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Catarrhine primate divergence dates estimated from complete mitochondrial genomes: concordance with fossil and nuclear DNA evidence

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Abstract

Accurate divergence date estimates improve scenarios of primate evolutionary history and aid in interpretation of the natural history of disease-causing agents. While molecule-based estimates of divergence dates of taxa within the superfamily Hominoidea (apes and humans) are common in the literature, few such estimates are available for the Cercopithecoidea (Old World monkeys), the sister taxon of the hominoids in the primate infraorder Catarrhini. To help fill this gap, we have sequenced the entire mitochondrial DNA (mtDNA) genomes from a representative of three cercopithecoid tribes, Cercopithecini (*Chlorocebus aethiops*), Colobini (*Colobus guereza*), and Presbytini (*Trachypithecus obscurus*), and analyzed these new data together with other catarrhine mtDNA genomes available in public databases.

Molecular divergence date estimates are dependent on calibration points gleaned from the paleontological record. We defined criteria for the selection of good calibration points and identified three points meeting these criteria: *Homo-Pan*, 6.0 Ma; *Pongo*-hominines, 14.0 Ma; hominoid/cercopithecoid, 23.0 Ma. Because a uniform molecular clock does not fit the catarrhine mtDNA data, we estimated divergence dates using a penalized likelihood and a Bayesian method, both of which take into account the effects of rate differences on lineages, phylogenetic tree structure, and multiple calibration points.

The penalized likelihood method applied to the coding regions of the mtDNA genome yielded the following divergence date estimates, with approximate 95% confidence intervals: cercopithecine-colobine, 16.2 (14.4-17.9) Ma; colobin-presbytin, 10.9 (9.6-12.3) Ma; cercopithecin-papionin, 11.6 (10.3-12.9) Ma; and *Macaca-Papio*, 9.8 (8.6-10.9) Ma. Within the hominoids, the following dates were inferred: hylobatid-hominid, 16.8 (15.0-18.5) Ma; *Gorilla-Homo + Pan*, 8.1 (7.1-9.0) Ma; *Pongo pygmaeus pygmaeus-P. p. abelii*, 4.1 (3.5-4.7) Ma; and *Pan troglodytes-P.*

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paniscus, 2.4 (2.0-2.7) Ma. These dates were similar to those found using penalized likelihood on other subsets of the data, but slightly younger than several of the Bayesian estimates.
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Introduction

Scenarios of primate evolutionary history increasingly rely on estimates of divergence dates derived from analyses of molecular data (e.g., Purvis, 1995; Pilbeam, 1996; Goodman et al., 1998; Stewart and Disotell, 1998). Molecule-based estimates of divergence dates provide supporting evidence for interpretations of the fossil record, aid the placement of evolutionary events in environmental and geographical context, and—when fossil evidence is lacking—provide the only data for estimating the chronology of evolutionary events. Molecule-based estimates have been widely used for interpreting hominoid (ape and human) evolutionary history. However, much less attention has been paid to the cercopithecoids (Old World monkeys) despite their importance in many biomedical studies, including those aimed at understanding the origin, spread, and adaptation of many human pathogens.

Molecular sequence data are commonly used for the inference of primate divergence dates, due to the overall "stochastic clocklike" nature of molecular sequence change (e.g., Kumar and Hedges, 1998; Arnason et al., 2000; Glazko and Nei, 2003; Yang and Yoder, 2003). It has been convincingly demonstrated that the underlying rates of nucleotide substitution often vary between groups of species (Bailey et al., 1991; Yi et al., 2002), and that such "local clock" effects must be corrected for when inferring divergence dates from sequence data. In addition, it is well known that proteins generally evolve in a less clock-like manner than do their genes—that is, the protein molecular clock is more "over-dispersed" (Gillespie, 1991). Indeed, it is now established that some protein families have experienced episodes of positive and negative selection that can be detected by phylogeny-based analyses (Messier and Stewart, 1997; for mitochondrial protein examples, see Schmidt et al., 1997; Wildman et al., 2002, 2003). The resulting speed-up and slow-down of protein sequence change (Goodman, 1982) can remain undetected by simple three-taxon relative rate tests, but revealed by more dense phylogeny-based analyses (Jolles et al., 1989). Thus, divergence dates estimated from only one or a few protein—or even gene—sequences should be considered potentially suspect, even if the molecules have passed simple rate constancy tests. Furthermore, an underlying assumption of all such studies is that the genes under study diverged at approximately the same time as the species that contained them, and thus the molecule-based age estimates provide good estimates for the time of divergence of the primate lineages.

In mammals, two organelles contain DNA, the nucleus and the mitochondria. Mitochondrial DNA (mtDNA) is commonly used in molecular evolutionary studies (Moritz et al., 1987; Harrison, 1989; Avise, 1998) because several of its properties make it preferable to nuclear DNA (nDNA) when studying fairly closely related species, such as the catarrhine primates (i.e., the hominoids and cercopithecoids). The high rate of mtDNA sequence evolution produces more phylogenetically informative sites per unit of sequence length than does the more slowly evolving nDNA. Evolutionary inferences are often simpler for the maternally inherited, non-recombining mtDNA genome than for the biparentally inherited, recombining nDNA genome. Furthermore, the smaller effective population size (Ne) of the uniparentally inherited mtDNA genome makes mtDNA-based phylogenies more likely to be congruent with the species tree than those derived from nDNA-encoded genes (Moore, 1995). Finally, the relative abundance and stability of mtDNA compared to nDNA often allow it to be sequenced from more

degraded samples, thus permitting mtDNA sequence analysis from old or otherwise rare specimens.

A practical difficulty of using mtDNA is that portions of the mitochondrial genome have been translocated into the nuclear genome numerous times during evolutionary history, creating multiple pseudogenes known as "numts" (for "nuclear-mitochondrial"; Lopez et al., 1996) that can be misidentified as "real" mtDNA. Many such numts have been demonstrated in the primates (Collura and Stewart, 1995; Collura et al., 1996; Bensasson et al., 2001; Thalmann et al., 2004). Fortunately, careful experimental design greatly reduces the likelihood of inadvertently amplifying numtDNA sequences.

In addition, individual mtDNA-encoded genes, while all linked, have been shown to exhibit variable rates of molecular evolution in different lineages, with several anthropoid primate genes and their protein products evolving faster than those of other primates (e.g., Adkins et al., 1996; Andrews et al., 1998; Wu et al., 2000). Similarly, entire mtDNA genomes can evolve more rapidly in some lineages than in others, and this appears especially true for the anthropoids (Gissi et al., 2000; Huelsenbeck et al., 2000; Hasegawa et al., 2003). Such rate variations have led some authors to exclude individual mtDNA loci from their analyses (e.g., COII and ND6, Arnason et al., 1996a), whereas others disavow the use of mtDNA genes and proteins entirely (Nei and Glazko, 2002; Glazko and Nei, 2003). Fortunately, such rate variations can be assessed statistically, and increasingly sophisticated divergence date estimation methods that compensate for lineage-specific rate differences in phylogenies (Sanderson, 1997, 2002; Thorne et al., 1998; Huelsenbeck et al., 2000; Thorne and Kishino, 2002; Hasegawa et al., 2003) can be applied, thus allowing increasingly more reliable analyses of mtDNA genome sequences.

Due to widespread interest in human evolution, numerous prior molecular studies have estimated divergence dates within the hominoid (ape and human) lineage. Over the past few decades, estimates of hominoid divergences have been derived from various molecular methods, as summarized in Table 1: quantitative immunological comparisons, DNA-DNA hybridization, nDNA or mtDNA-encoded protein sequences, and nDNA or mtDNA nucleotide sequences. Estimates of key hominoid divergence dates have varied widely between many of these studies. The furthest outliers among these estimates are based on analyses of mtDNA-encoded protein sequences by Arnason and colleagues (Arnason et al., 1998, 2000; Arnason and Janke, 2002), and involved extrapolation from non-primate calibration points (Fig. 1).

Without a doubt, one of the most significant factors in estimating divergence dates using molecular data is the choice of calibration points for the molecular clock (Lee, 1999; Yoder and Yang, 2000; Shaul and Graur, 2002; Graur and Martin, 2004). It is clear that at least some of the differences between the published divergence date estimates shown in Table 1 result from differences in the fossil calibration points chosen by the various authors. Notably, Arnason and colleagues contend that the primate fossil record is so poor that it cannot be used for calibration of primate molecular data (Arnason et al., 1996b, 1998, 2000). Instead, they use calibration points from other mammalian fossil lineages and extrapolate to the primate data. These researchers originally proposed the use of a calibration point taken from the divergence of two seal species, referred to as the "*Phoca* standard" (Arnason et al., 1996b). This calibration point produced hominoid divergence date estimates that are in line with many nDNA-based estimates, as well as primate fossil evidence (see Arnason et al., 1996b; entry in Table 1). However, Arnason and collaborators have since abandoned the *Phoca* standard in favor primarily of a calibration point, assumed to be 60 Ma, for the divergence of the whales from the ruminant artiodactyls, typically represented by the cow (Arnason et al., 1996b, 1998, 2000).

These particular studies estimate the divergence of the lineages leading to the cercopithecoids and the hominoids to have been between 47 and 74 Ma (Table 1), which is two to three times earlier than the known appearance of either lineage in the fossil record. Indeed, the 74 Ma date would require cercopithecoids and hominoids to have co-existed with the dinosaurs for millions of years prior to the Cretaceous-Tertiary (K/T) boundary!

Table 1
Molecular estimates of hominoid divergence dates (in Ma)

	Cercopithecoidea-Hominoidea (23.8-29.6)[1]	Hominidae-Hylobatidae (14.2-19.2)	Homininae-Ponginae (14.0-15.7)	Hominini-Panini (6.0-6.9)
Immunological data				
Sarich and Wilson, 1967	*(30)*[2]	10	8	5
DNA-DNA hybridization data				
Kohne et al., 1972	*(30)*	17.7		8.0
Sibley and Ahlquist, 1987	*(25)*	16.4	12.2	5.5
Caccone and Powell, 1989	*(25-30)*	17 - 21	*(12-16)*	6 - 8
nDNA sequence data				
Sakoyama et al., 1987 (ϵ globin)	*(30)*		17.3 ± 4.5	6.4 ± 2.6
Hasegawa et al., 1989 (ψη globin)	21.5 ± 1.9		12.3 ± 1.8	6.5 ± 1.1
Bailey et al., 1991(ψη globin)	*(25)*	17.3	13.5	6.5
Easteal and Herbert, 1997 (>10 loci)	20.0 ± 0.8	12.0	9.0 ± 0.8	4.0 ± 0.5
Goodman et al., 1998 (globins)	*(25)*	18	14	6
Stauffer et al., 2001 (up to 28 loci)	*(23)*	14.9 ± 1.0	11.3 ± 0.7	5.4 ± 0.6
nDNA encoded protein sequences				
Kumar and Hedges, 1998 (56, 4, 6, and 5 proteins, respectively)	23.3 ± 1.2	14.6 ± 2.8	8.2 ± 0.8	5.5 ± 02
Nei and Glazko, 2002 (12 proteins)	23.5 ± 4.1		14.2 ± 4.7	6.3 ± 3.9
Glazko and Nei, 2003 (13 proteins)	23		13	6
mtDNA sequence data				
Horai et al., 1995 (complete mtDNA)			*(13)*	4.9 ± 0.2
Huelsenbeck et al., 2000 (complete mtDNA)		23.2	17.8	7.0
Yoder and Yang, 2000 (Cytochrome *b*)	29.8 - 40.9	16.9 - 23.2	8.4 - 19.0	3.8 - 6.8
Yang and Yoder, 2003 (COII and Cytochrome *b*)			15.2 (12.1-18.6)[3]	7.1 (5.1-9.3)[3]
mtDNA encoded protein sequences (excluding COII and ND6)				
Arnason et al., 1996a		36.1 ± 4.1	24.4 ± 2.7	13.7 ± 2.5
Arnason et al., 1996b			16.5 - 19.6	5.2 - 6.9
Arnason et al., 1998	47 - 74	28 - 39	23 - 36	10 - 15
Arnason et al., 2000	52	35	30	13
Arnason and Janke, 2002	47	32	27	11
mtDNA encoded protein sequences (12 heavy strand proteins)				
Hasegawa et al., 2003	34.6 ± 1.6	21.7 ± 1.0		7.4 ± 0.7
rRNA sequences (12 nDNA and 2 mtDNA)				
Hasegawa et al., 2003	25.5 ± 2.7	15.6 ± 2.1		

[1] Full range of estimates from all methods in Table 3.
[2] Values in italics are calibration points.
[3] 95% credibility interval.

As currently understood, the earliest definitive catarrhine fossil, *Propliopithecus*, first appears in the fossil record around 35 Ma; this fossil is "primitive," having no defining characteristics of hominoids or cercopithecoids (Delson, 2000a; Rasmussen, 2002). Indeed, there are few, if any, plausible early *anthropoid* fossils prior to 47 Ma (Rosenberger and Delson, 2000). Thus, even considering likely gaps in the primate fossil record (Martin, 1993; Tavaré et al., 2002), a catarrhine divergence date as early as 47 to 74 Ma seems highly improbable, as all relevant lineages would have to be missing from the fossil record for the majority of their lifespans.

However, support for such seemingly implausible early primate divergence dates has been claimed

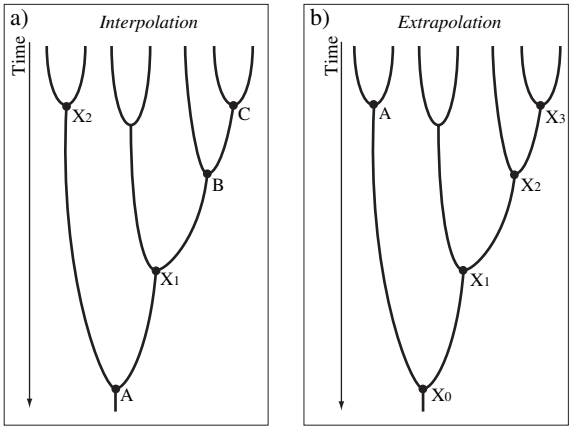

Fig. 1. Interpolation versus extrapolation in estimation of divergence dates in evolutionary trees. a) Interpolation. Fossil calibration points (A-C) within and bracketing the unknown divergence point (X_1) are most desirable. Such calibration points allow for mathematical interpolation of the unknown divergence dates—i.e., estimation of the unknown in the range of the known. Mathematically, interpolation requires fewer assumptions than extrapolation. Interpolation is also biologically preferable, because lineages often differ in generation time, body size, metabolic rate, and nucleotide composition—all of which may cause variation in molecular evolutionary rates and patterns (Kohne, 1970; Martin and Palumbi, 1993; Bromham et al., 1996; Li et al., 1996; Graur and Li, 2000). Unknown point X_2 is also interpolated because there is always an implicit zero calibration point (at time zero, there is no divergence). b) Extrapolation. Fossil calibration point A, which falls outside of the lineage of interest is used to first estimate unknown divergence point X_0 by extrapolation—estimation of the unknown outside the range of the known. As practiced by Arnason and colleagues (e.g., 1998), extrapolated point X_0 is then used to estimate unknown divergence points X_1-X_3 through interpolation. Such extrapolation followed by interpolation is less desirable than straightforward interpolation, both mathematically and biologically, as it assumes that the relevant processes are stationary over all lineages.

based on computer simulations that suggest that the primate fossil record is dramatically incomplete (Martin, 1993; Tavaré et al., 2002). These simulations have been interpreted as providing evidence that the fossil record underestimates major primate divergences by tens of millions of years, and thus supports the early primate divergence dates proposed by Arnason and colleagues (Martin, 1993; Tavaré et al., 2002). Many paleontologists would seem to disagree with this interpretation of the primate fossil record, however (see, e.g., Szalay and Delson, 1979; Carroll, 1988; Delson et al., 2000; Hartwig, 2002). As discussed below, the primate fossil record is relatively abundant and provides several independent calibration points for estimating molecular divergence dates.

In addition, many published studies may not have adequately compensated for the dramatic rate and pattern differences in the evolution of the mtDNA-encoded proteins from divergent mammalian taxa, particularly for the speed-up seen in anthropoid mtDNA and proteins compared to other primates and most other mammals (Adkins et al., 1996; Andrews et al., 1998; Andrews and Easteal, 2000; Hasegawa et al., 2003). Importantly, although some of Arnason's analyses attempted to compensate for the increased rate of primate mitochondrial protein evolution compared to the artiodactyls (Arnason et al., 1996a, 1998, 2000), the correction was applied evenly to the entire primate clade, rather than limiting it to the anthropoids, and no compensation was made for differing rates within the cetartiodactyla. Furthermore, local clock corrections were not applied to the mitochondrial sequences within the anthropoids in these studies, as we have shown here is necessary. Thus, the unrealistically early primate divergence date estimations produced by Arnason and colleagues (Arnason et al., 1996b, 1998, 2000) probably are the result of compounded errors in their analyses. To begin to address these issues, Huelsenbeck et al. (2000) did pioneering phylogeny-based Bayesian analyses on mammalian mtDNA data that better compensated for rate differences between lineages, and thereby found more reasonable divergence date estimates for the primates (see Table 2), even using a whale-cow divergence date of 56.5 Ma to calibrate the tree.

In the case of nDNA-derived divergence dates, the relatively modest difference in mutation rate between hominoid and cercopithecoid nDNA—about 1.4-fold, the so-called "hominoid slowdown" (Goodman, 1961; Goodman et al., 1971; Li and Tanimura, 1987; Li et al., 1996)—has been better accommodated by some authors (e.g., Goodman et al., 1998). As a result, most nDNA-derived estimates (Table 1) for the divergence dates of hominoid lineages are generally consistent with current interpretations of the primate fossil

Table 2
Molecular estimates of cercopithecoid divergence dates (in Ma)

	Cercopithecinae-Colobinae (13.5-20.7)[1]	Colobini-Presbytini (8.9-13.5)	Cercopithecini-Papionini (9.4-13.7)	Macacina-Papinonina (7.7-12.1)
Immunological data				
Cronin and Sarich (1976)	10	4	6	5
DNA-DNA hybridization data				
Kohne et al., 1972			11.7	
nDNA sequence data				
Goodman et al., 1998 (globins)	14	10	10	7
mtDNA sequence data				
Disotell and Raaum, 2003 (COII and "896 bp" region)			13.5	12.0
mtDNA encoded protein sequences (excluding COII and ND6)				
Arnason et al., 2000				25
Arnason and Janke, 2002				23

[1] Full range of estimates from all methods in Table 3.

record (see, Goodman et al., 1998; Stewart and Disotell, 1998; Pilbeam, 2002).

Relatively few molecular studies have estimated divergence dates within the cercopithecoids, despite the fact that these primates are the closest sister-group to the hominoids and are used widely in biomedical studies (Carlsson et al., 2004). The few estimates available (Table 2) derive from limited immunological data (Cronin and Sarich, 1976), DNA-DNA hybridization data (Kohne et al., 1972), mtDNA gene sequence data (Disotell and Raaum, 2003), and nDNA sequence data (reviewed in Goodman et al., 1998). To help fill this gap in the understanding of primate evolutionary history, our laboratories are sequencing complete mtDNA genomes and several unlinked nDNA loci from various genera of cercopithecoids.

Many divergence date estimates have been reported without confidence intervals (see Tables 1 and 2), making it impossible to evaluate their precision. In some cases, confidence intervals have been miscalculated, and are typically highly optimistic (Graur and Martin, 2004). Establishing the confidence interval for a divergence date estimate from a phylogeny requires combining the fractional statistical uncertainty from three separate processes: 1) the uncertainty of the estimate of the branch length (Sanderson, 2002); 2) the stochastic nature of the evolutionary process itself (Gillespie, 1991); and 3) the uncertainty associated with the calibration (Graur and Martin, 2004).

Choosing appropriate calibration points is absolutely required for meaningful estimates of divergence dates (Lee, 1999; Shaul and Graur, 2002). As with any mathematical calibration process, it is best to have multiple fossil calibration points that are as reliable and independent as possible (Yang and Yoder, 2003). This is tricky to do, given the incompleteness of the fossil record and the challenges involved in its interpretation. Ideally, there also should be congruence between the molecular and morphological evidence for the branching order of the lineages around the calibration points. Furthermore, there should either be a consensus among paleontologists regarding the phylogenetic position of the fossil specimens on which a given calibration point is based, or the alternative interpretations should not affect the timing of the divergence (see Fig. 2). Whenever possible, each calibration point should be bracketed by fossil specimens attributed by independent researchers (i.e., researchers in addition to the original discoverers of the fossils in question) to both descendent lineages and plausible fossil members of the ancestral lineage (Fig. 2).

To address such calibration issues for the catarrhine primates, we have done the following: 1) as explained in Fig. 1, we have estimated molecular divergence dates through a process involving

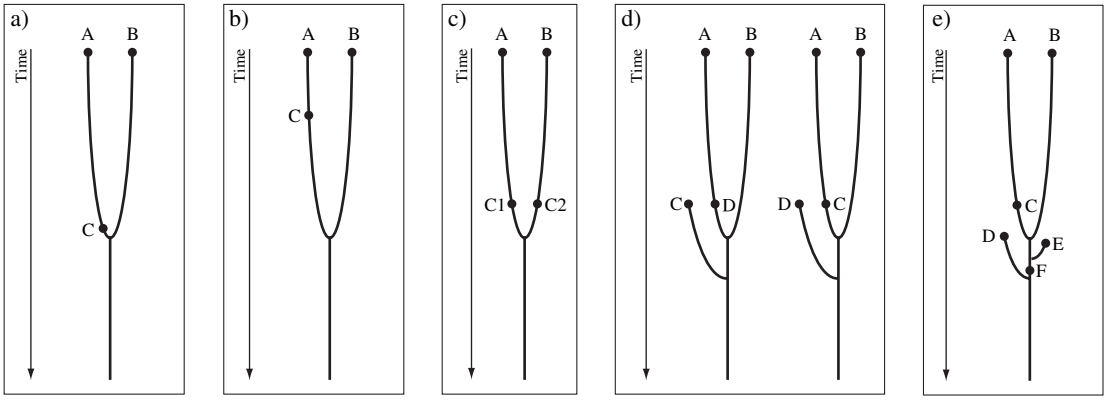

Fig. 2. Criteria for choosing fossils to calibrate divergence dates of extant lineages. We have applied the following principles to the fossil data shown in Fig. 3 to identify three calibration points in the catarrhine fossil record that most closely meet the ideal conditions outlined in (e) below. Given two extant taxa (A, B) and fossil specimens (C-F), when do the fossils provide relevant data for calibration? a) If fossil C is a member of the lineage leading to extant taxon A, then clearly the lineages leading to A and B must have diverged before the date of the earliest known specimen of C. For establishing a calibration point, it is desirable for the fossil to date just after the split. b) However, it is generally difficult to know just how close to the split the known fossil specimens are, as they may significantly postdate the split. c) It does not matter if it is unclear whether fossil taxon C is a member of the A (C1) or B (C2) lineage, as the earliest known specimen of C will provide a latest date for the divergence of A and B in either case. d) Given contemporaneous fossil taxa (C, D), one of which is likely to be on the lineage leading to the extant taxon A, it does not matter if there is debate over which actually is on the lineage leading to A, because the same calibration date will be chosen in either case. e) It is best to have fossils (C) attributed to one or the other, or both, of the extant lineages, as well as other fossil specimens from around the time of the split (D and E); here fossil C provides a conservative *lower bound* for calibration. Ideally, a plausible ancestor (F) is also available to provide an *upper bound* to the estimated date; it is notoriously difficult to establish such upper bounds based on the fossil record (see Stewart and Disotell, 1998). When good lower bound fossils and potential upper bound fossils exist, this implies a relatively well-sampled fossil record for the divergence in question.

interpolation within primates rather than extrapolation from distantly related mammals; 2) as outlined in Fig. 2, we have established consistent criteria for selecting fossil calibration points; 3) as summarized in Fig. 3, we have extensively surveyed the paleontological literature to identify catarrhine fossils and their time ranges; and 4) from the data presented in Fig. 3, we have identified three independent fossil calibration points that best meet the selection criteria outlined in Fig. 2. These aspects of the study are explained in greater detail below. These three fossil calibration points allow us to apply rigorous, phylogeny-based methods of estimating unknown divergence dates that account for nucleotide substitution heterogeneity along different lineages (Sanderson, 1997, 2002; Thorne and Kishino, 2002). We are able to estimate confidence intervals for these divergence dates in a manner that reflects the uncertainty of the branch length estimates and the stochastic nature of the molecular evolutionary process. Combined, the above approaches address most of the deficiencies of previous mtDNA-based studies aimed at estimating catarrhine primate divergence dates.

The present study reports the first systematic analysis of complete mtDNA genome sequences from species representing each of the four major groups of cercopithecoids: 1) the African colobines (guereza); 2) the Asian colobines (dusky leaf monkey); 3) the African cercopithecins (vervet); and 4) the African (baboon) and predominantly Asian (macaque) papionins. The mtDNA genomes from the first three groups listed were sequenced for this project, and are presented here for the first time. In addition, we also have made an independent estimate of the divergence of the mtDNA genome of the hylobatids (gibbon) from the hominids (great apes and humans) using previously published sequence data. Assuming that these mitochondrial genomes and the species that contained them diverged at the same time, then these

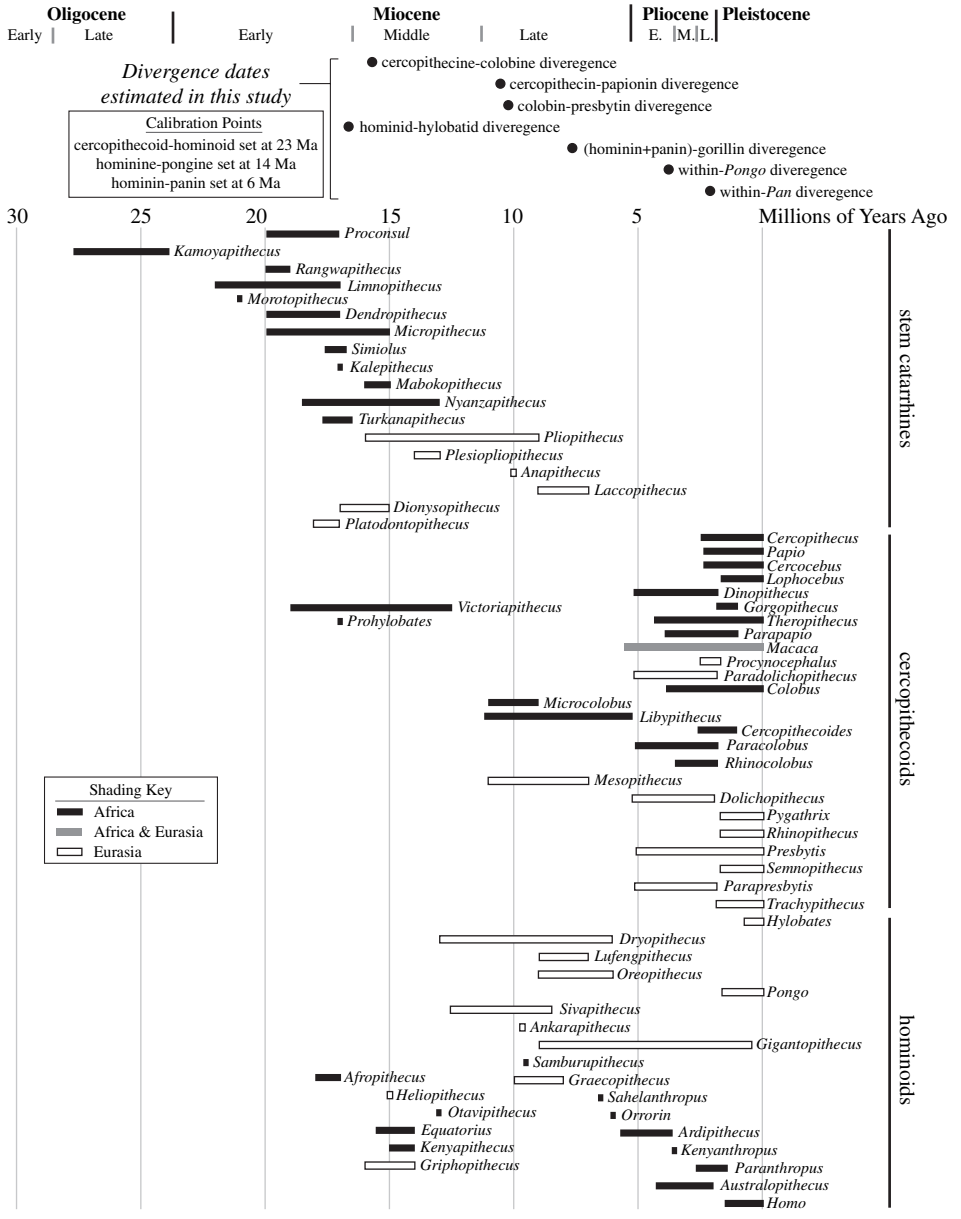

Fig. 3. Time ranges of fossil catarrhine genera. All genera listed as catarrhines in the taxonomy of Delson et al. (2000) with attributed fossil remains during the last 30 million years are shown. Time ranges are as collected from Delson et al. (2000) and from Hartwig's (2002) edited volume. The ranges presented here are generous, as we have joined the earliest and latest dates given in the source volumes for each taxon. All ranges are approximate, but the cercopithecoid ranges are the most so, as most dates presented in the source volumes for the cercopithecoids are to epochal stages rather than to empirical dates of given fossils. See text for details of calibration point choice and divergence date estimations.

mtDNA-based dates provide good estimates for the divergence times of these lineages of primates.

Materials and methods

DNA purification

To complete a phylogenetically balanced dataset that includes representatives of all major catarrhine lineages, three Old World monkey species were selected for complete mitochondrial genome sequencing: *Chlorocebus aethiops*, the vervet (an African guenon representing the tribe Cercopithecini); *Colobus guereza*, the guereza or Abyssinian black-and-white colobus (an African colobine representing the tribe Colobini); and *Trachypithecus obscurus*, the dusky langur (an Asian colobine representing the tribe Presbytini). The colobus (''Flat-top''; liver) and langur (liver) tissues were kindly provided by B. Lester at the Houston Zoological Gardens. High molecular weight cellular DNAs (containing both nNDA and mtDNA) were prepared from frozen colobine tissues by R. Holt and J. Schienman using standard protocols; these procedures were performed under the auspices of C.-B. Stewart's IACUC protocol at the University at Albany, SUNY. Vervet DNA from Ethiopia (VE98007) was a gift of C. Jolly at NYU.

Amplification and sequencing

The polymerase chain reaction (PCR) was used to amplify long fragments of the mtDNA genomes of each species, employing a long-range PCR kit (Expand Long Template PCR System, Roche). Two independent sets of amplification primers that amplify the entire mtDNA genome (Fig. 4) were designed for each species. All primer sequences are available upon request from T. R. Disotell. The first set of primers amplified three fragments—approximately 5, 7, and 7.5 kilobase pairs (Kb) in length—that overlap by about 500-1000 base pairs (bp). The second set of primers amplified two fragments (both about 10 Kb in length) that overlap by at least 1000 bp.

Both strands of these overlapping PCR products were sequenced directly using BigDye v3.0

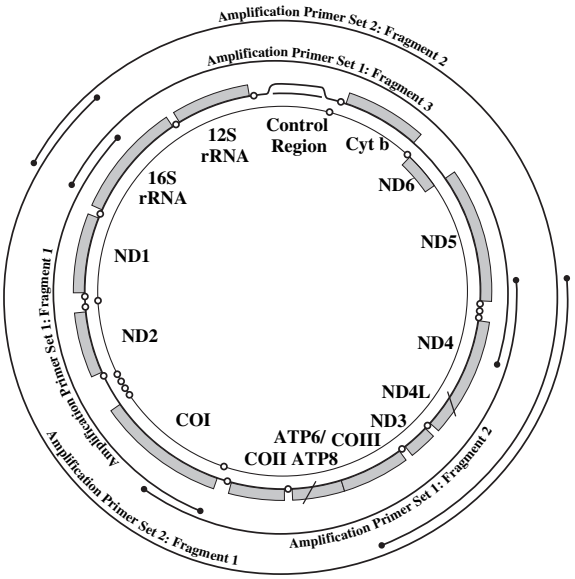

Fig. 4. Amplification strategy for the mtDNA genomes. The complete mtDNA genome of each species was amplified twice independently using a long-range PCR protocol with two sets of amplification primers (indicated by the dots at the ends of the fragments); see text for details. The first primer set produced three fragments (about 5, 7, and 7.5 Kb in length), whereas the second produced two fragments (both about 10 Kb in length). The mtDNA genome layout follows Taanman (1999).

(Applied Biosystems, Inc.) and a suite of sequencing primers spaced approximately 350 bp apart. The sequencing products were analyzed on an ABI PRISM 377 DNA Sequence Analysis System (Applied Biosystems, Inc.). The bases were called using *Sequencing Analysis v3.4* (Applied Biosystems, Inc.), and the individual sequences were assembled into the complete mtDNA genomes with the aid of the program *Sequencher v4.1* (Gene Codes Corp.). The redundant overlapping of the PCR fragments that were sequenced for each genome allowed for the identification of any sequences that might not match the rest of the assembled genome, and thus the sorting of the true mtDNA genome sequences from any contaminating numtDNA sequences (Thalmann et al., 2004).

Alignment and data partitions

These three newly generated Old World monkey mtDNA genomes were aligned with the following

catarrhine mtDNA genomes found in GenBank: *Papio hamadryas* (NC_001992), *Macaca sylvanus* (NC_002764), *Hylobates lar* (NC_002082), *Pongo pygmaeus pygmaeus* (NC_001646), *Pongo pygmaeus abelii* (NC_002083), *Gorilla gorilla* (NC_001645), *Pan paniscus* (NC_001644), *Pan troglodytes* (NC_001643), and *Homo sapiens* (NC_001807). *Cebus albifrons* (NC_002763), representing the New World monkeys, was included as an outgroup.

A master alignment that included all of the mtDNA-encoded genes (13 protein-coding loci, two rRNAs, and 22 tRNAs) was created. Initially, each individual gene was aligned using default settings in *ClustalX* (Thompson et al., 1994, 1997). Next, insertions and deletions of nucleotides were adjusted manually in the 13 protein-coding genes so that they were consistent with codon boundaries of the aligned amino acid sequences; the termination codons were excluded. These separate gene alignments were then assembled into a master alignment for each species that consisted of all mtDNA-encoded genes, and that encompassed the majority of each genome, minus the control region and a few intergenic bases. Finally, because many analytical applications are known to perform poorly when the input matrix includes gaps in any of the aligned sequences, all sites at which there were insertions or deletions in any species were excluded from the master alignment. This master alignment is 15,228 bp long, and is termed the "All Genes" alignment.

Because prior publications have suggested that the 12 protein-coding genes encoded by the heavy strand of the mtDNA genome have the most similar evolutionary properties (e.g., Gissi et al., 2000), we created a 10,828 bp alignment from the "All Genes" alignment that includes only these 12 genes. This heavy strand protein-coding alignment is herein referred to as the "HSP" alignment. Finally, because the third base position in codons may rapidly become saturated (Li, 1997), we derived a third alignment excluding these positions from the HSP alignment; this is the 7,222 bp "HS12P" alignment (heavy strand 1st and 2nd positions.) Nucleotide sequence data rather than translated protein sequence data are used in this study for reasons discussed in the Introduction; in addition, analytic flexibility would be reduced, as neither the All Genes alignment nor the HS12P alignment could be created using protein sequence data.

The concatenated alignments described above were used for evolutionary tree inference and for the penalized likelihood analyses. For the Bayesian analysis, the individually aligned protein-coding genes were used.

Choice of evolutionary model

Many different nucleotide substitution models have been developed for use in molecular evolutionary studies. These models mostly differ in the number of parameters involved (see Rodríguez et al., 1990). The simplest nucleotide substitution model has only one parameter, which is the probability of any one base changing to another (Jukes and Cantor, 1969). A slightly more complex model has two parameters, one for the probability of a transversion occurring and a second for the probability of a transition occurring (Kimura, 1980). Additional parameters can be added to increase the complexity of models, but may not increase the fit of the model to the data. In a maximum likelihood (ML) framework, each nucleotide substitution model has a likelihood value on a particular tree. The likelihood values of different models on a tree may be statistically tested to determine the best fitting model (Huelsenbeck and Rannala, 1997). These likelihood ratio tests (LRTs) were performed to determine the best-fit model(s) of nucleotide substitution using *ModelTest v3.5* (Posada and Crandall, 1998). The best-fit nucleotide substitution models were used for phylogenetic tree inference and for the penalized likelihood analyses described below.

Phylogenetic tree inference

Phylogenetic trees were inferred from the All Genes, HSP, and HS12P alignments using a variety of approaches (ML, parsimony, distance) available in *PAUP** (Swofford, 2004). The same tree topology was found for all methods; this topology was thus used in the penalized likelihood and Bayesian divergence date estimations described below.

Rate variation between lineages

Likelihood ratio tests were used to determine if the mtDNA tree topology is significantly better fit by a nucleotide substitution model (as chosen above) that allows rate variation or by a model that does not (i.e., a uniform molecular clock). The likelihoods of these two models on the mtDNA tree were estimated in *PAUP** (Swofford, 2004). These LRTs indicated significant rate heterogeneity in the phylogeny. Thus, tree-based relative rate tests (RRTs), as implemented in the *RRTree* software (Robinson-Rechavi and Huchon, 2000), were used to identify sister groups with significantly different nucleotide substitution rates.

Fossil calibration

Careful examination of the catarrhine fossil data compiled in Fig. 3 led us to conclude that there are three calibration points that meet the criteria set forth in Fig. 2. These calibration points are as follows: (1) the divergence of the Old World monkey and hominoid lineages by about 23 Ma, (2) the divergence of the orangutan and African ape lineages by about 14 Ma, and (3) the divergence of the human and chimpanzee lineages by about 6 Ma. The data and reasoning supporting these three calibration points are explained below.

(1) The cercopithecoid and hominoid lineages most likely diverged around 23 Ma. This estimate is supported by multiple independent lines of fossil evidence. First, many potential early hominoid fossils are dated to around 20 Ma (*Proconsul* at 19-20 Ma, Andrews, 1992; *Morotopithecus* at >20 Ma, Gebo et al., 1997, and MacLatchy et al., 2000; *Ugandapithecus* at 19-20 Ma, Senut et al., 2000). Second, the earliest cercopithecoid specimen, referred to *Victoriapithecus*, is dated to 19 Ma (Benefit and McCrossin, 2002). Together, these hominoid and cercopithecoid fossils require that these two lineages diverged before 20 Ma. Other possible hominoid fossils include *Kamoyapithecus*, dating from 24 to 28 Ma (Leakey et al., 1995; Harrison, 2002), and the Meswa Bridge specimen, dating from 22 to 23 Ma (Andrews et al., 1981); if so, the divergence date of the hominoid and cercopithecoid lineages could have occurred by 28 Ma. However, these specimens have also been interpreted as likely stem catarrhines (Kelley, 2002; Harrison, 2002); if so, they would place an upper bound on the hominoid-cercopithecoid divergence at about 22-23 Ma. Taken together, we conservatively estimate that the divergence of these two lineages occurred by 23 Ma.

(2) The divergence of the orangutan and African ape lineages by about 14 Ma is supported by the following data. The pongine (orangutan) lineage first appears in the fossil record with *Sivapithecus* at about 12-13 Ma (Kelley, 2002). Contemporaneous with *Sivapithecus* is *Dryopithecus*, which is found in Europe and western Asia. Cladistic analysis of *Dryopithecus* morphological characters suggests that it is an early representative of the lineage leading to African apes (Begun, 2000). If these phylogenetic placements of *Sivapithecus* and *Dryopithecus* are correct, then they clearly suggest that the divergence of the pongine and African Ape lineages occurred prior to about 13 Ma (see Stewart and Disotell, 1998), and thus we estimate this divergence point at 14 Ma, which is the current consensus among paleoanthropologists.

(3) Most fossil evidence supports the idea that the *Homo* and *Pan* lineages diverged around 6 Ma. Postcranial elements of *Ardipithecus*, dated to 4.5 to 5.8 Ma, likely belong to an early representative of the hominin lineage (Haile-Selassie, 2001). Unfortunately, there are no fossils that unambiguously fall on the *Pan* lineage, and thus a lower bound cannot be established based on this lineage. In addition, specimens tentatively dated to about 6 to 7 Ma are plausibly close to the common ancestor of *Homo* and *Pan* (i.e., *Orrorin*: Pickford and Senut 2001; Senut et al., 2001; *Sahelanthropus*: Brunet, 2002; Brunet et al., 2002). Although the original discoverers interpret *Orrorin* and *Sahelanthropus* as the earliest representatives of an exclusively human lineage (an idea that has been widely touted in the popular press), other paleoanthropologists who have made

their views known in print either reserve judgment or disagree with these interpretations (Wolpoff et al., 2002; Wood, 2002). As there are no independent published analyses of these specimens, the phylogenetic relationships of *Orrorin* and *Sahelanthropus* remain ambiguous. Nonetheless, these fossils lend additional support for dating the *Homo-Pan* divergence, as outlined in Fig. 2.

Estimation of divergence dates

The rate variation analyses described above revealed that statistically significant nucleotide substitution rate differences have occurred in the evolution of the catarrhine mtDNA genome. Therefore, to most accurately estimate divergence dates, a variable-rate molecular clock must be applied in the sequence analysis. Furthermore, variable-rate sequence data are most powerfully analyzed in phylogenetic context (reviewed in Hasegawa et al., 1989). Two methods that take these two issues into account are the penalized likelihood method of Sanderson (1997, 2002) and the Bayesian method of Thorne and Kishino (Thorne et al., 1998; Thorne and Kishino, 2002). Both of these methods allow different rates on different lineages, but assume that rates are constant on individual branches (Thorne et al., 1998).

Sanderson (2002) implemented a semi-parametric method wherein each lineage has a separate rate that is limited from varying too much across the phylogeny. We used this method to estimate divergence dates using three datasets (All Genes, HSP, and HS12P), with confidence intervals calculated via a bootstrap procedure. Each alignment was re-sampled 100 times. For each of these 100 replicates, ML branch lengths were calculated using the *PAML* software package (Yang, 2003). Branch lengths estimated by *PAML* were employed in the penalized likelihood date estimation method implemented in the *r8s* computer program (Sanderson, 2003). The three fossil-derived divergence dates described above were used as "point estimates" to calibrate the ML trees. The bootstrap sample was tested for normality (Shapiro-Wilks test for normality; Royston, 1995). Since the sample passed the normality test, we estimated the 95% confidence interval for the uncertainty resulting from statistical error in branch length estimation and stochasticity in the molecular evolutionary process as 2.576 times the standard deviation of the bootstrap sample for each node. To this value a fractional error of 10% was added as an estimate of uncertainty surrounding the dates of the calibration points used. Thus, the error values we present here are an approximation of the true uncertainty, but should not be expected to have all the statistical qualities of true confidence intervals; we thus refer to them as "credibility" intervals.

The Bayesian method of Thorne and Kishino (Thorne et al., 1998; Thorne and Kishino, 2002) derives the posterior distribution of rates and times using a Markov chain Monte Carlo (MCMC) procedure that applies a probabilistic model to the estimation of change in nucleotide substitution rate over time. Following Yang and Yoder (2003) and employing the guidelines offered by Rutschmann (2004), we estimated the F84 (Felsenstein, 1984; Hasegawa and Kishino, 1989) model parameters separately for each of the 13 protein-coding genes (the "observed data") using the *baseml* program in the *PAML* package (Yang, 2003). These 13 sets of parameter estimates were then used to estimate the ML branch lengths for the catarrhine mtDNA tree and the variance-covariance matrix using the *estbranches* program for each alignment (Thorne et al., 1998). Using these combined data and parameters, the *multidivtime* program (Thorne and Kishino, 2002) was used to estimate divergence dates, with the three fossil-based dates discussed above used as constrained lower bound priors to calibrate the trees. Results were obtained using a burn-in of 100,000 iterations, followed by 10,000 samples taken every 100 iterations. Posterior probability distributions were thus estimated, and serve as the basis for Bayesian inferences of the primate divergence dates.

Results

Mitochondrial genome sequences

Direct sequencing of the long-range PCR products produced a single, unambiguous mtDNA

genome sequence for all three species. Each of the assembled mtDNA genomes averaged four-fold coverage. Each mtDNA genome was annotated by reference to the curated NCBI reference mtDNA genome sequences (see, for example, *Papio hamadryas*, NC_001992). Complete mtDNA genome sequences for *Chlorocebus aethiops* (16,389 bp, AY863426), *Colobus guereza* (16,648 bp, AY863427), and *Trachypithecus obscurus* (16,560 bp, AY863425) have been deposited in GenBank.

There are no insertions or deletions affecting amino acid number in the protein coding regions of the three newly sequenced genomes presented here. The gene order in all three genomes conforms to that seen in other eutherians. Base composition is similar to that seen in currently available cercopithecine mtDNA genomes.

Phylogeny

A single mtDNA tree topology was inferred (Fig. 5), regardless of tree inference method or dataset. This mtDNA genome tree is congruent with phylogenetic inferences based on morphological studies of the catarrhines (see entries in Delson et al., 2000, and Hartwig, 2002), thus meeting one of the criteria outlined above for estimating divergence dates.

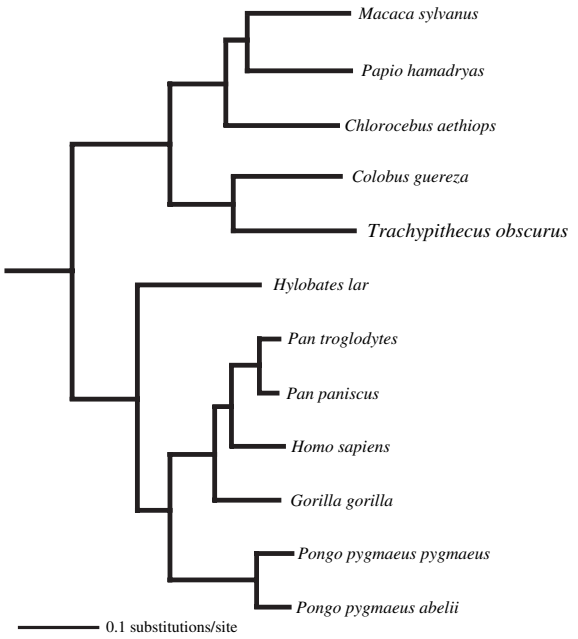

Fig. 5. Mitochondrial DNA-based phylogeny of the species used in this study. The branch lengths on the underlying tree were estimated by the maximum likelihood method for the "All Genes" alignment (13 protein-coding genes, 2 rRNAs, and 22 tRNAs). Branch lengths represent the estimated number of nucleotide substitutions per site along each lineage, as estimated by the GTR + I + G (General Time Reversible with Invariant sites and 4 Gamma-distributed rate classes) in *PAUP** (Swofford, 2004). The tree was rooted by the *Cebus albifrons* (New World monkey) sequence (not shown).

Model choice

The best-fit evolutionary model selected by *ModelTest* (Posada and Crandall, 1998) for the All Genes and HSP alignments was the GTR + I + G model (general time reversible, with invariant sites and a gamma distribution of site specific rates). The TVM + I + G model (1 transition rate and 4 transversion rates, with invariant sites and a gamma distribution) was the best-fit for the HS12P alignment.

Assessment of rate variation

Visual inspection of the ML tree shown in Fig. 5 reveals an apparent nucleotide substitution rate difference between the hominoid and the cercopithecoid mtDNA genome clades. To test to see if this apparent difference was statistically significant, we calculated log likelihood scores for this tree topology using each alignment, both with and without a molecular clock assumption. A LRT was performed and showed that the data were statistically significantly better described by the models without a clock ($df = 11$ for all three alignments; All Genes, $\Lambda = 62.7$, $p < 0.001$; HSP, $\Lambda = 57.2$, $p < 0.001$; HS12P, $\Lambda = 55.8$, $p < 0.001$).

Given the statistically significant variation in nucleotide substitution rate in the evolution of the catarrhine mtDNA genomes, we then used RRTs to determine which lineages differed significantly. Specifically, we compared (1) all Hominoidea to all Cercopithecoidea relative to *Cebus albifrons*, (2) Hylobatidae to all Hominidae relative to *Papio hamadryas*, (3) Ponginae to all Homininae relative

to *P. hamadryas*, (4) *Gorilla* to *Homo* and *Pan* relative to *P. hamadryas*, (5) *Homo* to *Pan* relative to *P. hamadryas*, (6) all Cercopithecinae to all Colobinae relative to *Homo sapiens*, (7) Papionini to Cercopithecini relative to *H. sapiens*, and (8) Presbytini to Colobini relative to *H. sapiens*. We found that the hominoids and cercopithecoids exhibited significantly different relative nucleotide substitution rates in all three alignments (All Genes, HSP, and HS12P), while the cercopithecines and colobines exhibited significantly different rates in the All Genes and HSP alignments. No other comparisons revealed significantly different rates of nucleotide substitution by RRTs.

Date estimates

The divergence date estimates calculated by the penalized likelihood and Bayesian methods are summarized in Table 3, and the full ranges of these estimates appear in the relevant columns in Tables 1 and 2. Regardless of which alignment was used, the date estimates produced by the penalized likelihood method were quite similar to one another, differing by less than 1 myr in all cases. Because the All Genes alignment used the greatest amount of the sequence data, we will primarily use the dates from these analyses to discuss the penalized likelihood results. For this dataset, we estimate that the cercopithecine-colobine divergence occurred about 16.2 Ma (with an approximate 95% confidence interval of 14.4-17.9 Ma), the cercopithecin-papionin divergence about 11.6 (10.3-12.9) Ma, the *Macaca-Papio* divergence about 9.8 (8.6-10.9) Ma, the colobin-presbytin divergence about 10.9 (9.6-12.3) Ma, the hylobatid-hominid divergence about 16.8 (15.0-18.5) Ma, the *Pongo pygmaeus pygmaeus-P. p. abelii* divergence about 4.1 (3.5-4.7) Ma, the *Gorilla-Homo + Pan* divergence about 8.1 (7.1-9.0) Ma, and the *Pan troglodytes-P. paniscus* divergence about 2.4 (2.0-2.7) Ma.

The date estimates based on the Bayesian analysis are, for the most part, slightly older than those from the penalized likelihood method, although most of the estimates are within the 95% credibility intervals. This result may be explained, in part, by the fact that the fossil calibration points were used as lower bound priors in the Bayesian analysis, whereas they were used as fixed point estimates in the penalize likelihood analyses. Thus, in the Bayesian analysis the three original calibration points themselves were estimated in the posterior distributions. In all three cases, the estimated dates are older than the original calibration dates. Notably, the hominoid-cercopithecoid divergence date estimate is 26.5 Ma, with the lower end of its 95% confidence interval (23.8 Ma) being slightly above the original calibration date of 23 Ma. Thus, it is possible that our original

Table 3
Estimates of cercopithecoid divergence dates from mitochondrial genome sequence data (in Ma)

	Penalized Likelihood			Bayesian 13 protein-coding genes
	All Genes	HSP	HS12P	
Hominoidea-Cercopithecoidea	**23.0**[1]	**23.0**	**23.0**	26.5 (23.8-29.6)[3]
Hominidae-Hylobatidae	16.8 (15.0-18.5)[2]	16.7 (14.9-18.5)[2]	16.1 (14.2-18.1)[2]	17.5 (16.3-19.2)
Homininae-Ponginae	**14.0**	**14.0**	**14.0**	14.5 (**14.0**-15.7)
Within *Pongo*	4.1 (3.5-4.7)	4.3 (3.6-4.9)	4.5 (3.6-5.4)	3.8 (3.3-4.4)
Homo+Pan-Gorilla	8.1 (7.1-9.0)	8.2 (7.2-9.2)	8.1 (7.0-9.2)	8.6 (7.9-9.4)
Homo-Pan	**6.0**	**6.0**	**6.0**	6.3 (**6.0**, 6.9)
Within-*Pan*	2.4 (2.0-2.7)	2.4 (2.0-2.7)	2.7 (2.1-3.3)	2.3 (2.0-2.7)
Cercopithecinae-Colobinae	16.2 (14.4-17.9)	16.3 (14.4-18.1)	15.6 (13.5-17.7)	17.9 (15.3-20.7)
Colobini-Presbytini	10.9 (9.6-12.3)	11.4 (9.9-12.8)	10.7 (8.9-12.5)	11.5 (9.6-13.5)
Cercopithecini-Papionini	11.6 (10.3-12.9)	10.9 (9.4-12.3)	11.3 (9.7-13.0)	11.7 (9.8-13.7)
Macaca-Papio	9.8 (8.6-10.9)	9.5 (8.2-10.7)	9.2 (7.7-10.8)	10.2 (8.5-12.1)

[1] Values in **boldface** are assumed calibration points (see text).
[2] Values are given as Estimate (approximate 95% confidence interval).
[3] Values are given as Estimate (Bayesian 95% credibility interval).

calibration point of 23 Ma for this divergence is overly conservative, and the true divergence date of the hominoids and cercopithecoids was slightly older, as has been assumed or estimated by many previous researchers (see Table 1).

Discussion

Complete mtDNA genome sequences were acquired for an exemplar of all currently unrepresented catarrhine tribes, including *Chlorocebus aethiops* (Cercopithecini), *Colobus guereza* (Colobini), and *Trachypithecus obscurus* (Presbytini), in order to estimate divergence dates for the major catarrhine lineages. These data were collected by direct sequencing of long-range PCR products, which has proven to be an effective and efficient method of determining mtDNA genome sequences. Long-range PCR and direct sequencing has the added advantage of virtually eliminating the possibility of mistaking nuclear pseudogenes of mtDNA for the real mitochondrial genome (Thalmann et al., 2004). While not eliminating the possibility of *amplifying* nuclear insertions, because numts are unlikely to assemble into a circular genome, they are likely to be *detected* by this method. This approach is also less tedious than identifying the real mtDNA genomic sequence versus numt-DNA sequences through reverse transcriptase-coupled PCR of mitochondrial messenger RNAs, as has been done for some Old World monkey species (Collura et al., 1996). RNA-based methods also require higher quality tissue samples than do standard DNA-based PCR methods; thus, the "long PCR" method employed here is applicable to a wider range of specimens.

Mitochondrial nucleotide substitution rates have varied across taxa during catarrhine evolution. Most strikingly, the hominoids and cercopithecoids have had statistically significantly different mtDNA nucleotide substitution rates. The mtDNA nucleotide substitution rate is about 1.4 times faster in the cercopithecoids than in the hominoids, which is approximately the same rate difference as seen for nDNA of these taxa (Goodman et al., 1998; Yi et al., 2002). We do not know whether the 1.4 fold difference in rate seen for both the mtDNA and nDNA is merely a coincidence, or is the result of some common underlying process governing the nucleotide substitution rate of the nuclear and mitochondrial genomes in the catarrhines. This rate difference in nDNA sequence evolution has long been suggested to reflect a "hominoid slow-down" in the rate of nucleotide substitution (Goodman, 1961; Goodman et al., 1971; Li et al., 1987; Li, 1997). Based on the available data, at present it is not possible to tell whether the rate difference in mtDNA evolution is due to a "hominoid slow-down" or a "cercopithecoid speed-up"—or both. It is difficult to determine the evolutionary polarity of nucleotide substitution rate changes because there is no universal rate of nucleotide substitution (Yi et al., 2002), and thus no "normal" nucleotide substitution rate. Cladistically, one way to polarize evolutionary changes is to examine outgroups (Sober, 1988). However, within the primates, there have been several accelerations and/or decelerations in the rate of nucleotide substitution in mtDNA. For instance, evidence of an episodic pattern of substitution rate acceleration and deceleration has been inferred from the sequences of mtDNA- and nDNA-encoded protein subunits involved in the mitochondrial electron transport chain (Wu et al., 2000; Grossman et al., 2001; Wildman et al., 2002; Goldberg et al., 2003). A further complication in the assessment of the polarity of the change in rate is that cercopithecoids and hominoids have been characterized by a significantly slower rate of nucleotide substitution than most other mammals in nDNA sequences from at least the time of the catarrhine diversification (Yi et al., 2002). Thus, at present we cannot determine if the mtDNA rate difference between hominoids and cercopithecoids is due to a slowdown in the former or a speed-up in the latter.

The new date estimates presented here are difficult to compare directly to previously published estimates for a variety of reasons, including the use of different types of data and molecules, the addition of newly sequenced cercopithecoid mtDNA genomes, different assumed calibration points, and different methods of analysis. However, certain generalizations can be made for some cases.

Recent reviews of the fossil history of the catarrhine primates place the divergence of the living taxa well within the last 30 million years (entries in Delson et al., 2000; chapters in Hartwig, 2002). All well-established fossil catarrhine taxa fall within this time range—with the vast majority dating from the last 20 million years (Fig. 3). The divergence dates we have estimated based on the sequences of the mitochondrial genomes (Table 3; the results from the "All genes" analysis summarized in Fig. 6) coincide completely with the known fossil history of the catarrhine primates (Fig. 3), as well as many previous molecular estimates, particularly those based on nDNA analyses (Tables 1 and 2).

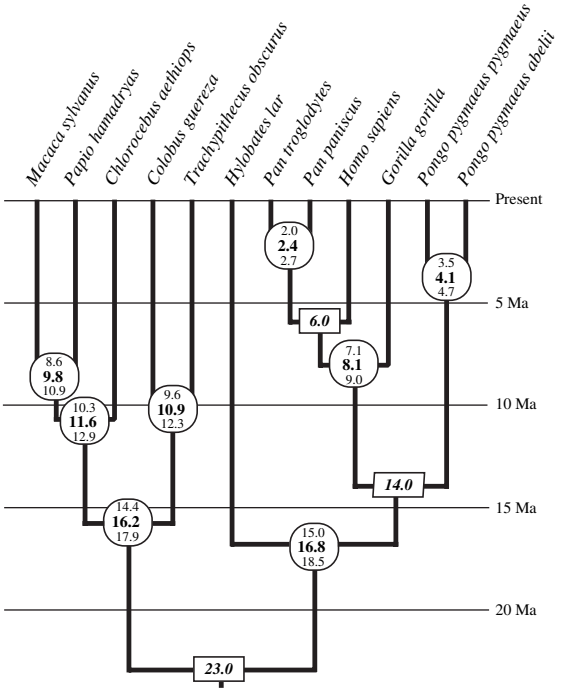

Fig. 6. Catarrhine divergence date estimates from complete mtDNA genome sequences. The statistically significant nucleotide substitution rate difference between the hominoids and cercopithecoids was taken into account by likelihood-based rate correction methods (penalized likelihood; Sanderson, 2002). Calibration points (in squares) were chosen on the basis of substantial fossil evidence in the primate record using the criteria set forth in the text. The estimates resulting from analysis of the All Genes dataset (see text) are given, flanked above and below by their approximate 95% confidence intervals.

Many prior publications have estimated hominoid divergence dates from both genetic and paleontological data (reviewed in Pilbeam, 2002). The mtDNA-based divergence dates presented here are consistent with estimates derived from relatively long nDNA sequences, such as those reviewed by Goodman et al. (1998). While our estimates are broadly comparable to other nDNA-derived estimates, several studies that analyzed multiple loci have suggested more recent pongine-hominine and hylobatid-hominid divergence dates (Easteal and Herbert, 1997; Kumar and Hedges, 1998; Stauffer et al., 2001; Hedges and Kumar, 2003, 2004). In these studies, the date estimates for the within-hominoid divergences are younger than generally suggested by the fossil record. Because the first appearance of a lineage in the fossil record provides an upper bound of that lineage's origin, divergence dates estimated from molecular data that are more recent are clearly problematic.

Few cercopithecoid divergence dates have been reported, whether based on paleontological or molecular data. The micro-complement fixation approach to immunological systems of Cronin and Sarich (1976) was the earliest attempt to use molecular data to estimate cercopithecoid divergence dates. One result of that study was the first report of molecular support for the polyphyly of the then-single genus of mangabeys—now split into the *Cercocebus* and *Lophocebus* mangabeys (Disotell, 2000). However, the divergence dates derived from these immunological data were substantially younger than other within-cercopithecoid divergence date estimates, including the results from this study (see Table 2). In fact, all divergence date estimates from these immunological data were too recent (see entry for Sarich and Wilson, 1967, in Table 1). Micro-complement fixation titers are now known to be difficult to apply to divergence date estimation because they comprise complex, continuous data that often show non-reciprocal results depending on the source species for the antiserum (Sarich and Wilson, 1967; Cronin and Sarich, 1976; Disotell 2000).

Some within-cercopithecoid divergence date estimates derived from analysis of a smaller segment (~1600 bp) of the mitochondrial genome

(Disotell and Raaum, 2003) are on the older side of the ranges reported here (Table 2). However, Disotell and Raaum (2003) discussed difficulties with the relatively small size of the dataset and problems of consistency with the fossil record not present in the new, expanded mitochondrial dataset. Our results are consistent with both the single within-cercopithecoid result from DNA-DNA hybridization experiments (Kohne et al., 1972) and the nDNA globin sequence estimates (Goodman et al., 1998; Table 2).

Our estimated cercopithecoid divergence dates are also consistent with what is known about the cercopithecoid fossil record (Delson, 1975, 1979, and updates in Delson et al., 2000). Setting aside the single upper premolar found in Germany with questionable colobine affinity and a suggested date of 10-11 Ma (Delson, 2000c), the oldest known fossil colobine was present in Africa by around 10 Ma (*Microcolobus*; Delson, 2000c) and the earliest Eurasian fossil colobine appeared around 9 Ma (*Mesopithecus*; Delson, 2000c). The ages for these specimens are concordant with our estimate of the divergence date for the African and Asian colobine lineages of about 11 Ma. These fossils and our estimated dates are also consistent with the hypothesis that the ancestral colobine was African and subsequently dispersed into Eurasia around 10 Ma (Stewart and Disotell, 1998). The earliest fossil evidence for the cercopithecines consists of isolated teeth found in North Africa, often referred to *Macaca*, at around 8 Ma (Arambourg, 1959; Delson, 1975, 2000b); our molecular estimates of the divergence of the *Macaca* and *Papio* lineages at about 9-10 Ma is consistent with this interpretation of the fossil record. Our results also support the current understanding of the phylogenetic position of the extinct Old World monkey genus, *Victoriapithecus* (Delson, 2000b; Benefit and McCrossin, 2002); while clearly cercopithecoid, *Victoriapithecus* can be assigned neither to the colobines nor to the cercopithecines.

Robust molecular dates calibrated with reliably interpreted fossil evidence are essential for consistent biogeographic scenarios (e.g., Begun and Gülec, 1998; Stewart and Disotell, 1998; Jaeger et al., 1998). For example, the identity and geographic origins of the African hominoid lineage is still under great debate. Stewart and Disotell (1998) used molecule-based divergence dates along with phylogenetically analyzed fossils to suggest an Asian origin for the lineage leading to modern African hominoids. Related studies testing hypotheses of environmental effects on catarrhine evolution (e.g., Jaeger and Hartenberger, 1989; Potts, 1998; Frost, 2001; Bobe et al., 2002; Pickford, 2002) require temporal data for the origins of lineages that may not be sufficiently documented by the fossil record. The natural history of the Old World monkeys may provide good models for interpreting human evolution (Jolly, 1970, 2001); accurate divergence date estimates enhance such models. Finally, the use of properly inferred and concordant divergence date estimates is a crucial component for the reconstruction of the evolutionary histories of the primates and their pathogens, which, in many cases, may have co-evolved for millions of years.

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