding additional upstream sequence data of the sample species, we performed MP analysis on several subsets of all available ε-globin data. Using all 42 taxa in the study, we analyzed (1) the downstream sequences combined with the upstream sequences for 14 species represented in both portions of the genome and (2) the downstream sequences only. Using only the 14 species in the upstream sequence, we analyzed (1) a tandem alignment of both upstream and downstream data, (2) the upstream data only, and (3) the downstream data only.

We constructed neighbor-joining (NJ) trees based on noncoding sequences, and using the appropriate programs of PHYLIP. We modified the alignments constructed for the MP analysis for NJ analysis by deleting the protein coding regions and truncating a portion of both 5′ and 3′ ends so that all sequences began and ended at nearly the same point. Accordingly, the branch lengths in the NJ trees provide a measure of the amount of nucleotide substitution that occurred in noncoding sequences along each lineage. We constrcted neighbor-joining trees using (1) noncoding downstream sequences of all 42 species and (2) both upstream and noncoding downstream sequences for the 14 species in the upstream data set.

We used branch lengths of the neighbor-joining trees to calculate estimated divergence times according to a local molecular clock (Bailey *et al.*, 1991, 1992b).

RESULTS

We deposited DNA sequences in GenBank under the accession numbers in Table I. Newly determined sequences are in Fig. 2, aligned with those of human, capuchin, and rabbit. The full alignment of 42 taxa is available from the authors on request, or can be accessed through the internet at http://ns.med.wayne.edu/.

Thirteen species of primates and one species of rabbit have now been sequenced across approximately 1.6–1.9 kb ending at a point just 5′ of the coding region of the ε-globin gene. We determined the maximum-parsimony trees (Fig. 3) for these 14 species, using the complete data set, including both the ε-globin gene and the 5′ flanking region. We also determined maximum-parsimony trees for them, using (1) only the sequences upstream of the ε-globin gene and (2) only the downstream sequences. We divided the upstream and downstream sequences at the 5′ end of the annealing site of primer A (Figs. 1 and 2). The trees (not shown) produced only from the upstream or downstream data a similar to the tree generated from the complete data set, except that in the upstream tree, *Cacajao* is

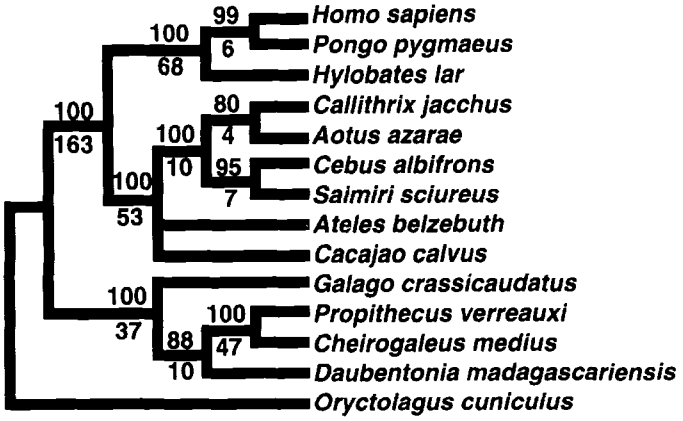

Fig. 3. Consensus of two maximum-parsimony trees for 14 mammalian species based on aligned ε-globin sequences and 5′ flanking sequences. Numbers above each node represent percentages of bootstrap replications supporting that node (1000 replications). Numbers below each node are strength of grouping values.

the sister group to the remaining ceboids, with *Ateles* joining the clade formed by *Callithrix*, *Aotus*, *Cebus*, and *Saimiri*, and in the most parsimonious tree generated from the downstream data, the ceboids form a tree ((*Ateles*, *Cacajao*), (*Saimiri*, (*Aotus*, (*Cebus*, *Callithrix*)))), and *Daubentonia* joined with *Galago* rather than the lemuroids.

The consensus maximum-parsimony tree for 40 taxa of primates (with goat and rabbit as outgroups) is shown in Fig. 4. It is based on an alignment of all available sequence data: the ε-globin gene proper for all species, as well as the upstream flanking region for the 14 species represented in that portion of the genome. We determined a similar tree (not shown) for the same 42 taxa using only the downstream data. This tree differed from that produced by the complete data set (Fig. 4) in that the orangutan is the sister group to a clade consisting of gibbon, gorilla, chimpanzee, and human, and the aye-aye (*Daubentonia*) joins the lorisoids (*Perodicticus*, *Nycticebus*, and *Galago*) rather than the lemuroids.

Neighbor-joining trees are shown in Figs. 5 and 6.

DISCUSSION

Our study expands the previously examined ε-globin data set (Schneider *et al.*, 1993, 1996; Harada *et al.*, 1995; Porter *et al.*, 1995) in

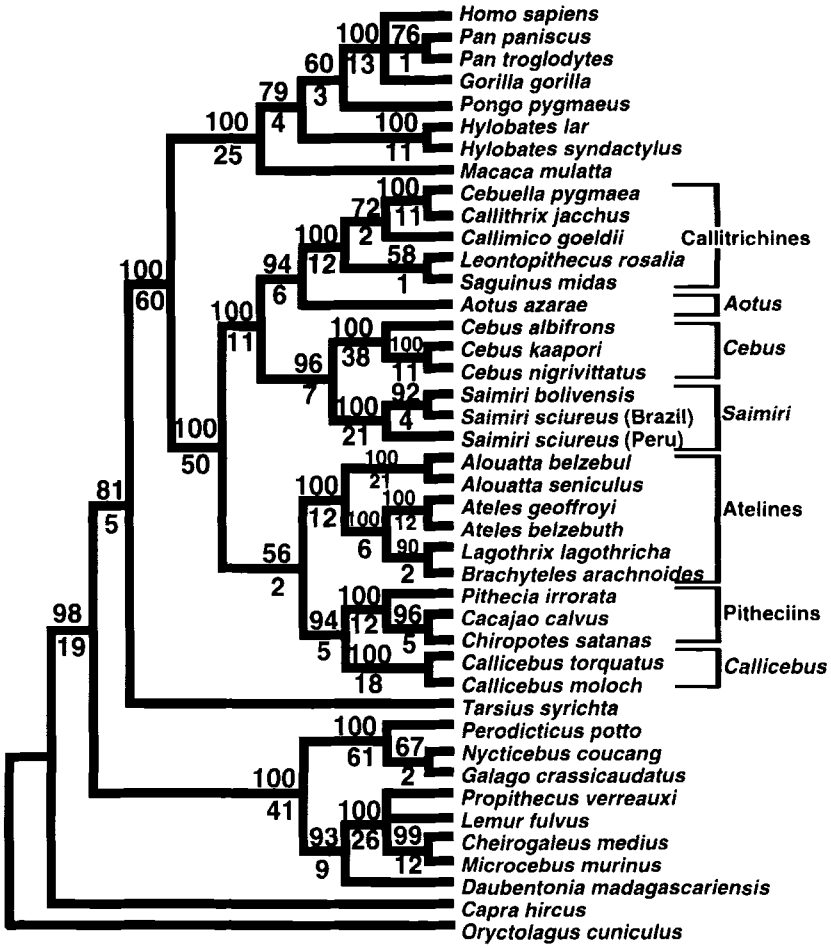

Fig. 4. Consensus of four maximum-parsimony trees for 42 mammalian taxa based on aligned ε-globin sequences and (for 14 of the species) 5′ flanking sequences. Numbers above each node represent percentages of bootstrap replications supporting that node (1000 replications). Numbers below each node are strength of grouping values. Brackets to the right of the tree designate the seven monophyletic ceboid clades.

two directions. First, we have increased the number of species represented in both the upstream and the downstream regions. Second, we have added upstream sequence data for species previously represented only in the downstream region. For the 14 species in the upstream data set, we analyzed the upstream and downstream data sets separately (trees not shown),

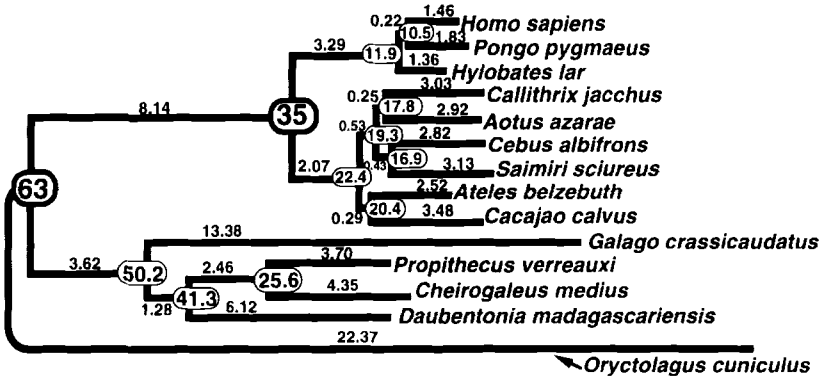

Fig. 5. Neighbor-joining tree for 14 mammalian taxa based on aligned noncoding sequences associated with the ε-globin locus and 5′ flanking sequences. Numbers on each branch represent branch length expressed in percentage nucleotide change. Encircled numbers at branch points represent estimated divergence time Ma, based on 63 Ma for the strepsirhine–haplorhine split (Gingerich and Uhen, 1994) and 35 Ma for the platyrrhine–catarrhine split (Gingerich, 1984; Fleagle, 1988). These fixed points are indicated by numbers in large print.

as well as the upstream and downstream data combined (Fig. 3). This expanded data set allows us to determine with greater certainty, the amount of confidence that can be placed in the various phylogenetic hypotheses.

Hominoids

The results of the present study generally support the traditional relationships among the hominoid primates and, as expected, are congruent with previous results of studies involving the ε-globin gene. These ε-globin data provide no resolution of the chimpanzee/gorilla/human trichotomy. The (*Homo*, (*Pan*, *Gorilla*)) and ((*Homo*, *Pan*), *Gorilla*) trees are equally parsimonious, and the consensus of these maximum-parsimony trees is shown as a trichotomy in Fig. 4. In extensive stretches of noncoding sequence from the β-globin gene cluster, the ((*Homo*, *Pan*), *Gorilla*) grouping is favored (Goodman *et al.*, 1994). The present ε-globin data set differs from previous ones by the addition of sequence data from siamang (*Hylobates syndactylus*) in addition to white-handed gibbon (*H. lar*). The siamang is quite distinct from other species of lesser apes, and has sometimes been placed in a distinct genus, *Symphalangus*. The downstream data, considered alone, support the tree (((*Homo*, *Pan*, *Gorilla*), *Hylobates*), *Pongo*) (Harada *et al.*, 1995). However, a tree of (((*Homo*, *Pan*, *Gorilla*), *Pongo*), *Hylobates*)

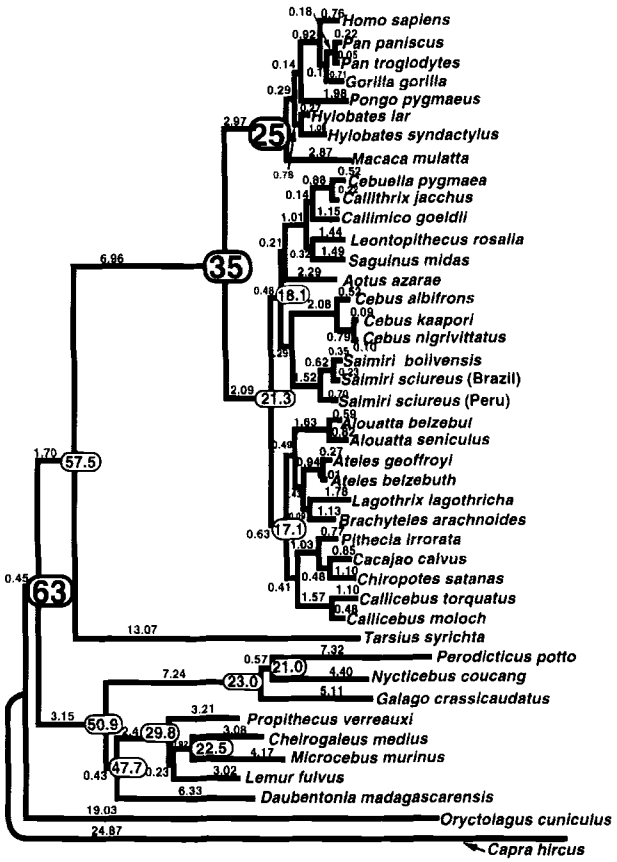

Fig. 6. Neighbor-joining tree for 42 mammalian taxa based on aligned noncoding sequences associated with the ε-globin locus. Numbers on each branch represent branch length expressed in percentage nucleotide change. Encircled numbers at branch points represent estimated divergence time Ma, based on 63 Ma for the strepsirhine–haplorhine split (Gingerich and Uhen, 1994), and 35 Ma for the platyrrhine–catarrhine split, and 25 Ma for the hominoid-cercopithecoid split (Gingerich, 1984; Fleagle, 1988). These fixed points are indicated by numbers in large print. Divergence times not shown here are in Table II.

is supported when the upstream data are included (Fig. 4) or, in the NJ tree, when the protein coding sequences are deleted from the downstream data (Fig. 6). The tree of (((*Homo*, *Pan*, *Gorilla*), *Pongo*), *Hylobates*) is more congruent with other evidence (Goodman *et al.*, 1990, 1994; Bailey *et al.*,

1992), and we regard the tree of (((*Homo, Pan, Gorilla*), *Hylobates*), *Pongo*) to be an anomaly peculiar to the downstream coding sequences of the ε-globin locus.

Ceboids

Previous morphological and molecular studies support grouping the 16 living genera of New World monkeys into seven monophyletic clades. However, the relationships of the genera within these clades and the relationships among them are arguable. The seven clades as defined in Schneider *et al.* (1993) are (1) the callitrichines, including *Cebuella* (pygmy marmoset), *Callithrix* (marmosets), *Callimico* (Goeldi's monkey), *Leontopithecus* (lion tamarin), and *Saguinus* (tamarins); (2) the atelines, including *Alouatta* (howling monkeys), *Ateles* (spider monkeys), *Lagothrix* (woolly monkeys), and *Brachyteles* (woolly spider monkey); (3) the pitheciins, including the genera *Pithecia* (sakis), *Cacajao* (uakaris), and *Chiropotes* (bearded sakis); (4) the genus *Aotus* (night monkeys); (5) the genus *Cebus* (capuchins); (6) the genus *Saimiri* (squirrel monkeys); and (7) the genus *Callicebus* (titi monkeys).

Neotropical primates have commonly (Simpson, 1945; Nowak and Paradiso, 1983; Groves, 1993) been classified into families Callitrichidae (marmosets and tamarins) and the Cebidae (the remainder of the neotropical primates). However, this classification does not reflect the actual phylogeny of New World monkeys. Fleagle (1988) divided the Cebidae by recognizing a separate family—Atelidae—for the atelines and pitheciins.

Morphological studies (Rosenberger, 1984; Ford, 1986; Kay, 1990) have produced a variety of ceboid trees. Like Feagle (1988), Ford (1986) and Rosenberger (1984), agreed on a close relationship of the atelines and pitheciins in the same major branch of the ceboids, although Rosenberger (1984) placed *Aotus* and *Callicebus* within this branch, closest to the pitheciins. Rosenberger (1984) placed *Cebus* and *Saimiri* as the sister group to the callitrichines, while Ford (1986) placed the callitrichines as the sister group to the ateline-pitheciin clade with the remaining four genera being more anciently derived. Like Rosenberger (1984), Ford (1986) placed *Saimiri* either as a sister group to *Cebus* or instead as the sister group to *Aotus* and *Callicebus*. Kay (1990) placed the atelines and callitrichines in a clade with *Aotus* and *Saimiri*, with *Callicebus*, *Cebus*, and the pitheciins branching from the basal portion of the tree.

Past studies of the ε-globin gene (without the upstream region) supported the monophyly of each of the seven clades (Schneider *et al.*, 1993, 1996; Harada *et al.*, 1995; Porter *et al.*, 1995). These ε-globin gene results

divided the ceboids into two major branches. The first consists of the callitrichines, *Aotus*, *Cebus*, and *Saimiri*. The relationships among these four clades was not resolved by Schneider *et al.* (1993), but a subsequent study by Harada *et al.* (1995), included additional species of both *Cebus* and *Saimiri*, showed that they form a monophyletic group, with *Aotus* being most closely related to the callitrichines. Schneider *et al.* (1993, 1996) and Harada *et al.* (1995) found that the ε-globin gene sequences grouped the remaining eight genera into the second branch of the ceboids, with the atelines being a sister group to a clade consisting of the pitheciins and *Callicebus*.

Porter *et al.* (1995) analyzed nucleotide sequences from seven primate species in the complete data set associated with the ε-globin gene and the upstream region. They include four anthropoids—human, orangutan, white handed gibbon, and capuchin—and three strepsirhines: bush baby, mouse lemur, and aye-aye. Upstream data are now available from five additional ceboid species: marmoset, squirrel monkey, night monkey, uakari, and white-bellied spider monkey. They, along with the white-fronted capuchin (*Cebus albifrons*) previously studied, represent six of the seven recognized ceboid clades. We used sequences from them to determine if previous ceboid phylogenies based on ε-globin sequences (Schneider, *et al.*, 1993, 1996; Harada *et al.*, 1995; Porter *et al.*, 1995) are supported by the upstream data.

For the six ceboid species now represented in the upstream region, we found that MP analysis of the combined upstream and downstream sequences produced (Fig. 3) a branching pattern that is identical to that from a more restricted data set of the ε-globin gene proper (Schneider *et al.*, 1993, 1996; Harada *et al.*, 1995; Porter *et al.*, 1995). The addition of upstream data for six ceboid species greatly strengthened support for several clades but weakened support for the pitheciin-ateline association.

Upstream sequences increased support for the *Cebus–Saimiri* clade from 78% (Harada *et al.*, 1995) to 95% (Fig. 3) or 96% (Fig. 4) of the bootstrap replications, and the strength of grouping for the clade increased from 3 (Harada *et al.*, 1995) to 7 (Figs. 3 and 4). The addition of upstream sequences increased support for the family Cebidae [as defined by Harada *et al.*, (1995), including *Cebus*, *Saimiri*, *Aotus*, and the callitrichines] to 100% (Figs. 3 and 4) from 94–98% (Schneider *et al.*, 1993; Harada *et al.*, 1995; Porter *et al.*, 1995) of the bootstrap replications. The strength of grouping for the cebids increased from 5 (Harada *et al.*, 1995) to 10 (Fig. 3) or 11 (Fig. 4).

The position of *Aotus* within the Cebidae was not resolved by Schneider *et al.* (1993) or Porter *et al.* (1995). The analysis of Harada *et al.* (1995) grouped *Aotus* as the sister group to the callitrichines, but with only minimal support. Confidence in the callitrichine–*Aotus* clade has in-

creased from a strength of grouping of 2 (Harada *et al.*, 1995) to 4 (Fig. 3) or 6 (Fig. 4). Although the bootstrap support for this group slightly decreased from 85% (Harada *et al.*, 1995) to 80% when the data set with only 14 species (Fig. 3), it increased to 94% when we used the data set with all 42 species (Fig. 4). Support for the atelines has increased from 8 (Harada *et al.*, 1995) to 12 (Fig. 4), with the bootstrap values indicating extremely strong support in both studies.

Considered alone, the upstream data (tree not shown) support the same tree of ((*Cebus*, *Saimiri*), (*Aotus*, *Callithrix*)) that is supported by the combined data (Fig. 3). The upstream data support the clade of *Cebus–Saimiri* with a strength of grouping of 6, and with 96% of bootstrap replications. The clade of *Aotus–Callithrix* is supported by this data set with a strength of grouping of 4, and with 85% of bootstrap replications. This level of support is comparable to that in the combined data set (Fig. 3). In contrast, the downstream data support a clade consisting of these same four genera, but with very low support (51–54% bootstrap values, and strength of grouping values of 1) for the internal nodes within the clade. In general, it appears that the upstream sequences were more successful in resolving relationships among major groups of ceboids.

A comparison of the trees produced by the downstream data only shows, except in the ceboids, an identical branching sequence regardless whether 14 or 42 taxa were included. When we analyzed only 14 species, the downstream data alone did not resolve the relationships among *Aotus*, *Callithrix*, *Cebus*, and *Saimiri*. However, with the inclusion of additional species, the downstream data produced a ceboid tree identical in topology to that produced by the complete data set. Accordingly, it would appear that additional species can help to resolve some relationships. This was illustrated by the results of Harada *et al.* (1995), who support a clade of *Cebus–Saimiri* by including additional species that had not been studied by Schneider *et al.* (1993). Recall that the tree including 42 taxa in the downstream region differs in only two respects from the same tree (Fig. 4) with upstream sequences added: *Daubentonia* weakly joins the lorisoids, and the positions of *Hylobates* and *Pongo* are interchanged.

Although the grouping of the pitheciins and *Callicebus* with the atelines was supported by data from the ε-globin gene proper (Schneider *et al.*, 1993; Porter *et al.*, 1995), sequences of intron 1 of the interphotoreceptor retinoid binding protein (IRBP) gene (Harada *et al.*, 1995; Schneider *et al.*, 1996; Barroso *et al.*, 1997) provide evidence joining the clade of *Callicebus*-and the pitheciins to a clade formed by *Aotus*, *Cebus*, *Saimiri*, and the callitrichines. An MP analysis of tandemly combined ε-globin and IRBP sequences (Schneider *et al.*, 1996) supports the clade of atelines, pitheciins, and *Callicebus*, but only very weakly. When all species are included in the

data—the upstream and downstream regions for the 14 species and the downstream region for the remaining 28 taxa—the clade is supported by only 56% of bootstrap replications and a strength of grouping of 2 (Fig. 4). When the analysis includes only the 14 species represented in the complete data set (Fig. 3), the tree supporting an ateline–pitheciin clade is only one of two equally parsimonious trees. *Callicebus* has not been sequenced in the 5′ flanking region of ε-globin so we are unable to confirm the pitheciine clade of *Callicebus* and pitheciins.

The support for a pitheciine–ateline affinity is undermined both by analysis of the upstream sequence in the ε-globin data set and, also, by IRBP sequences (Harada *et al.*, 1995; Schneider *et al.*, 1996; Barroso *et al.*, 1997). Because the enlarged ε-globin data set does not support the grouping of *Callicebus*, pitheciins, and atelines, it is possible that the previous result was an anomaly based on the limited data only from the ε-globin gene proper. The sequence data upstream of ε-globin and the IRBP data both weaken support for this clade. However, our additional upstream data strengthen support for the grouping of *Cebus* and *Saimiri* and for the clade consisting of *Cebus, Saimiri, Aotus*, and the callitrichines. Based on the available molecular evidence, we regard as unresolved, a basal trichotomy of three major clades of ceboids: (1) *Callicebus* and the pitheciins; (2) the atelines; and (3) *Aotus*, *Cebus*, *Saimiri*, and the callitrichines.

The ε-globin and IRBP intron 1 data strongly support recognizing clade 3 as a monophyletic family, Cebidae. Analysis of the ε-globin gene proper supports the joining of clades 1 and 2 into a the Atelidae, but this relationship receives no support from either IRBP or the 5′ flanking region of ε-globin. Until this trichotomy can be resolved, it is probably best to divide the ceboids into three families as defined above, with clade 1 being the Pitheciidae, and clade 2 the Atelidae.

Strepsirhines

Our data strongly support the grouping of lemuroids and lorisoids into the strepsirhines. The tarsiers, represented only by the Philippine species, *Tarsius syrichta*, joined fairly strongly with the anthropoids, rather than with the strepsirhines. The ε-globin data also provide strong support for monophyletic Malagasy (lemuroid) and continental (lorisoid) clades within the strepsirhines. Indeed, the lorisoid clade was supported by a strength of grouping of 61, the greatest support for any node on the tree (Fig. 4). The bootstrap support for the Malagasy clade decreased to 88% (Fig. 3), with the addition of Verreaux's sifaka (*Propithecus*) to the upstream data set. However, the strength of grouping indicated strong

support for this lemuroid clade. Contrary to some behavioral and morphological evidence (Charles-Dominique and Martin, 1970; Szalay and Katz, 1973; Groves, 1974; Schwartz and Tattersal, 1985), our data strongly support all primates of Madagascar having descended from a single colonizing lineage, with the aye-aye being the most primitively derived member of the Malagasy clade. This result supports the findings of Yoder (1994) and Yoder *et al.* (1996). In the full data set (Figs. 3 and 4), the cheirogaleids join very strongly with *Lemur* and *Propithecus*. The downstream data place *Daubentonia* very weakly with the lorisoids, instead of the lemuroids. The evidence for monophyly of Malagasy strepsirhines therefore comes principally from upstream data, rather than from sequences of ε-globin proper.

The analysis of the complete data set also included dwarf lemur (*Microcebus*) and brown lemur (*Lemur fulvus*), and in this analysis, the cheirogaleids joined strongly with the other lemurs of Madagascar [including representatives of the lemuroid families Indriidae (*Propithecus*) and Lemuridae (*Lemur*)], but no resolution was obtained among the families Cheirogaleidae, Indriidae, and Lemuridae.

The lorisoids are now represented in the ε-globin gene proper by three species: potto (*Perodicticus potto*), slow loris (*Nycticebus coucang*), and thick-tailed greater bush baby (*Galago crassicaudatus*). Fleagle (1988) and Groves (1993) follow the classification of Simpson (1945) in dividing the lorisoids into families (or subfamilies) including the pottos and lorises of Africa and Asia, respectively, in one group and the bush babies of Africa in the other. However, the potto–loris association has not received universal support, and is not corroborated by our data; the slow loris appears to be most closely related to the bush baby, rather than to the potto. However, support for the clade is weak. Based on immunological data, Dene *et al.* (1976b) divided the lorisoids into three families represented by *Galago*, *Perodicticus*, and *Nycticebus*. Dene *et al.* (1976a) also followed the three-family arrangement and presented evidence that the galagids and lorisids are more closely related and that *Perodicticus* represents the earliest diverging branch of the lorisoids. Sarich and Cronin (1976) also recognized three distinct lorisoid lineages but were unable to resolve the relationships of these taxa via albumin and transferrin. Our MP results are consistent with the findings of Dene *et al.* (1976a), but the data are insufficient to falsify the potto–loris clade. Given the difficulty in resolving the relationships within the lorisoids, it seems likely that the three major lineages diverged at nearly the same time, and it is probably best to recognize three families.

Rates of Molecular Evolution

The sequences used to construct the NJ trees (Figs. 5 and 6) are from noncoding regions only. A small portion of the sequence used in the analysis shown in Fig. 5 includes the ε-globin promoter region, which functions in the initiation of transcription. However, the majority of the sequences in the NJ analysis would be expected to be under few selective pressures and, thus, would accumulate mutations at a rate largely unaffected by selection.

Rates of molecular evolution are not identical in the various primate lineages (Britten, 1986; Goodman, 1985; Koop *et al.*, 1989; Bailey *et al.*, 1991; Porter *et al.*, 1995). Via DNA hybridization studies, Bonner *et al.* (1980, 1981) found low substitution rates in lemuroids versus other primates. This finding agrees with results from analysis of noncoding sequences from the β-globin gene cluster (Koop *et al.*, 1989), including previous studies of the ε-globin gene (Porter *et al.*, 1995). The slower rate of nucleotide substitution of noncoding sequences of lemuroid ε-globin is evidenced by the shorter branch lengths in the NJ trees (Figs. 5 and 6). Koop *et al.* (1989) suggested that the difference may be due to the fact that the lemuroids may have encountered less selective competition in the insular environment of Madagascar. The lorisoids, on the other hand, evolved in a continental environment, with more competition from other species. A higher mutation rate may have been adaptive in these circumstances.

The rate of molecular evolution also appears to have occurred at different rates during various periods of evolutionary history, with nucleotide substitution often occurring at a faster rate in earlier lineages (Bailey *et al.*, 1991). The effects of this phenomenon can be seen in the branch lengths of the NJ trees (Figs. 5 and 6). The branch lengths of both trees indicate that fewer substitutions per year have become incorporated during the past 35 million years since the platyrrhine–catarrhine split, than occurred during the preceding 28 million years since the divergence of the strepsirhines.

Estimated Divergence Times

We estimated divergence times for some lineages (Figs. 5 and 6) based on local molecular clock calculations as described by Bailey *et al.* (1991, 1992b) and Schneider *et al.* (1993). They are based on branch lengths in the NJ trees. We calibrated strepsirhine divergence on a date of 63 Ma (million years ago) for the haplorhine-strepsirhine split (Gingerich and Uhen, 1994). Other estimations of this divergence are as ancient as 80 Ma (Sarich and Cronin, 1976; Martin, 1993). However, we selected the 63 Ma

date to provide a direct comparison with similar calculations based on this reference date by Yoder *et al.* (1996), who used cytochrome *b* sequences. Of course, if an earlier date is used for the baseline, then the estimated divergence times calculated from it will be proportionally more ancient. Within anthropoids, we calibrated the local clock using a date of 35 Ma for the divergence of platyrrhines and catarrhines and 25 Ma for the divergence of hominoids and cercopithecoids (Gingerich, 1984; Fleagle, 1988). Compared with a global clock, the local molecular clock gives more reliable estimates of primate branching times (Bailey *et al.*, 1991, 1992b).

Although the rate of nucleotide substitution varies greatly between lorisoid and lemuroid lineages, we do not know whether molecular evolution in the ancestral strepsirhines occurred at the fast rate typical of lorisoids or the slower rate typical of lemuroids. If the lemuroids experienced a slowdown as a result of their insular environment, then the ancestral strepsirhines would have incorporated mutations at a rate more similar to that seen in the descendants that remained in a continental environment. The branch lengths seen in the NJ trees (Figs. 5 and 6) suggest that the rate of molecular evolution in lorisoids is more or less comparable to that in primates general. Contrarily, lemuroids, evolve at a conspicuously slower rate. For these reasons, we estimated the time of the lemuroid-lorisoid split based on the average rate of substitution in lorisoid lineages from a haplorhine–strepsirhine divergence 63 Ma to the present. This procedure gives a minimum estimation of the time between the haplohrine–strepsirhine and the lemuroid–lorisoid divergences. Based on rates of substitution in noncoding sequences associated with ε-globin, we estimate the lemuroid–lorisoid split to be 50.2 Ma, based on upstream and noncoding downstream sequences (Fig. 5), or 50.9 Ma, based on noncoding downstream sequences only (Fig. 6). If the ancestral strepsirhine incorporated substitutions at a rate more similar to that seen in the lemuroid lineages, then the lemuroid–lorisoid split would be more recent than we have estimated. Our estimation places this split within the Eocene, and considerably more recent than the 62 Ma calculated by Yoder *et al.* (1996) based on cytochrome *b* sequences.

Sequences of cytochrome *b* (Yoder, 1996) place the most recent common ancestor of Malagasy primates ≤54 Ma. Assuming the lemuroid–lorisoid divergence to be 50.2 (Fig. 5) or 50.9 (Fig. 6) Ma, we used the average rate of evolution in lemuroid lineages to estimate the time of divergence of *Daubentonia* from other lemuroids.—between 41.3 (Fig. 5) and 47.7 (Fig. 6) Ma, which places it well within the Eocene, versus the Paleocene date indicated by the cytochrome *b* data.

Using the average substitution rate in the lorisoid lineage, we calculated the latest common ancestor of living lorisoids to be 27 million years

more recent than the lemuroid–lorisoid divergence, or 23.0 Ma (Fig. 6). Again, this is considerably more recent than the 55-Ma date estimated from cytochrome *b* (Yoder *et al.*, 1996). A fossil lorisoid lived in the early Oligocene (Simons *et al.*, 1987). It probably represents an undifferentiated lorisoid precedent to diversification of the lineages leading to modern lorisoid families, but after the separation of lemuroid strepsirhines.

The dates calculated from noncoding ε-globin sequences differ considerably from those calculated from cytochrome *b* (Yoder *et al.*, 1996). The difference can most likely be attributed to variation in the rates of nucleotide substitution in nuclear versus mitochondrial sequences. If substitutions were incorporated at a faster rate in mitochondrial as compared with nuclear sequences, then the length of ancient branches would be underestimated by the use of mitochondrial data sets. The unusually ancient dates postulated by Yoder *et al.*'s (1996) data may be an artifact of this effect.

Divergence dates estimated within the ceboid radiation (Figs. 5 and 6, Table II) are similar to those calculated by Schneider *et al.* (1993) from downstream ε sequences. However, when upstream sequences were included, the estimated divergence date for the separation of *Ateles* and *Cacajao* increased to 20.4 Ma (Fig. 5) from the 17.1 Ma found by us (Fig. 6) and by Schneider *et al.* (1993) based on downstream sequences only.

Bailey *et al.* (1992b) performed local molecular clock divergence estimates on hominoids. Their analysis was also calibrated with the 25-Ma divergence date for the separation of hominoids from the Old World monkeys but included data from the γ-globin and ψη-globin gene regions. Like us, they dated the division of bonobos and chimpanzees in the vicinity of 3 Ma (Table II). We included data from a second species of gibbon, which had not previously been included in phylogenetic studies of globin genes. We estimate a fairly ancient date (9.9 Ma; Table II) for the separation of *Hylobates syndactylus* from *H. lar*, which are thought to represent the most anciently separated gibbon taxa.

Compared with the γ- and ψη-globin noncoding sequences (Bailey *et al.*, 1992b), the ε-globin data suggest a slightly earlier date (21.3 Ma; Table II) for the divergence of the lesser apes from the great ape–human lineage and a substantially earlier date (19.6 Ma; Table II) for the split of the African and Asian lineages within the great apes. The estimates of Bailey *et al.* (1992b) (17.4–19.1 Ma for the gibbon–great ape divergence and 13.6–14.9 Ma for the divergence of the orangutan from the African hominoids) are probably more reliable because they are based on a much larger sample of noncoding sequences from the β-globin cluster. Because no cercopithecoid datum was available for analysis,

Table II. Estimated Times of Origin of Selected Primate Clades, Based on Local Molecular Clock Calculations[a]

Clade	Ma
Pan	2.7
Pan–Gorilla–Homo	7.7
Pan–Gorilla–Homo–Pongo	19.6
Hylobates	9.9
Hominoids	21.3
Cebuella–Callithrix	2.7
Cebuella–Callithrix–Callimico	9.0
Leontopithecus–Saguinus	8.1
Callitrichines	10.1
Aotus–callitrichines	16.7
C. kaapori–C. nigrivitattus	0.5
Cebus	4.3
S. bolivensis–S. sciureus (Brazil)	1.8
Saimiri	5.8
Cebus–Saimiri	16.3
Alouatta	4.1
Ateles	1.3
Lagothrix–Brachyteles	9.7
Lagothrix–Brachyteles–Ateles	10.2
Atelines	13.6
Cacajao–Chiropotes	5.0
Pithiciins	7.9
Callicebus	4.8
Callicebus–pithiciins	14.5

[a]Divergence dates in millions of years ago (Ma) are based on local molecular clock calculations of branch lengths in Fig. 6. Dates are based on a fixed estimate of 25 Ma for the hominoid–cercopthecoid branch point and 35 Ma for the catarrhine–platyrrhine divergence (Gingerich, 1984; Fleagle, 1988). Other estimated divergence dates are in Fig. 6.

hominoid divergence times in Fig. 5 are likely to be less reliable due to the clock being calibrated at the platyrrhine–catarrhine branch point, rather than the more recent calibration used in the analysis shown in Fig. 6. If nucleotide substitution occurred at a faster rate in earlier lineages, then we would expect that dates estimated for the more recent nodes of the tree would be more recent than the actual times of divergence. Because they are substantially removed from the time of the calibration point, the estimated times shown in Fig. 5 for the origin of the *Hylobates* and *Pongo* clades are probably too recent, and the times shown in Table II may be more reliable.

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