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Mitochondrial relationships and divergence dates of the African colobines: evidence of Miocene origins for the living colobus monkeys

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Abstract

The African colobines represent a neglected area of cercopithecid systematics. Resolving the phylogenetic relationships and estimating divergence dates among the living forms will provide insight into the evolution of this group and may shed light upon the evolution of other African primates as well. This is the first molecular assessment of the evolutionary relationships among the modern colobus monkeys, which are comprised of the black-and-white, olive, and red colobus groups. Over 4,000 base pairs of mitochondrial DNA were amplified and sequenced in over 40 colobus monkey individuals incorporating representatives from all commonly recognized species. Gene trees were inferred using maximum likelihood and Bayesian inference, and penalized likelihood was employed to estimate mitochondrial divergence dates among the sampled taxa. The results are congruent with some aspects of previous phylogenetic hypotheses based on morphology and vocalizations, although the relationships among several West and Central African taxa differ to some degree. The divergence date analysis suggests that the black-and-white, olive, and red colobus had diverged from one another by the end of the Miocene, and that by the Plio-Pleistocene many of the species lineages were already present. This demonstrates that the initial extant colobus monkey diversification occurred much earlier than previously thought and was likely part of the same adaptive radiation that produced the diverse colobine taxa seen in the African Plio-Pleistocene fossil record. The lack of early members from the modern lineages in fossiliferous deposits suggests that they resided in part in the forests of Central and West Africa, which also currently harbor the highest levels of colobus monkey diversity. These forests should not be ignored in models of Plio-Pleistocene human and nonhuman primate evolution.

Keywords: Colobine; Colobine phylogeny; Colobine systematics; *Colobus*; *Procolobus*; Molecular systematics; Mitochondrial DNA

Introduction

The diversification of African colobines represents a major component of African primate evolution whose details remain largely unresolved. Very few of the extinct forms can be reliably connected to the living ones, and the relationships and timing of divergence events among the extant taxa are unclear. Analogous trends between the evolution of this group and that of humans suggest that elucidation of colobine evolutionary history may provide insight into our own origins. Indeed, fossil colobines often co-occur with hominins at the same localities as the two radiations show similar temporal depth and geographic distribution.

The earliest colobine in the African fossil record is found in deposits dating to the late Miocene (9–8.5 Ma; Kingston et al., 2002). Colobines are then scarce in Africa until the Pliocene, at which time there is a diverse radiation of these animals, most of which were particularly large-bodied, adapted to partial terrestriality, and named as distinct genera. Colobines morphologically similar to the extant forms do not appear until the early Pleistocene, with specimens directly assignable to the modern

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species found in late Pleistocene deposits (reviewed in Jablonski, 2002 and Frost and Alemseged, 2007). Some have thus suggested that the living African colobines have recent origins and differentiated after the diverse Plio-Pleistocene forms had gone extinct (Delson, 1994; Leakey et al., 2003). Furthermore, since the earliest known cercopithecoid (*Victoriapithecus*; Benefit and McCrossin, 2002) and some early colobines (e.g., *Mesopithecus*, *Cercopithecoides*, *Paracolobus*; Birchette, 1982; Leakey, 1982; Delson, 1994; Ting, 2001; Frost and Delson, 2002; Jablonski, 2002) are known to be at least partly terrestrial, the modern colobine condition of being predominantly arboreal has been inferred to have recent origins as well (Leakey et al., 2003). However, a recent molecular study with a small African colobine sample ($n = 2$) suggests that the extant African colobine radiation began much earlier (Sterner et al., 2006). Estimation of divergence dates among all living colobus monkey species will provide better resolution of this issue.

The living African colobines are represented by three distinct groups distributed across the African rainforest belt—the black-and-white colobus, the olive colobus, and the red colobus. As ''leaf-eating monkeys,'' they have adaptations to a folivorous diet, including a multi-chambered ruminant-like stomach, and are distinguished from their Asian relatives by morphological features such as a reduced pollex and midtarsal shortening (Delson, 1975; Strasser and Delson, 1987). Of the three groups, black-and-white colobus monkeys (*Colobus*) have the most continuous distribution (Fig. 1), with five commonly recognized species dispersed throughout equatorial Africa (Oates and Trocco, 1983; Grubb et al., 2003). Red colobus monkeys [*Procolobus* (*Piliocolobus*)] are also distributed across equatorial Africa, from the Gambia to Zanzibar (Fig. 2), but in a more fragmented manner, and they possess an unstable taxonomy with little consensus on the number of species that should be recognized. The olive colobus monkey [*Procolobus* (*Procolobus*)] is monotypic and restricted to the Guinean coastal forests of West Africa, ranging from Sierra Leone to Nigeria (Fig. 3; Oates et al., 1994)

Phylogenetic hypotheses concerning the living African colobines have been developed using analyses of morphology, pelage, and vocalizations. The current hypothesis of relationships among the three groups is a sister taxon relationship between the olive colobus and red colobus to the exclusion of the black-and-white. The former two share certain features that set them apart, such as female sexual swellings, a small larynx, and discontinuous male ischial callosities (Hill, 1952; Kuhn, 1972; Napier, 1985; Strasser and Delson, 1987). However, the polarity of these traits is unknown and the states listed may be plesiomorphic, thus leaving some room for doubt concerning how the three groups are related to one another.

Among the black-and-white colobus monkeys, Schwarz (1929) and Hull (1979) used pelage and craniometrics, respectively, to discern four groups that are now commonly recognized as different species (*C. polykomos, C. guereza, C. angolensis,* and *C. satanas*). Oates and Trocco (1983) used male loud call variation to elevate a fifth form, *Colobus vellerosus*, to species rank. This taxon was formerly considered to be a subspecies of *C. polykomos* but the vocalization data suggest it has closer affinities with *C. guereza*. The black-and-white colobus monkey relationships displayed in Fig. 4 are hypotheses based on studies of male loud call variation (Oates and Trocco, 1983) and morphology (Groves et al., 1993).

Within the red colobus monkey group, relationships among the numerous forms (at least 18) remain one of the longest standing unresolved issues in African primate taxonomy. They display a level of diversity that exceeds what is typically seen in a single primate species but present a complex pattern

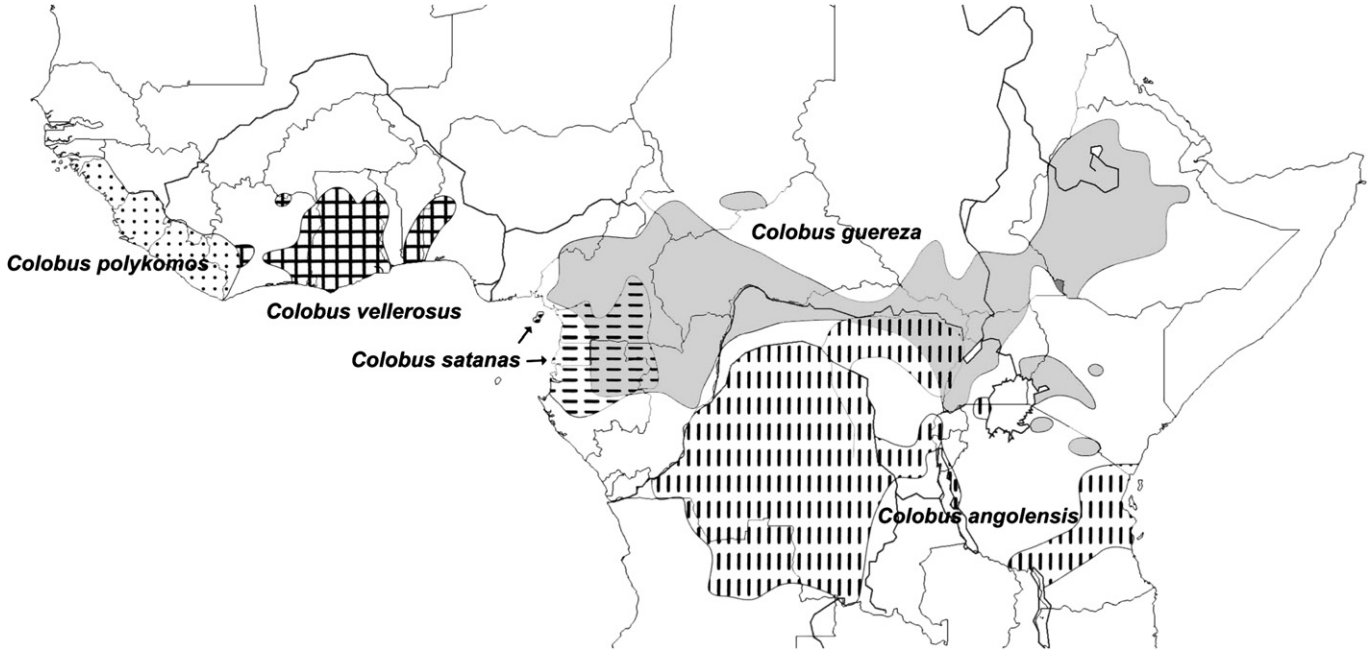

Fig. 1. Distribution of black-and-white colobus (*Colobus*) species. Classification follows Grubb et al. (2003). Adapted from Oates and Trocco (1983) and Oates et al. (1994). Dots = *C. polykomos*; squares = *C. vellerosus*; horizontal hatch = *C. Satanas*; vertical hatch = *C. angolensis*; grey = *C. guereza*.

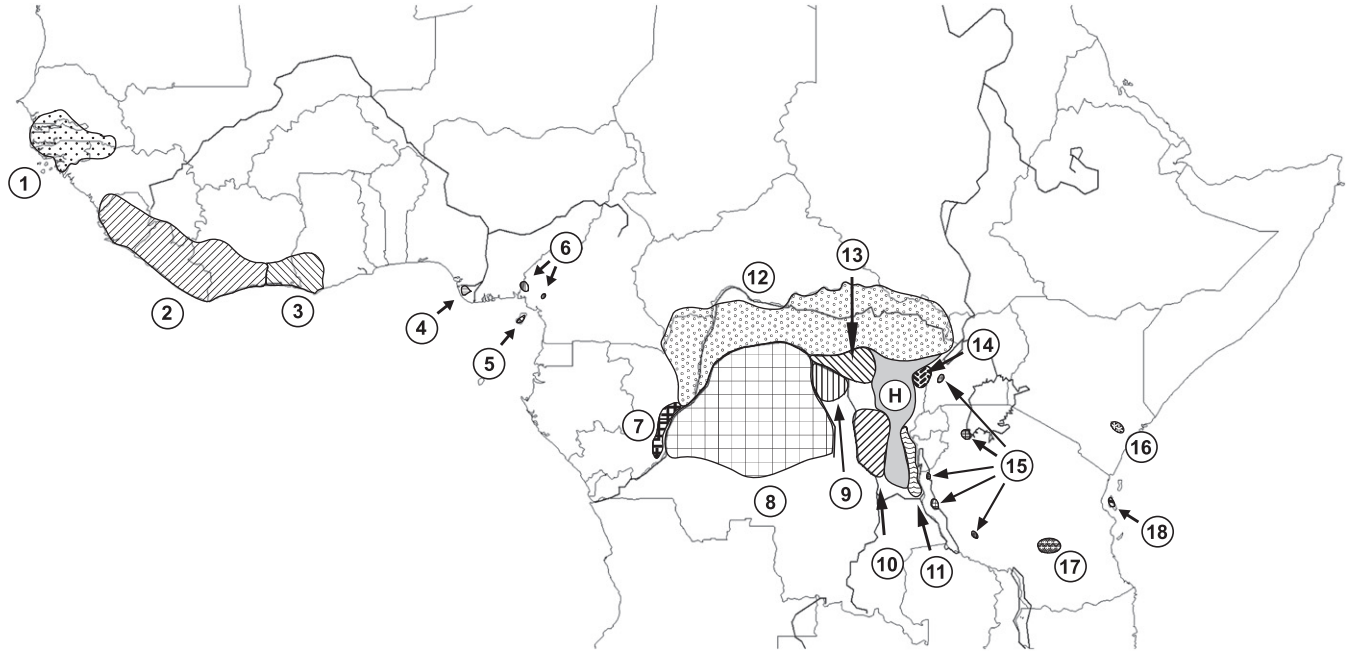

Fig. 2. Distribution of red colobus [*Procolobus* (*Piliocolobus*)] taxa. Subspecies are shown due to the uncertainty in red colobus species level classification. Area marked with an H refers to a putative zone of hybridization between adjacent taxa. Symbols indicate the range of the species referred to by the adjacent number. 1: *P. badius temminckii*; 2: *P. b. badius*; 3: *P. b. waldroni*; 4: *P. b. epieni*; 5: *P. b. pennantii*; 6: *P. b. preussi*; 7: *P. b. bouvieri*; 8: *P. b. tholloni*; 9: *P. b. parmentieri*; 10: *P. b. lulindicus*; 11: *P. b. foai*; 12: *P. b. oustaleti*; 13: *P. b. langi*; 14: *P. b. ellioti*; 15: *P. b. tephrosceles*; 16: *P. b. rufomitratus*; 17: *P. b. gordonorum*; and 18: *P. b. kirkii*. Classification follows Oates et al. (1994). Distributions from Colyn (1991, 1993), Oates et al. (1994), Grubb and Powell (1999), and author's own notes.

of variation that obscures evolutionary relationships, thus making the diagnosis of multiple species difficult. Although their classification at the subspecies level is relatively stable, there is no current consensus on how many species should be recognized, and the assignment of species names to certain forms remains contentious. Between one and sixteen different species have been recognized (e.g., Rahm, 1970; Napier, 1985; Groves, 2001, 2007), and the most recent assessment of African primate diversity was unwilling to even attempt assignment of species names to some forms (Grubb et al., 2003). Because there is no broad consensus on how to classify these animals, all red colobus taxa are assigned here to the species

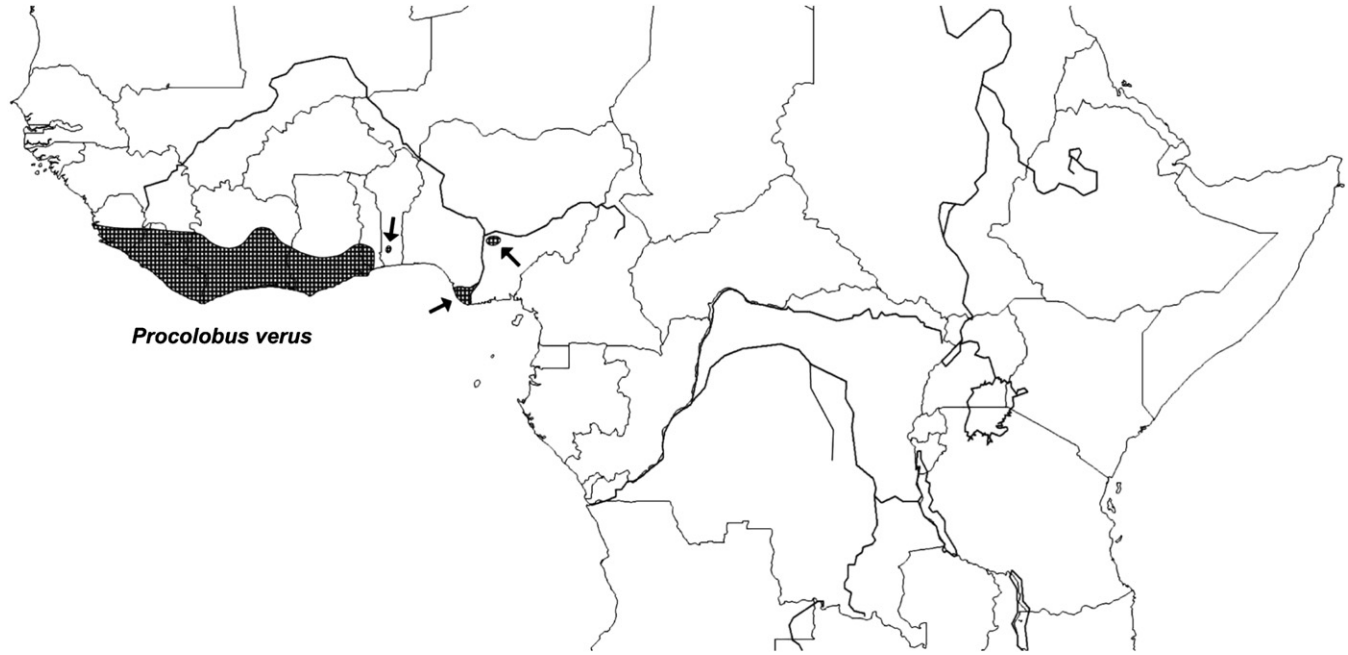

Fig. 3. Distribution of the olive colobus monkey [*Procolobus* (*Procolobus verus*)]. Adapted from Oates et al. (1994) with updated data from Oates (1996).

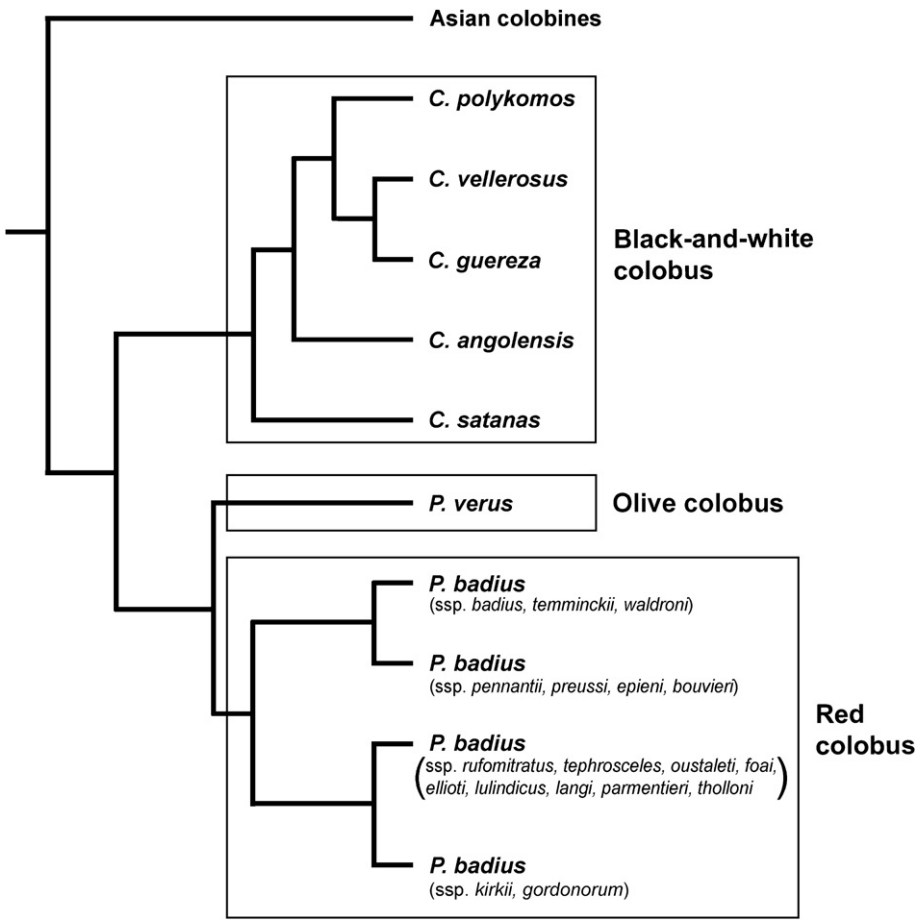

Fig. 4. Current hypotheses concerning the relationships among the living African colobines based on morphology, pelage, and vocalizations (Struhsaker, 1981; Oates and Trocco, 1983; Groves et al., 1993; Grubb et al., 2003). Classification follows Oates et al. (1994) and Grubb et al. (2003).

Procolobus badius, following Oates et al. (1994). It must be emphasized that this is *only* done for the sake of simplicity and is an unsatisfactory course of action. However, for the purposes of this study, taking a conservative one species approach is preferred over arbitrarily choosing a classification to follow. Pelage and vocalization data loosely assemble the red colobus forms into phylogenetic groups displayed in Fig. 4.

None of the hypotheses proposed for the relationships among the living African colobines (Fig. 4) have been tested using molecular methods, which have been used to provide new insights into the evolutionary history of other Old World monkey groups (e.g., Harris and Disotell, 1998; Evans, 1999; Tosi et al., 2003, 2004; Sterner et al., 2006; Whittaker et al., 2006; Ting et al., 2008). Nearly 4,000 base pairs of mitochondrial DNA were amplified, sequenced, and analyzed in order to test hypotheses concerning relationships among the living colobus monkeys and provide divergence date estimates for these taxa.

Materials and methods

Samples

All species of African colobine recognized by Grubb et al. (2003) are included in this study (Table 1). Figure 5 shows the various localities where samples were collected. It is important to note that the sampling focus for the black-and-white colobus group was at the species level while the sampling focus for the red colobus group was at the subspecies level since the species level classification is so problematic. Samples were from a variety of biomaterials (e.g., tissue, blood, feces), and all of the individuals used were from the wild with the exception of some *C. guereza* and *C. angolensis* zoo specimens. Effort was made to sample throughout the geographic range for each taxon. The data from one *P. badius badius* individual and various outgroup taxa were obtained from GenBank.

Molecular marker

The marker surveyed here is of mitochondrial origin. Mitochondrial DNA was chosen because of its fast rate of mutation, small effective population size, and quick time to lineage fixation. These characteristics make it more likely to track the species phylogeny compared to nuclear genes, which are generally poor at resolving short internodes (Moore, 1995). Furthermore, mitochondrial gene trees in particular are expected to reflect the population history of an organism when geographical distributions are restricted and females transfer from their natal groups. The red colobus and olive colobus

Table 1
Individuals and taxa sampled. Individuals excluded from analysis because of sequence data identical to others are marked with an asterisk (*). Classification follows Oates et al. (1994) and Grubb et al. (2003)

Taxon	Origin/Locality	Genbank #
Cebus albifrons	Unknown	NC002763
Homo sapiens	Unknown	NC001807
Pan troglodytes	Unknown	NC001643
Papio hamadryas	Unknown	NC001992
Theropithecus gelada	Unknown	EU580083
Presbytis melalophos	Unknown	DQ355299
Colobus angolensis spp.	Unknown, zoo specimen	EU580046
Colobus angolensis ssp.	Wild born zoo specimen, ssp. *palliatus?*	EU580047
Colobus angolensis palliatus	Udzungwa Mountains, Tanzania	EU580048
Colobus guereza ssp.	Unknown, zoo specimen, ssp. *caudatus?*	EU580049
Colobus guereza ssp.	Unknown, zoo specimen, ssp *kikuyuensis*?	EU580050
Colobus guereza matschiei	Kakamega Forest, Kenya	EU580051
Colobus guereza occidentalis	Unknown locality, Cameroon	EU580052
Colobus polykomos	Taï National Park, Côte d'Ivoire	EU580053
*Colobus polykomos**	Taï National Park, Côte d'Ivoire	–[a]
*Colobus polykomos**	Taï National Park, Côte d'Ivoire	–[a]
Colobus satanas satanas	Bioko Island, Equatorial Guinea	EU580054
Colobus satanas satanas	Bioko Island, Equatorial Guinea	EU580055
*Colobus satanas satanas**	Bioko Island, Equatorial Guinea	–[a]
Colobus vellerosus	Boabeng-Fiema Monkey Sanctuary, Ghana	EU580056
*Colobus vellerosus**	Boabeng-Fiema Monkey Sanctuary, Ghana	–[a]
*Colobus vellerosus**	Boabeng-Fiema Monkey Sanctuary, Ghana	–[a]
*Colobus vellerosus**	Boabeng-Fiema Monkey Sanctuary, Ghana	–[a]
Procolobus (*Procolobus*) *verus*	Taï National Park, Côte d'Ivoire	EU580082
P. (*Piliocolobus*) *badius badius*	Unknown locality, Sierra Leone	DQ355301
P. (*Piliocolobus*) *badius badius*	Taï National Park, Côte d'Ivoire	EU580057
P. (*Piliocolobus*) *badius badius*	Taï National Park, Côte d'Ivoire	EU580058
P. (*Piliocolobus*) *badius gordonorum*	Matundu Forest, Udzungwa Mountains, Tanzania	EU580059
P. (*Piliocolobus*) *badius gordonorum*	Ndundulu Forest, Udzungwa Mountains, Tanzania	EU580060
P. (*Piliocolobus*) *badius gordonorum*	Mwanihana Forest, Udzungwa Mountains, Tanzania	EU580061
P. (*Piliocolobus*) *badius kirkii*	Zanzibar Island, Tanzania	EU580062
P. (*Piliocolobus*) *badius kirkii*	Zanzibar Island, Tanzania	EU580063
P. (*Piliocolobus*) *badius kirkii*	Zanzibar Island, Tanzania	EU580064
P. (*Piliocolobus*) *badius kirkii*	Zanzibar Island, Tanzania	EU580065
P. (*Piliocolobus*) *badius kirkii*	Zanzibar Island, Tanzania	EU580066
P. (*Piliocolobus*) *badius oustaleti*	Badane, Central African Republic	EU580067
P. (*Piliocolobus*) *badius parmentieri*	Democratic Republic of Congo	EU580068
P. (*Piliocolobus*) *badius pennantii*	Bioko Island, Equatorial Guinea	EU580069
P. (*Piliocolobus*) *badius pennantii*	Bioko Island, Equatorial Guinea	EU580070
P. (*Piliocolobus*) *badius pennantii*	Bioko Island, Equatorial Guinea	EU580071
P. (*Piliocolobus*) *badius preussi*	Korup National Park (North), Cameroon	EU580072
P. (*Piliocolobus*) *badius preussi*	Korup National Park (South), Cameroon	EU580073
P. (*Piliocolobus*) *badius preussi*	Korup National Park (South), Cameroon	EU580074
P. (*Piliocolobus*) *badius rufomitratus*	Tana River, Kenya	EU580075
P. (*Piliocolobus*) *badius temminckii*	Abuko Nature Reserve, The Gambia	EU580076
P. (*Piliocolobus*) *badius temminckii*	Njassang Forest Park, The Gambia	EU580077
P. (*Piliocolobus*) *badius tephrosceles*	Kibale National Park, Uganda	EU580078
P. (*Piliocolobus*) *badius tephrosceles*	Gombe National Park, Tanzania	EU580079
P. (*Piliocolobus*) *badius tholloni*	Salonga National Park, Democratic Republic of Congo	EU580080
P. (*Piliocolobus*) *badius tholloni*	Salonga National Park, Democratic Republic of Congo	EU580081

[a] This sequence is identical to another individual submitted to GenBank.

taxa, and perhaps some of the black-and-white colobus populations, tend to show these distributions and social organization (Newton and Dunbar, 1994; Oates, 1994).

A 3,831 base-pair fragment of mitochondrial DNA encompassing the NADH3, NADH4L, NADH4, and NADH5 genes was analyzed. Whole mitochondrial genomes were amplified, sequenced, and aligned in the few taxa from which high quality biomaterials were available (see Extraction, amplification, and sequencing). From this alignment, the chosen gene regions were identified as having an appropriate amount of variation for phylogenetic analysis. Whole mitochondrial genomes were not used in the final analysis because those data were not available for the majority of the individuals (e.g., those whose biomaterials were of degraded origin). The length of the fragment analyzed was found to be near the maximum limit of mitochondrial DNA that can be reliably amplified in

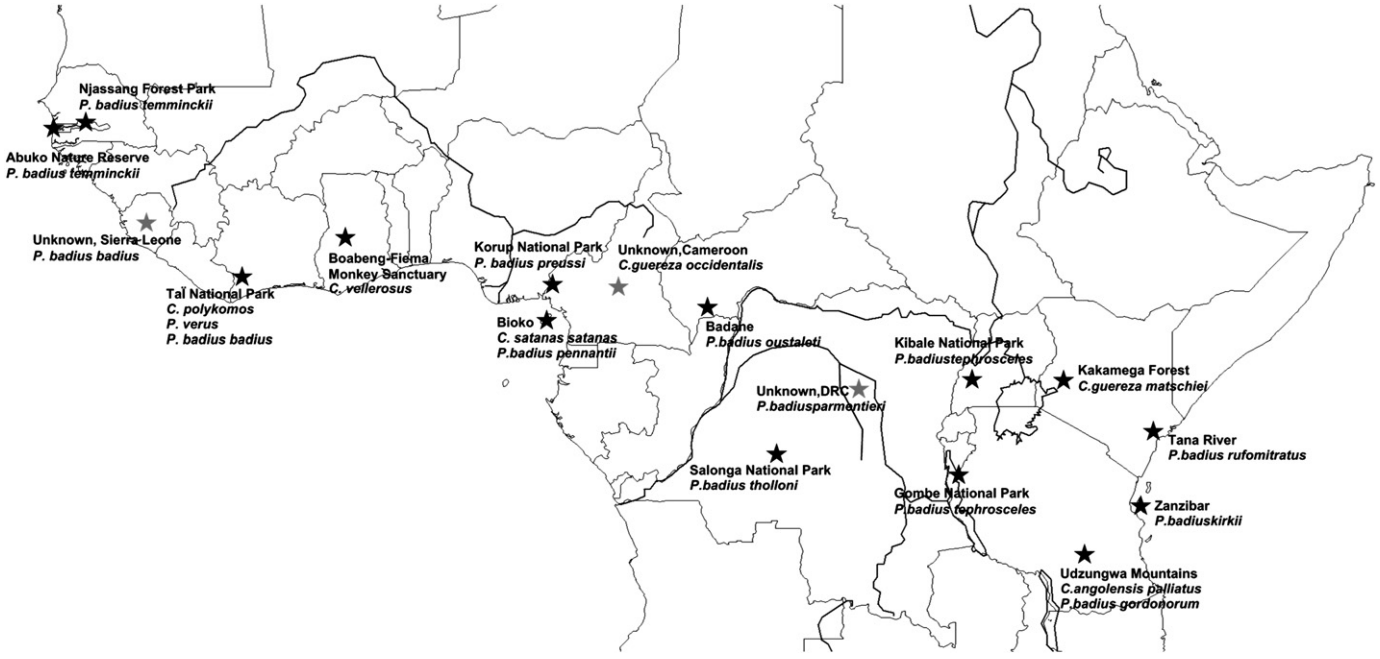

Fig. 5. Map of localities for black-and-white (*Colobus*), olive [*Procolobus (Procolobus*)], and red [*Procolobus* (*Piliocolobus*)] colobus monkey samples. Beneath each locality name are the species collected there. Gray stars indicate countries where samples were collected but the exact origins are unknown.

one piece from fecal samples. It comprises over a third of the mitochondrial protein-coding regions, all of which evolve at a similar rate and together make up the majority of the mitochondrial genome (Sterner et al., 2006). The chosen marker should thus provide a reliable estimate of the mitochondrial gene tree.

Extraction, amplification, and sequencing

Total genomic DNA was extracted using protocols from the QIAamp DNA Blood mini kit (Qiagen, cat. No. 51104), the DNeasy Tissue kit (Qiagen, cat. No. 69504), and the Qiagen DNA stool kit (Qiagen cat. No. 51504). The Expand Long Template PCR system (Roche, cat. No. 1681834) was used to perform long-range amplifications because all PCR targets were 3,000+ base-pairs long. This amplification strategy was specifically employed to avoid nuclear pseudogenes of mitochondrial origin. These are fragments of mitochondrial DNA that, through evolutionary time, have inserted themselves into the nuclear genome. The presence of these pseudogenes is problematic as they resemble mitochondrial DNA and can be accidentally amplified when targeting mitochondrial markers. If unrecognized, inclusion of these sequences in a dataset will lead to false inferences concerning the mitochondrial gene tree. Thus, it is essential to ensure that sequences of true mitochondrial origin are being preferentially amplified in comparison to nuclear pseudogenes.

When high-quality tissue samples were available, the mitochondrial genome was amplified in two 10,000 base-pair segments that overlapped on one another at each end, and the overlapping regions were sequenced to ensure identical reads (see Fig. 6 for amplification strategy). This method increases

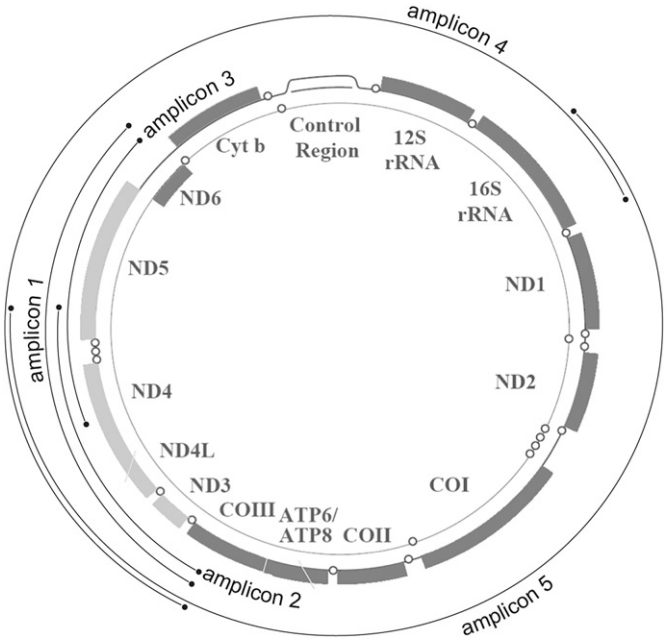

Fig. 6. Amplification strategy. Analyzed region (ND3, ND4L, ND4, ND5) is shown in light gray. Long-range PCR was employed in an attempt to avoid amplification of nuclear pseudogenes. Amplicon 1 (primers 9210F/14103R) represents a 5,000 base-pair amplification of the entire target region. For more degraded samples, this region had to be broken into two 3,000 base pair fragments: Amplicon 2 (primers 9210F/12576R) and Amplicon 3 (primers colFE1 and 14103R). For samples of particularly high quality, the whole mitochondrial genome was amplified in two 10,000 base-pair segments that overlapped with one another on each end: Amplicon 4 (9210F/2730R) and Amplicon 5 (1970F/12576R). The overlapping regions were sequenced to ensure that they were identical. See Table 2 for amplification primer sequences.

the likelihood of obtaining a target template that is circular and, thus, of mitochondrial origin (as opposed to nuclear DNA, which is linear; Thalmann et al., 2004; Raaum et al., 2005). In more degraded samples such as fecal specimens, the target region was amplified in one 5,000 base-pair amplification or two 3,000 base-pair amplifications. Since nuclear DNA exists at much lower copy numbers than mitochondrial DNA, preferentially amplifying such large regions of the former over the latter from degraded samples is very unlikely.

PCR primers (Table 2) and sequencing primers (available upon request) were from Sterner et al. (2006) or designed from a known African colobine template. Amplified products were cleaned using exonuclease I and shrimp alkaline phosphatase (Hanke and Wink, 1994). Cycle sequencing was performed using the Big Dye kit (Big Dye v3.1, ABI, cat. No. 4337456) following the manufacturer's protocol for diluted reactions, and products were run on an ABI PRISM 3730 DNA Sequencer. Complementary strands were sequenced from multiple PCR products to ensure the fidelity of the data, and the sequences were edited and assembled using Sequencher v4.5 (Gene Codes Corp.). These data have been deposited in GenBank under accession numbers found in Table 1.

Phylogenetic analysis

The intervening tRNAs between the chosen gene regions were excluded from the analysis as their model of evolution is different from the protein-coding regions. The NADH3, NADH4L, NADH4, and NADH5 genes were individually aligned using the program ClustalW (Chenna et al., 2003), adjusted by eye to correct for spurious insertions/deletions, then translated in MacClade 4.08 (Maddison and Maddison, 2005) to ensure that there were no stop codons or frameshift mutations typical of nuclear pseudogenes. These alignments were then reassembled into one dataset. Certain taxa sampled had different individuals with identical sequence data. In these cases, only one of these individuals was used to ease the computational intensity of the analysis.

Maximum likelihood (PAUP 4.0b10; Swofford, 2002) and Bayesian inference (Mr. Bayes 3.1; Ronquist and Huelsenbeck, 2003) were used to infer mitochondrial gene trees. The evolutionary model that best fit the data was determined using Modeltest 3.6 (Posada and Crandall, 1998). When analyzed under the Akaike Information Criterion (AIC, the more optimal of the two model comparison methods employed in Modeltest 3.6, see Posada and Buckley, 2004) the data were best fit by the general time reversible (GTR) model with invariant sites (I) and a gamma distribution (G) of site-specific rates. For maximum likelihood, 100 bootstrap replicates were performed under a heuristic search with random taxa added to the current node of the search tree and all other parameters left as default values. For the Bayesian analysis, the Markov Chain Monte Carlo (MCMC) chain was run for 1,000,000 generations sampled every 100 generations with a burnin value of 300. Nodes that were supported by a bootstrap value lower than 85 and a posterior probability value lower than 0.90 were manually collapsed.

Table 2
Amplification primers. Those marked with an asterisk (*) are from Sterner et al. (2006). Primer combinations and amplification strategy explained in Fig. 6

Primer name	Sequence
1970F*	CCCCGCCTGTTTACCAAAAACATCA
12576R*	GCTGCTGTGTTGGCATCTGTT
9210F*	CTCAGAGTATTATGAAGCACCCTTTACC
2730R*	TTTTATGCAATTACCGGGCTCTGCCATCTTAACAA
14103R	TCTTCTAAGCCTTCTCCAATTTATGG
colFE1	TCCTCCGTAAGCCACATAGCCCTA

Divergence date analysis

Following the methods of Raaum et al. (2005), penalized likelihood was used with a truncated Newton algorithm to estimate mitochondrial divergence dates in the program r8s (v1.71; Sanderson, 2003). This is a semiparametric approach that combines a parametric model with varying rates on different branches of the tree with a nonparametric penalty for the model if rates change too fast between branches. Thus, rates that strongly diverge from a clock-like model get "smoothed over" by the penalty function. Because r8s has difficulty handling unresolved nodes and the inferred tree had numerous polytomies at its terminal ends, the dataset was pruned so that each taxon was represented by only one individual. The one exception was the red colobus form *P. b. tholloni*, which had two individuals retained in the analysis because they fell on two divergent branches of the tree. In order to develop confidence intervals, the dataset was resampled 100 times and standard deviations were calculated for the sample of dates at each node. Calibration points were chosen based on the fossil record because using secondary points derived from other molecular datasets can compound the error in the date estimates (Graur and Martin, 2004). Ideally, multiple points that are bracketed and lie within the clade of interest are used (Raaum et al., 2005). Unfortunately, since the evolutionary relationships of the fossil colobines are so poorly understood, there is no calibration point within the colobine clade to anchor a divergence date analysis. The taxa most closely related to the colobines that have a well-dated split are *Papio* and *Theropithecus*, as the latter appears in the fossil record by 4–3.5 Ma (Leakey, 1993; Delson, 2000). Thus, the calibration points used were a *Papio-Theropithecus* split at 4 Ma, a hominoid-cercopithecoid split at 23 Ma, and a *Pan-Homo* split at 6 Ma. The latter two were chosen based on reasoning outlined in Raaum et al. (2005). It should be noted that these fossil calibrations are relatively young. Thus, for a given node, the analysis will infer a date (with confidence intervals) that corresponds to the latest mitochondrial date that a split could have occurred given these data.

Results

The divergence date results show some cases where branch lengths are so short that confidence intervals from neighboring

nodes show overlap, but this should not be interpreted as the later node possibly splitting before the earlier one. Instead, since support for all shown nodes is strong, this can be interpreted as mitochondrial lineages splitting consecutively and relatively quickly one after the other.

The higher-level catarrhine relationships and divergence dates generated by the data (Fig. 7) are consistent with previous studies that used all protein-coding regions on the heavy strand of the mitochondrial genome (Raaum et al., 2005; Sterner et al., 2006). The modern African colobine radiation seems to have started by the late Miocene with the black-and-white colobus having split from the other colobus groups by 7.5 Ma. The red colobus and olive colobus share a sister taxon relationship and had also diverged from one another by the late Miocene (6.4 Ma).

Among the black-and-white colobus species (Fig. 8), *C. satanas* is the first to diverge, followed by *C. angolensis* and then *C. guereza,* leaving *C. polykomos* and *C. vellerosus* as sister taxa. These species lineages had diversified by the end of the Pliocene and beginning of the Pleistocene, with *C. satanas* diverging by 3.5 Ma, *C. angolensis* by 2.1 Ma, *C. guereza* from *C. polykomos*/*C. vellerosus* by 1.6 Ma, and *C. polykomos* from *C. vellerosus* by 200,000 years ago.

Within the red colobus group (Fig. 9) there exist some mitochondrially paraphyletic or polyphyletic groupings. For example, the subspecies *P. b. badius* is mitochondrially paraphyletic as allelic lineages of *P. b. temminckii* are phylogenetically nested among those of *P. b. badius*. Meanwhile, *P. b. tholloni* is a mitochondrially polyphyletic taxon, as it possesses allelic lineages that are phylogenetically interspersed with respect to other taxa in the gene tree. Overall, there are three major clades in the red colobus group. One contains the West African subspecies *P. b. badius* and *P. b. temminckii*. Another contains the western equatorial subspecies (*P. b. pennantii* and *P. b. preussi*) and two individuals from taxa that reside in the Congo Basin (*P. b. tholloni* and *P. b. parmentieri*). A second individual of one of these Congo Basin forms (*P. b. tholloni*) appears in the third clade in a group that contains *P. b. oustaleti*, *P. b. rufomitratus,* and *P. b. tephrosceles*. The third clade also contains a sister taxon relationship between *P. b. kirkii* and *P. b. gordonorum*. In both likelihood and Bayesian analyses, the latter two major clades were sisters to the exclusion of the first, but this node was collapsed due to questionable bootstrap support (bootstrap value 73, posterior probability 0.96). The three main red colobus mitochondrial clades had separated by 3.0 Ma. Two other particularly deep splits also occurred, with *P. b. pennantii* and *P. b. preussi* separating from *P. b. tholloni* and *P. b. parmentieri* by 2.3 Ma, and *P. b. gordonorum* and *P. b. kirkii* diverging from the other East and Central African taxa by the early Pleistocene (1.4 Ma). The remaining red colobus mitochondrial lineages sampled diverged by the mid-late Pleistocene.

Discussion

Colobus phylogeny

Mitochondrial relationships among the black-and-white colobus species are similar to the ones based on male loud call variation with one exception (Oates and Trocco, 1983; Fig. 10). While the vocalization data place *C. vellerosus* as sister to *C. guereza*, the mitochondrial data suggest that it is very closely affiliated with *C. polykomos*, which is consistent with some previous hypotheses and classifications (Hull, 1979). Because mitochondrial DNA and male loud calls are presumably inherited through different systems of genetic transmission, these conflicting signals could be explained by differential lineage sorting of either mitochondrial or nuclear alleles (e.g., those affecting male loud calls). Differential lineage sorting occurs at a given locus when an ancestral population contains multiple alleles and the one that goes to fixation (through random processes) does not track the organismal phylogeny. Alternatively, ancestral hybridization between *C. guereza* and *C. polykomos* could explain the incongruence documented here. *C. guereza* males could have moved into a population of *C. polykomos* and selectively outcompeted the resident males over time. If this population subsequently became isolated, its descendants (*C. vellerosus*) would have mitochondrial alleles that affiliate with *C. polykomos* and at least some paternally linked traits (e.g., male loud calls) that indicate a close relationship to *C. guereza*. Female *C. polykomos* moving into *C. guereza* populations could also explain the patterns here but this scenario is less likely because of the predominantly female philopatric nature of black-and-white colobus monkeys. Although the geographic ranges and pelage patterns of these animals do not preclude these hypotheses, they must remain speculative until more data are collected. Additional sampling is required as the individuals for some

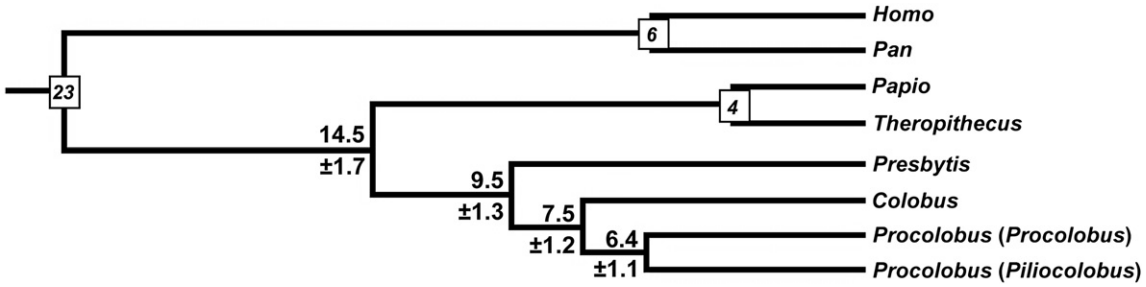

Fig. 7. Catarrhine mitochondrial likelihood and Bayesian tree based on NADH3, NADH4, NADH4L, and NADH5 genes (3,831 base pairs). All nodes supported by bootstrap values ≥85 and posterior probabilities ≥0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Calibration points are boxed and italicized. *Cebus* was the outgroup taxon. Classification follows Grubb et al. (2003).

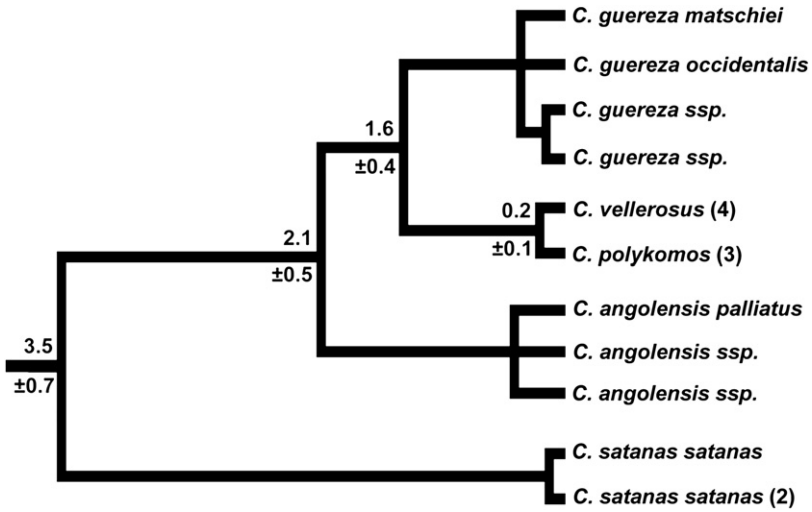

Fig. 8. Black-and-white colobus (*Colobus*) mitochondrial likelihood and Bayesian tree. All nodes supported by bootstrap values ≥85 and posterior probabilities ≥0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Numbers in parentheses indicate individuals with identical sequence that were not included in the analysis. Classification follows Grubb et al. (2003).

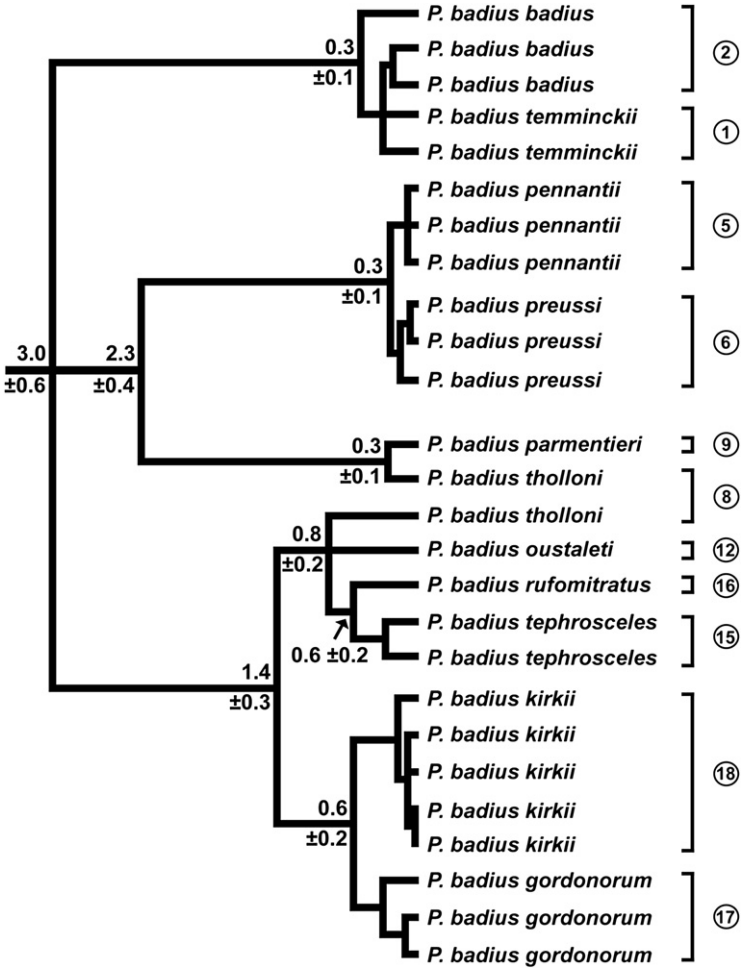

Fig. 9. Red colobus [*Procolobus* (*Piliocolobus*)] mitochondrial likelihood and Bayesian tree. All nodes supported by bootstrap values ≥85 and posterior probabilities ≥0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Numbers correspond to range distributions from Fig. 2. Classification follows Oates et al. (1994).

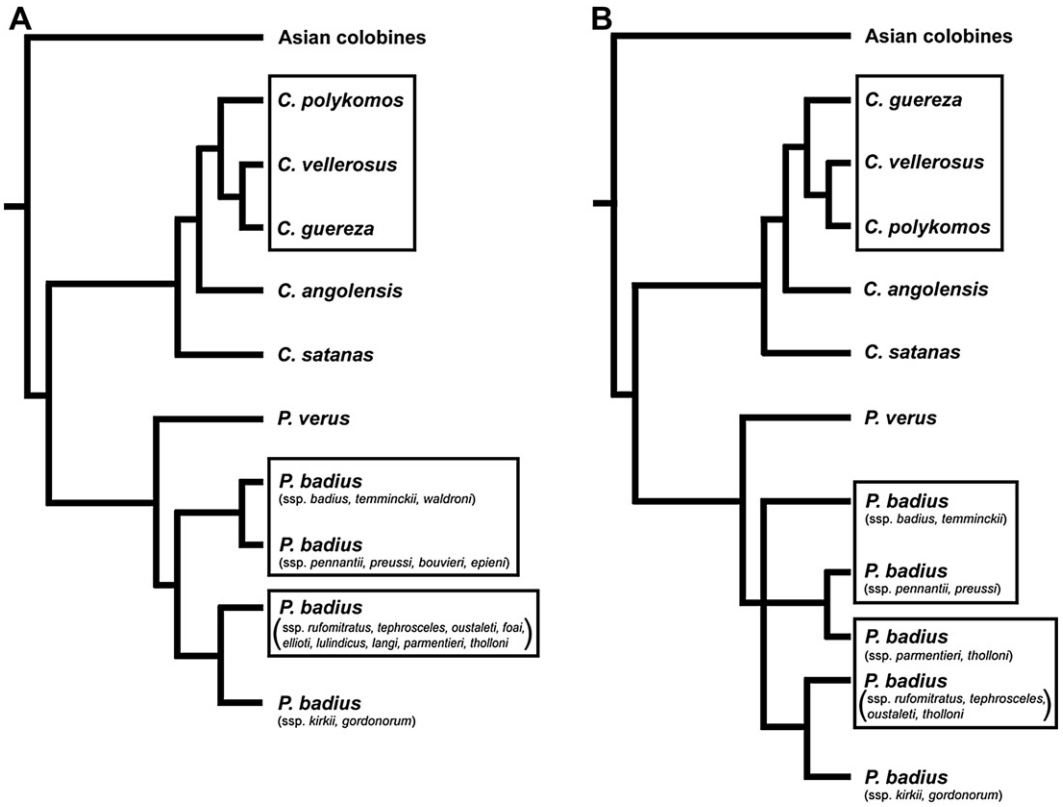

Fig. 10. Cladograms showing incongruent relationships between current phylogenetic hypotheses and mitochondrial relationships among the living African colobines. A) Phylogenetic hypotheses based on morphology, pelage, and vocalizations (Struhsaker, 1981; Oates and Trocco, 1983; Groves et al., 1993; Grubb et al., 2003). B) Mitochondrial relationships inferred in this study. Boxed taxa highlight the differences between the cladograms. Notice differences between the sister taxon to *C. vellerosus* and the phylogenetic groupings of the *P. badius* subspecies. Some subspecies of red colobus were not sampled. Classification follows Oates et al. (1994) and Grubb et al. (2003). See Discussion for details.

species are derived from only one locality each, and the results only reflect the mitochondrial relationships. Finding informative nuclear markers would be desirable, as are more field studies to determine social organization and how genetic lineages may have moved through populations. Until more data are collected, it is recommended to retain *C. vellerosus* at the species level as its evolutionary origins may have been distinct and quite complex.

Procolobus phylogeny

The sister taxon relationship between the red colobus and olive colobus found here is consistent with that based on other forms of data and can be combined with mitochondrial gene trees inferred by Sterner et al. (2006) and Whittaker et al. (2006) to support the hypothesis of reciprocal monophyly among the Asian and African colobines (Fig. 10). Within the red colobus group [*Procolobus (Piliocolobus)*], congruence between the mitochondrial relationships and those based on pelage and vocalization data are most evident in the grouping of *P. b. gordonorum* with *P. b. kirkii*, *P. b. badius* with *P. b. temminckii*, and *P. b. pennantii* with *P. b. preussi*. The paraphyly seen in *P. b. badius* with respect to *P. b. temminckii* may indicate that these animals shared gene flow until very recently, which gains credibility as their ranges are poorly documented and possibly still overlap.

There are also several areas of the red colobus inferred mitochondrial tree that are incongruent with current phylogenetic hypotheses (Fig. 10). The *P. b. pennantii*/*P. b. preussi* pair does not seem to have any particular affinity with the *P. b. badius*/*P. b. temminckii* pair; rather, they seem to be more closely related to some, if not all, of the taxa found further east. *P. b. parmentieri* grouping away from most of the Central African forms is unexpected as it shares with them many similarities in pelage (Colyn and Verheyen, 1987; Groves, 2001). It is also *very* unexpected to find individuals of *P. b. tholloni* in two divergent clades, as this is not a polytypic taxon and the samples were collected at the same time and locality. Thus, the polyphyly seen in *P. b. tholloni* is most likely a mitochondrial phenomenon and does not correspond to the organismal phylogeny. This type of pattern can be indicative of hybridization and/or the retention of ancestral lineages, and is possibly due to the complex biogeographic history of the Congo Basin. Further sampling of red colobus forms in Central Africa is needed to test the extent of this pattern and to elucidate the complex evolutionary history of this group in that region.

Mitochondrial divergence dates suggest that phylogenetic groups within the red colobus complex have been genetically

isolated from one another since the Pliocene. This is as long as the isolation seen between some black-and-white colobus species and lends further support to the notion that multiple red colobus species should be recognized. However, much more work is necessary to build a classification that accurately reflects the diversity of these animals. More samples from Central Africa, as well as samples from *P. b. waldroni* and *P. b. epieni*, must be added to this dataset, nuclear markers should be surveyed, and a thorough morphological study needs to be performed. Until the diversity and relationships within this group are further assessed, a modified version of the Grubb et al. (2003) classification that designates the species name *P. rufomitratus* to their Central Assemblage is consistent with the results here, although other alternatives exist (e.g., Groves, 2007). Contrasting red colobus classifications are bound to persist due to philosophical differences in how to diagnose species (e.g., biological species versus phylogenetic species).

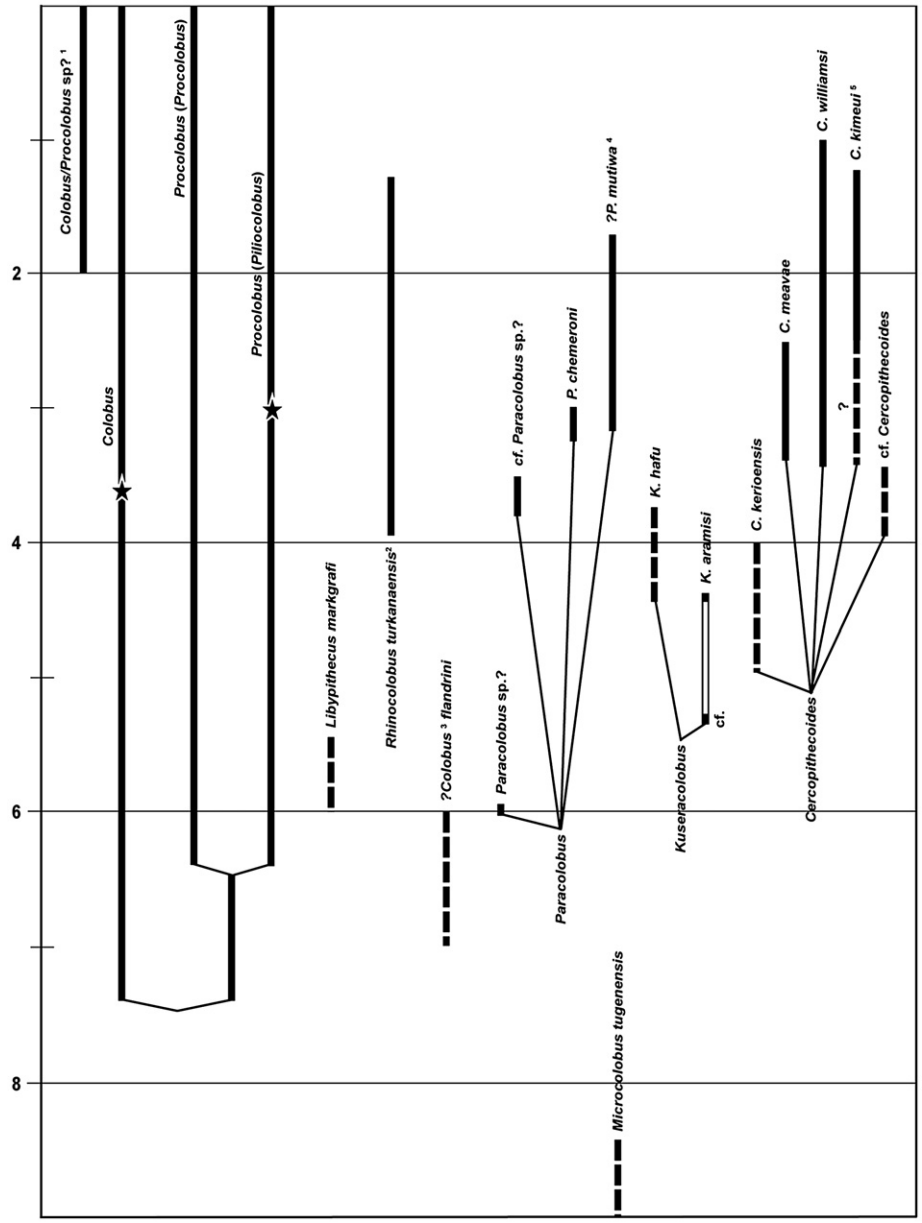

Fig. 11. Chart diagramming the existence of the living colobus monkey lineages and the fossil African colobines through time. Stars indicate the start of the modern *Colobus* and *Procolobus* (*Piliocolobus*) radiations based on molecular evidence in this paper. Fossil taxa represented by solid bars are well-dated throughout a time-span, while dashed bars represent taxa whose specimens are insecurely dated. 1) *Colobus* sp.? indicates specimens tentatively assigned to black-and-white colobus from various localities with undetermined species affinities. It is possible that some of these specimens also represent *Procolobus* (*Piliocolobus*). 2) This includes specimens tentatively allocated to *Rhinocolobus turkanaensis* found in different intervals at Koobi Fora. 3) ?*Colobus* is not used here to indicate the modern genus, but an early African colobine. 4) There is some dispute as to whether specimens attributed to this species should remain in *Paracolobus* or be placed in a different genus. 5) Early specimens attributed to *C. kimeui* may not belong to this species. Fossil data compiled from Birchette (1982), Kalb et al. (1982), Leakey (1982, 2007), Harrison and Harris (1996), Frost (2001), Ting (2001), Deino and Hill (2002), Frost and Delson (2002), Jablonski (2002), Frost et al. (2003, 2007), Leakey et al. (2003), Hlusko (2006, 2007), Frost and Alemseged (2007).

Divergence dates and the modern African colobine radiation

The alternating Pleistocene intervals of glacial maxima have been used to infer recent origins for the living colobus monkeys (e.g., Grubb, 1978, 1982; Hull, 1979; Struhsaker, 1981; Oates and Trocco, 1983). The fossil record has been interpreted as suggesting the same, with the oldest specimens attributable to an extant lineage found in early Pleistocene deposits (Leakey, 2007). The results of this study show that the modern African colobines had started to diversify much earlier than this, and are consistent with Sterner et al.'s (2006) finding that this radiation had begun by the late Miocene. They also show that all three colobus clades had differentiated by the end of that epoch, and that by the Pliocene and early Pleistocene many of the extant species lineages were already present. These dates reveal that the modern lineages coexisted with the Plio-Pleistocene diversification of now extinct African colobines (Fig. 11), thus raising the possibility that at least some of these fossil taxa are phyletically related to the extant lineages. Although the evolutionary relationships of the fossil African colobines are currently unclear, it is possible to use the molecular tree produced here to polarize morphological traits and bring some phylogenetic order to the extinct forms.

It has been suggested that African colobines only recently invaded the arboreal niche (Leakey et al., 2003). However, it is possible that the biased nature of the fossil record has skewed perceptions concerning the evolution of this group. Thus far, the extinct forms are known nearly exclusively from East Africa and South Africa, whereas the extant taxa currently find their greatest diversity in Central and West Africa. Furthermore, there exist smaller specimens that date to the late Miocene whose poor preservation precludes proper diagnosis; these represent a major aspect of the colobine radiation about which we know very little. Small-bodied arboreal colobines may have existed since the Miocene but we have been unable to recognize them as such, while others perhaps occurred in areas such as Central and West Africa where there are no fossil deposits. The divergence times estimated here support this notion, with the three extant groups already distinct by the late Miocene. It seems improbable that all African colobines were terrestrial until the Pliocene, as it would mean arboreal adaptations arose independently in each of the three living colobus groups and in the Asian colobines as well. Instead, it is most likely that some of the earliest colobines were adapted to life in the trees (Delson, 1975; Szalay and Delson, 1979; Hlusko, 2006).

Conclusions

This study represents the first taxonomically comprehensive molecular survey of the African colobines. Further evidence is found for a sister taxon relationship between the olive colobus and red colobus, and this can be combined with results from other studies (e.g., Sterner et al., 2006; Whittaker et al., 2006) to support a hypothesis of reciprocal monophyly among the Asian and African colobines. It is apparent that the position of *C. vellerosus* needs further clarification and additional work is required to elucidate the evolution of the red colobus group. Despite the choice of relatively conservative calibration points, the mitochondrial divergence dates produced here show that the modern African colobine radiation began much earlier than previously thought, with the three major groups present by the late Miocene and many of the species lineages diversifying by the Pliocene. Using earlier calibration points or a range of dates at each point would only serve to push the inferred dates further back in time. These lineages thus coexisted with the large-bodied African Plio-Pleistocene fossil colobines and were likely part of the same adaptive radiation.

Given gene coalescence times that date back to the late Miocene, the general lack of morphologically modern colobines in the Miocene and Pliocene African fossil record remains curious. The extant guenon and African ape radiations show similar temporal depths based on molecular data (e.g., Raaum et al., 2005; Tosi et al., 2005) and are likewise rare or absent in fossil deposits. Most members of these clades are adapted to a closed environment and find their greatest living diversity in Central and West Africa. This was most likely the case in the past as well and may explain why early members of their clades are rarely found. These areas are often neglected in discussions of Plio-Pleistocene primate evolution but should not be ignored. Indeed, if humans initially evolved in a forested environment (Senut, 2006), it is possible that early human lineages have also gone undetected and the forests of Central and West Africa were past areas of hominin diversity.

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