MOLECULAR SYSTEMATICS OF RED COLOBUS MONKEYS (*PROCOLOBUS* [*PILIOCOLOBUS*]): UNDERSTANDING THE EVOLUTION OF AN ENDANGERED PRIMATE

by

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Abstract

Molecular Systematics of Red Colobus Monkeys (*Procolobus* [*Piliocolobus*]): Understanding the Evolution of an Endangered Primate

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The phylogeny and classification of the red colobus monkeys (genus *Procolobus*; subgenus *Piliocolobus*) remains one of the longest standing unresolved issues in African primate taxonomy. Despite widespread agreement that variation within this group exceeds what is typically seen in a single species, complex patterns of variation and an allopatric distribution have made the recognition of more than one red colobus species difficult. I used a mitochondrial marker to elucidate the phylogenetic relationships and timing of divergence events among the extant colobus monkeys, focusing on the red colobus group. The results were used to provide insight into the evolutionary history of the African colobines, the biogeographic history of the African rainforest, the classification of red colobus species, and the designation of conservation priorities for the red colobus monkeys. Mitochondrial divergence dates for the three major extant colobus monkey groups (black-and-white, olive, red) extended into the Miocene, with several black-and-white and red colobus mitochondrial lineages diverging by the Pliocene. Relationships among the colobus monkeys were generally congruent with previous hypotheses with the exception of several West and Central African forms. Specifically, among the red colobus monkeys neither the *preussi* nor *waldroni* forms were found to have particularly close affinities with the *badius* and *temminckii* forms, and the *epieni* form was more closely related to taxa found in East and Central Africa than its nearest neighbors. Furthermore, certain red colobus taxa in Central Africa were found to be mitochondrially polyphyletic, and lumping Grubb et al.'s (2003) Central Assemblage together created a mitochondrially paraphyletic group. However, at least some of these complex patterns in Central Africa are not inferred to be tracing true population history and are likely due to the complex history of the Congo Basin. Biogeographic hypotheses supported by the red colobus phylogeny include a close relationship between the tropical forests of Central and East Africa with past northern and southern connections. Also, it seems that certain large rivers within the Congo Basin were not always barriers to gene flow for some rainforest dwelling animals. Among the 18 commonly recognized red colobus monkey subspecies, I recognized five species (*P. badius*, *P. epieni, P. pennantii*, *P. rufomitratus, P. kirkii*) that had molecular lineages as divergent as those seen between well agreed upon black-and-white colobus species. Application of this classification to red colobus monkey conservation planning gives the highest conservation priority to several distinct species in western Africa that are threatened primarily by human hunting and extremely limited distributions. Extinction of these species would result in a major loss of colobus monkey evolutionary history.

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Dedication

To my parents
Thank you for your trust and support,
I could not have done it without you…

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Chapter 1: Introduction and Background

Introduction

Red colobus monkeys (*Procolobus,* subgenus *Piliocolobus*) represent one of the three major extant groups of African Colobinae and are distributed across the rainforest belt in numerous allopatric forms. They display a level of diversity that exceeds what is typically seen in a single primate species but present a complex pattern of variation that obscures evolutionary relationships, thus making the diagnosis of multiple species difficult. There is no current consensus on how many species should be recognized, and the assignment of species names to certain forms remains contentious. This has made their classification one of the thorniest issues in African primate taxonomy (Grubb et al. 2003). Red colobus monkeys also rank among the most endangered of all living primates (Oates 1996a; Struhsaker 2005), but the confused state of their classification has hampered the designation of conservation priorities for these animals. This is the first study to address red colobus monkey systematics using molecular phylogenetic methods. Understanding the evolution of this group will aid in building a classification that accurately reflects red colobus monkey diversity and provide valuable insight into colobine evolutionary history and African rainforest biogeography.

Red colobus monkeys – A general description

The extant Old World monkeys have been divided into two major groups which diverged from one another in the mid-Miocene – the Cercopithecinae and the Colobinae (Delson 1994; Sterner et al. 2006) The latter are also known as “leaf eating monkeys,” and range across the tropics and subtropics of Africa and Asia. The living African colobines, or colobus monkeys, can be split into three different groups – the black-and-white colobus (*Colobus*), the olive colobus (*Procolobus* [*Procolobus*]), and the red colobus (*Procolobus* [*Piliocolobus*]). Like all living colobines, red colobus monkeys are quadrupedal, primarily adapted to an arboreal niche, and have a multi-chambered stomach adapted to processing foliage. As African colobines, they are distinguished from their Asian relatives by features such as an extremely reduced pollex. The olive colobus and red colobus share the presence of a male perineal organ, female sexual swellings, ischial callosities that remain separate in both sexes, a small larynx (compared to the black-and-white colobus), and a sagittal crest in adult males (Kuhn 1972; Napier 1985; Strasser and Delson 1987).

Red colobus monkeys are medium sized monkeys with females that range from 4.4 – 9.9 kg and males that range from 6.3 – 12.5 kg (Delson et al. 2000). Their hindlimbs are slightly longer than their forelimbs as indicated by an intermembral index of 86 (Groves 2001). All populations have varying amounts of red, black, white, brown, and gray in their pelage, with certain forms differing at the individual level while others are relatively uniform (Kingdon 1997). They have a complex and continuously graded vocal system so that it is difficult to classify their vocalizations into discrete categories, which is different than what is seen in the black-and-white colobus group (e.g., black-and-white colobus male roars) (Struhsaker 1975). Red colobus monkeys are capable of exploiting a diverse array of ecological niches, including lowland moist forest, medium altitude (submontane) forest, and fragmented forests with a considerably long dry season. They tend to feed on some of the tallest and most common trees in their habitat, and their preferred food items are young leaves, immature fruits and seeds, and floral parts, while mature leaves are consumed as a fallback resource (Oates 1994). Some populations have been noted for their relatively high intake of fruit (Galat-Luong and Galat 2005), their exploitation of edge vegetation (Mbora and Meikle 2004; Wahungu et al. 2005), or their variation in feeding ecology within and between groups on spatial and temporal scales (Chapman et al. 2002). Social groups vary in size from 12 – 80 individuals, with a typical group size of 25 – 40. Red colobus females commonly disperse from natal groups while male membership is relatively stable.

Research questions

The overall goal of this dissertation is to use molecular data to infer the relationships and timing of divergence events within the African colobine clade, focusing specifically on the various red colobus forms. The inferred relationships and divergence dates are used to test hypotheses concerning the classification and evolutionary history of these animals with implications for biogeography and conservation. Specifically, these three research questions are addressed:

1) ***What are the higher-level relationships and divergence dates among the living African colobines?***

Molecular methods have been used to investigate the evolutionary history of nearly all Old World monkey groups, including baboons, macaques, guenons, and Asian colobines (Harris and Disotell 1998; Evans 1999; Tosi et al. 2003; Geissmann et al. 2004; Newman et al. 2004; Tosi et al. 2005; Sterner et al. 2006; Whittaker et al. 2006; Xing et al. 2007; Ting et al. 2008). However, these methods have not yet been used to test hypotheses concerning the evolutionary relationships and divergence dates among the extant African colobines. Phylogenetic hypotheses concerning these animals have been generated using morphology

(Colyn 1993), vocalizations (Struhsaker 1975, 1981), and pelage patterns (Colyn 1993), but the colobus monkey relationships remain unresolved. Furthermore, fossil specimens reliably linked to the extant African colobines are rare and do not appear until the Late Pliocene and Early Pleistocene, suggesting relatively recent origins for this group (Delson 1994). Molecular data reveal this radiation may have started much earlier (Sterner et al. 2006), but the time depths of lineages within the group are still unknown.

2) ***What are the evolutionary relationships within the red colobus group, and what do they reveal about the biogeographic history of the African rainforest?***

The evolutionary relationships within the red colobus group in particular are unresolved. Past attempts to elucidate this issue have used a variety of methods such as craniometrics, vocalizations, and pelage patterns (Verheyen 1957, 1962; Struhsaker 1981; Colyn 1991; 1993). However, these studies contained a limited sampling of red colobus forms and did not take primitive and derived character states into account. Molecular phylogenetic methods have never been used to address red colobus monkey phylogeny and have been suggested as critical in resolving the relationships among the numerous red colobus forms (Oates et al. 1994; Grubb et al. 2003).

Patterns of modern species distributions and phylogenetic relationships have been used to generate hypotheses concerning the biogeographic history of the African rainforest (e.g., Hamilton 1976; Grubb 1982). Inferring evolutionary relationships among the red colobus monkeys will aid in testing these hypotheses. Support may be provided for certain evolutionary models if the red colobus monkeys can be shown to share a history of vicariance and dispersal with other rainforest taxa. Alternatively, other models may be needed to explain red colobus monkey distribution and phylogeny if they do not fit the previously inferred patterns.

3) ***What red colobus classification is most consistent with their phylogeny and molecular diversity, and how does it affect the designation of red colobus monkey conservation priorities?***

It is currently unclear how the three major living colobus monkey groups should be ranked. Classifications have used one (Groves 1970; Delson 1975), two (Kuhn 1967; Brandon-Jones 1984; Grubb et al. 2003), and three genus arrangements (Groves 2001). Although most authors now separate the black-and-white colobus into its own genus, there is still disagreement on whether the red colobus and olive colobus are distinct enough from one another to each merit genus rank (e.g., Groves 2001; Grubb et al. 2003).

Numerous taxonomic schemes have been offered in an attempt to classify the various red colobus forms. Between one and nine species are commonly recognized, and the most recent assessment of African primate diversity did not even attempt to assign species names to some of the forms because it was unclear how they are related to one another (Grubb et al. 2003). It is unknown which classification, if any, reflects phylogenetic distinctiveness and relationships within the group. Molecular techniques have been used in cercopithecids to both resolve evolutionary relationships and provide taxonomic suggestions in species-level diagnosis (e.g., Harris and Disotell 1998; Md Zain 2001; Tosi et al. 2003; Li et al. 2004; Tosi et al. 2004), but these approaches have not yet been taken for the colobus monkeys. This study will thus aid in determining which (if any) existing classification best represents red colobus relationships and molecular diversity, or will suggest taxonomic revisions that might be needed. A classification that best reflects the diversity in this group, while staying consistent with how its closest relatives are ranked, will likely be the most useful.

Red colobus monkeys rank among the most endangered African primates, and choosing a species level classification for this group has important implications for planning conservation strategies. Conservation biologists often use species as a fundamental unit in describing biodiversity, and thus species definitions must reflect biological variation if conservation strategies are to be effective. If not, limited conservation resources can be diverted away from distinct and threatened populations in need of protection. One focus of this dissertation is to explore how different species level classifications affect the designation of conservation priorities, especially in regard to the red colobus group.

Overview of dissertation

The remainder of this first chapter will review the relevant background information needed to address the three main questions outlined above. This includes systematic theory and previous hypotheses concerning colobus monkey systematics. The second chapter addresses phylogenetic relationships and divergence dates among the living African colobines. It reports the analysis of a 3,831 base pair fragment of mitochondrial DNA from the olive colobus, all species of black-and-white colobus, and over half of the red colobus forms. Chapter 3 provides a more detailed investigation into red colobus systematics by analyzing an 897 base pair fragment of mitochondrial DNA from nearly all red colobus forms. Chapter 4 discusses the implications of the inferred red colobus phylogeny for the biogeographic history of the African rainforest. Chapter 5 contains a branch length analysis of the Chapter 3 dataset in order to identify divergent red colobus molecular lineages. These are then used to aid in the diagnosis of red colobus species. Chapter 6 is a discussion of the relationship between systematics and conservation biology and focuses on how the species level classification proposed in Chapter 5 affects conservation planning for the red colobus group. Chapter 7 is a summary of the results and conclusions of this dissertation.

Systematics – Issues in taxonomy and classification

Classification above the species level

Linnaeus (1758) defined a hierarchical structure of groups (classes) into which organisms could be assigned, and this structure remains the foundation for modern taxonomic theory and biological classification. This is a character-based system where supraspecific taxa are defined by certain attributes. Therefore, two organisms are placed into the same taxon when they share the defining characters of that taxon. A taxon is then ranked into the Linnean hierarchy based on the degree of (traditionally, morphological) difference it shows with respect to other taxa. The ranking of a taxon is therefore somewhat subjective, which has led to taxonomic categories that are often arbitrarily defined and classifications that are inconsistent in the way biological variation is distributed. This in turn has made the testing of competing taxonomic hypotheses difficult.

Some authors have advocated the use of a temporal scheme to delineate taxonomic ranks in order to standardize the Linnaean hierarchical categories, particularly within primates (Hennig 1966; Goodman et al. 1998; Groves 2001; Wildman et al. 2003). Once established, this method would reduce confusion in supraspecific classification and allow novel approaches in studying rates of evolution across various taxa. However, Avise and Johns (1999), who advocate eventually implementing such a system across all organisms, point out numerous difficulties in pursing this course of action. The biggest one is the establishment of temporal ranges for each taxonomic rank. Goodman et al. (1998) conveniently chose ranges that produced relatively little change within primates. However, these dates will not produce the same result when applied to other groups of organisms. Indeed, Avise and Johns (1999) point out that the genus *Drosophila* contains species that last shared a common ancestor over 40 Ma, while some genera of fishes have diverged within the past few thousand years. Drawing temporal boundaries for taxonomic ranks is an arbitrary exercise, and a consensus on where to draw these boundaries is unlikely. It is more likely that taxonomic specialists will come to a consensus on boundaries for their own groups of study (sensu Goodman et al. 2001). However, this would lead to the same problem inherent in the current system, namely the use of arbitrarily drawn yardsticks for taxonomic ranks that differ across groups of organisms. Although the concept of a universal system for classification is desirable, a time-based classification may be more useful for investigating different evolutionary questions and is not an appropriate replacement for the current methods of ranking a taxon into the Linnaean hierarchy.

Even if molecular divergence dates are not used for taxonomy, genetic data can still be of use for classifying organisms above the species level. One method involves comparing levels of genetic divergence at a common marker across taxa. Several researchers (Johns and Avise 1998; Bradley and Baker 2001) have done this with the mitochondrial cytochrome b gene to see how well distances correspond to taxonomic rank. Although they found a poor correlation across a wide range of taxa (e.g., all vertebrates or all mammals), Baker and Bradley (2006) note that genetic divergence may be of more predictive value for taxonomic rank if used in closely related taxa with common generation times, metabolic rates, and body sizes. Using genetic distances instead of a temporal standard is further desirable because it would remove the large margins of error involved in calculating divergence dates from molecular datasets and also avoids the difficulties in interpreting alternative dates inferred from different methodologies.

Classification at the species level

Species concepts – Species have long been used in evolutionary biology to describe the process of evolution and the biological diversity that it has created. Species have been called the principal units of evolution (Mayr 1996) and the currency of biology (Agapow et al. 2004). Indeed, Darwin's seminal 1859 publication that introduces the theory of natural selection is entitled "On the Origin of Species by Means of Natural Selection..." Despite this acknowledged importance of species, there is still no consensus among evolutionary biologists on what exactly species are and how they should be diagnosed. There is a voluminous literature pertaining to the species concept debate and a complete review is beyond the scope of this thesis (see Claridge et al. 1997; Howard and Berlocher 1998; Coyne and Orr 2004 for review). However, since the species level classification of red colobus monkeys is a focus of this research, a brief overview of the relevant concepts is necessary.

There are two major categories in which most species concepts fall – those that are based on the process of speciation and those that are based on the patterns that result from this process. Although the difference between the two may seem subtle, it has led to a major theoretical divide in how species should be defined and diagnosed. A dizzying number of concepts have been invoked, even within each category, in an attempt to further elucidate this issue. The best-known process-based species concept is the biological species concept (BSC), while the most commonly cited pattern-based concept is known as the phylogenetic species concept (PSC).

The theoretical foundation of the BSC is in the "Modern Synthesis"(Huxley 1942) and population genetics, and some have considered it a fundamental part of evolutionary theory (Szalay 1993). Usually credited to Mayr (1942), the BSC defines species as "*groups of interbreeding natural populations that are reproductively isolated from other such groups,*" and it only applies to sexually reproducing organisms (Mayr 1996; 264). Advocates of the BSC claim that species are real entities whose most important characteristic is gene pool integrity. Some authors (e.g., Bock 2004; Coyne and Orr 2004; Baker and Bradley 2006) have elaborated on this definition to indicate that "reproductively isolated" should be interpreted to mean "genetically isolated." Since genetic isolation can occur without strict reproductive isolation, this clarification allows for limited gene flow between two species as long as their respective gene pools are protected from one another. Under the BSC, species can be polytypic, and intraspecific variation between populations is commonly described with the use of subspecies. Numerous other species concepts have been formulated as modifications to the BSC, although it has been argued that some of them are redundant (Szalay 1993; Mayr 1996). These include the ecological species concept (Van Valen 1976), the recognition species concept (Paterson 1985), the evolutionary species concept (Simpson 1961), the cohesion species concept (Templeton 1989), and the genetic species concept (Baker and Bradley

2006), among others. These concepts group organisms into species based on characteristics (e.g., ecology, fertilization system, adaptive zone, etc.) that allow them to remain cohesive units through time.

The BSC has been criticized on several grounds (see Sokal and Crovello 1970), with one of its biggest shortcomings being the inability to diagnose species when populations do not overlap in distribution. Since there is no way of knowing with complete confidence whether or not allopatric populations can potentially interbreed, the BSC is unable to distinguish species boundaries in these situations. Mayr (1996) points out that scientific inference is needed to resolve this issue and a yardstick can be constructed to indicate whether reproductive isolation has occurred. Species status can be given to an isolated population whose morphological difference from other populations exceeds the amount of variation seen among conspecifics. Others have extended this reasoning to molecular markers, particularly mitochondrial DNA (Avise and Walker 1999; Bradley and Baker 2001; Baker and Bradley 2006). This is especially relevant to the current research which surveys a mitochondrial marker, and because red colobus monkeys are distributed primarily in an allopatric manner.

The main conceptual competitor to the BSC is the PSC. There has been considerable confusion over the nature of the PSC because there are multiple species concepts based on phylogenetic systematics, and they all tend to be labeled as “the PSC” even though their methods for species diagnosis are very different from one another. Reference to the PSC in this dissertation is reserved for Cracraft’s (1983) definition of a phylogenetic species, which is "*the smallest diagnosable cluster of individual organisms within which there is a parental pattern of ancestry and descent*" (Cracraft 1983). The

PSC in this view is a character-based approach that identifies populations with unique fixed character combinations (species) to delimit minimal units appropriate for cladistic analysis (Davis and Nixon 1992). In this sense, it is not so much a "species concept," but a criterion for the diagnosis of species (Mayr 1996; Goldstein and DeSalle 2000). Indeed, the strength of the PSC lies in its ability to diagnose species consistently across taxa. Unlike in the BSC, there is no room for subspecies in the PSC because subspecies are typically raised to species status under this concept (although see Groves 2004). As a result the PSC has been accused of an "over-diagnosis" of species (Agapow et al. 2004; Isaac et al. 2004), and it has been argued that the PSC is simply a reincarnation of the typological and morphological species concepts that predate evolutionary theory (Mayr 1996). Furthermore, since the only requirement for species recognition under the PSC is the presence of a unique shared fixed character, lineages that are particularly distinct get recognized at the same level as those that are not distinct, and evolutionary relationships between lineages are often not reflected.

What is clear is that there are multiple species concepts, species classifications will differ from one another depending on which concept is employed, and there is no foreseeable resolution to this debate since choosing between species concepts often comes down to a matter of philosophical preference. Advocates of the BSC place an emphasis on species being real entities that are reproductively isolated from one another. However, these advocates must be resigned to the fact that the recognition of biological species is not straightforward when populations are allopatric. Furthermore, it is possible that Mayr's (1996) suggested construction of a "yardstick" for allopatric populations is not an accurate predictor of reproductive isolation. Those who favor the PSC emphasize the value of a concept that stresses diagnosability, but they must accept the fact that the concept often fails to identify distinct lineages and evolutionary relationships. Regardless of what concept is chosen, it is clear that some amount of inference is needed for species diagnosis (Sites and Marshall 2004). This has led some to believe that arguing over how to define a species detracts from what they think is most important – collecting more data in order to understand biological variation and the process that creates it (e.g., Jolly, 1993; 2003; Hey, 2001). It is essential to keep in mind that classifications are best viewed as hypotheses to be tested, and they will change as our understanding of biodiversity grows.

Genus level classification of the Colobinae

Groves (1970) was one of the first to synthesize a colobine classification and proposed the recognition of five genera (*Colobus, Procolobus, Nasalis, Presbytis, Pygathrix*). Since then, between six and ten genera have been acknowledged in various classifications (e.g., Brandon-Jones 1984; Oates et al. 1994; Groves 2001), with the most recent taxonomic overviews accepting two African and seven Asian genera (Grubb et al. 2003; Brandon-Jones et al. 2004). The majority of morphological and molecular evidence supports the separation of the colobines into African and Asian clades (but see Groves 1989; Jablonski 1998), although there is still no consensus concerning the relationships within those groups (Delson 1975; Strasser and Delson 1987; Sterner et al. 2006; Whittaker et al. 2006).

Prior to 1980, all three living African colobine groups were usually considered to be congeneric and placed in the genus *Colobus*. However, shared traits between the red colobus and olive colobus, such as a four chambered stomach, a small larynx, separate ischial callosities, and the presence of a perineal organ in males and sexual swellings in females (Pocock 1936; Hill 1952) have led them to be separated from the black-and-white colobus (*Colobus*) at the generic level, even though it remains unclear whether these traits are synapomorphic or symplesiomorphic (Brandon-Jones 1984; Strasser and Delson 1987; Oates et al. 1994; Groves 2001; Grubb et al. 2003). Some authors (Kingdon 1997; Groves 2001) further separate the red colobus from the olive colobus into separate genera (*Piliocolobus* and *Procolobus*, respectively). A more conservative approach subdivides *Procolobus* into two subgenera (*Piliocolobus* and *Procolobus*) and recognizes the shared traits seen in the two groups. This latter arrangement is provisionally followed in this study.

Classification, distribution, and phylogeny of the living African colobines

Black-and-white colobus classification

The recognition of subspecies within black-and-white colobus monkeys is contentious but beyond the scope of this study. The classification of these animals at the species level, however, is relatively well resolved. Schwarz (1929) and Hull (1979) used pelage and craniometrics, respectively, to discern four groups which are now commonly recognized as different species (*C. polykomos, C. guereza, C. angolensis, C. satanas*). Oates and Trocco (1983) used male loud call variation to separate *Colobus vellerosus* from *Colobus polykomos* at the species level, which brings the total number of black-and-white colobus species commonly recognized to five.

Black-and-white colobus distribution

Black-and-white colobus monkeys are distributed across the African rainforest belt in a nearly continuous manner (Figure 1.1). *C. polykomos* ranges from Guinea-Bissau to the Sassandra River in Côte d'Ivoire. *C. vellerosus* is found east of the Bandama River in Côte d'Ivore to Western Nigeria. The area between the Sassandra and Bandama Rivers is inferred to be an area of past secondary integradation between these two taxa. *C. guereza* has the widest distribution of all colobus monkeys, ranging from Cameroon west of the Sanaga River, into Gabon, eastward north of the Congo River, and into East Africa and the Ethiopian highlands. *C. angolensis* is also widely distributed, particularly in the Congo Basin south of the river but also north of the river and in the southern mountains and coastal forests of East Africa. *C. satanas* is found east of the Sanaga River in Cameroon and into Gabon and mainland Equatorial Guinea, but also on Bioko Island. At least historically, *C. guereza* is sympatric with *C. angolensis* and *C. satanas* (Oates et al. 1994; Groves 2001).

Black and white colobus phylogeny

Schwarz (1929) believed *C. angolensis* to be the most primitive of the black-and white-colobus monkeys, originating in Central Africa and giving rise to *C. satanas*. A *C. satanas* population then dispersed into West Africa and gave rise to *C. polykomos*, while *C. guereza* differentiated from one of the western populations and dispersed towards East Africa. Grubb (1978; 1982) suggested a similar arrangement but with *C. satanas* being the most primitive and giving rise to *C. angolensis*. The phylogram Oates and Trocco

Figure 1.1: Distribution of black-and-white colobus (*Colobus*) species. 1 – *C. polykomos*, 2 – *C. vellerosus*, 3 – *C. satanas*, 4 – *C. guereza*, 5 – *C. angolensis*. Classification follows Grubb et al. (2003). Adapted from Oates and Trocco (1983) and Oates et al. (1994).

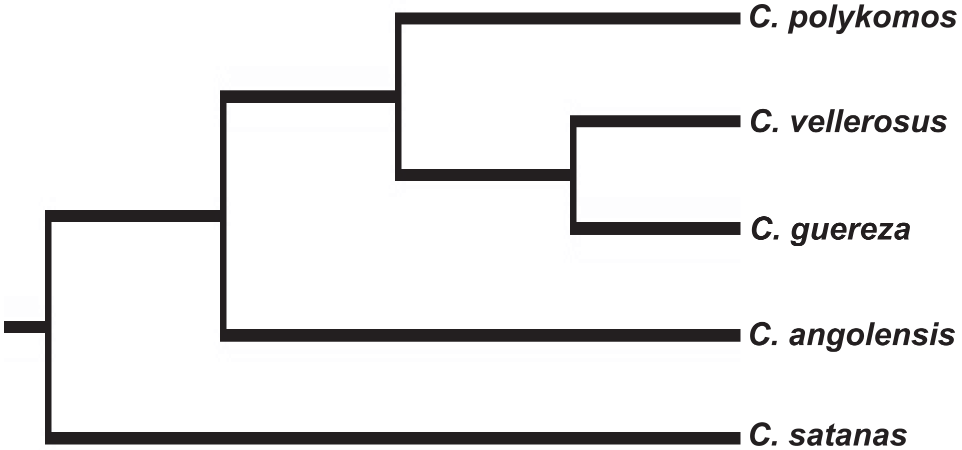

Figure 1.2: Inferred relationships between black-and-white colobus species based on male loud calls (Oates and Trocco 1983).

(1983) produced from male loud call variation (Figure 1.2) is generally consistent with Grubb's evolutionary scenario (apart from the position of *C. vellerosus*, which Grubb considered conspecific with *C. polykomos*), but they were not convinced that direct ancestor-descendent relationships existed between all these species.

Red colobus classification

Schwartz (1928) lumped all 34 red colobus taxa described between 1792 and 1924 into one species, *Colobus badius*, with 18 subspecies. Miss Waldron's red colobus (*P. b. waldroni*) was described shortly thereafter, but Dandelot (1968) further reduced the number of forms to 14. Three new forms have since been described (*P. b. parmentieri, P. b. semlikiensis,* and *P. b. epieni*), but one of them (*P. b. semlikiensis*) is likely a junior synonym of a previously named taxon (Grubb et al., 2003). Although there is still no consensus on how these forms should be classified at the species level, most authors at least agree with their recognition as subspecies (Rahm 1970; Kingdon 1971; Dandelot 1974; Napier 1985; Groves 2001; Grubb et al. 2003). Aside from controversy over some taxa in Central Africa (see below), the classification of this group seems to be stable at this level.

Most authors have recognized that the level of variation among these different forms exceeds that which is typical for a single primate species, but there has been difficulty in deciding how the variation should best be described. This has often led to "giving up" (in the words of Groves 2001) and combining all of them into one species, sometimes with the exception of a few distinct forms. Thus, there is no consensus on how many species of red colobus should be recognized. Numerous classifications have been proposed, and among these Schwartz (1928), Rahm (1970), Kingdon (1971), and Oates et al. (1994) recognized one species; Napier (1985) recognized two; Groves (1989) recognized four; Kingdon (1997) recognized eight; Groves (2001) recognized nine; and Groves (2007) recognized sixteen. Central to this taxonomic problem is the fact that the phyletic relationships among the different forms are poorly resolved. Table 1.1 displays various taxonomic arrangements that have been proposed. Until this dissertation reaches its own taxonomic conclusions (Chapter 5), I will label all red colobus forms as belonging to the single species *Procolobus badius* in order to avoid confusion. Also, for the sake of simplicity, references to the various forms will be by their subspecies names, omitting genus and species names (e.g., *P. b. waldroni* vs. *waldroni*) even though this is incorrect according to strict taxonomic practice.

Red colobus distribution

The 16-18 red colobus forms are distributed in a fragmented manner across the African rainforest belt, from the Gambia to Zanzibar (Figure 1.3). Given their ability to exist in a wide range of habitats, the large gaps in red colobus distribution are somewhat surprising; it is possible that the spatial distribution of food resources in some forests is unsuitable for these animals (Werre 2000; Mbora and Meikle 2004).

The most western red colobus taxa (*temminckii, badius, waldroni*) range from the Gambia to Ghana and may intergrade with one another. The southern limit of *temminckii* and northern limit of *badius* are not well documented, and Booth (1954) reported a zone of secondary intergradation between *badius* and *waldroni*.

Moving east, the next red colobus forms encountered are *epieni, preussi,* and

Table 1.1: Taxonomic arrangements for red colobus forms. Subspecies are listed on the far left followed by each author's species level diagnosis. Total number of species for each classification at bottom.

Taxon	Schwarz 1928	Dandelot 1974	Napier 1985	Grubb et al. 2003	Groves 2007
temminckii	*badius*	*badius*	*badius*	*badius*	*badius*
badius	*badius*	*badius*	*badius*	*badius*	*badius*
waldroni	undescribed	*waldroni*	*badius*	*badius*	*waldroni*
epieni	undescribed	-----	-----	*pennantii*?	*epieni*
preussi	*badius*	*preussi*	*badius*	*pennantii*?	*preussi*
pennantii	*badius*	*pennantii*	*badius*	*pennantii*?	*pennantii*
bouvieri	*badius*	*pennantii*	*badius*	*pennantii*?	*bouvieri*
tholloni	*badius*	*tholloni*	*badius*	?	*tholloni*
rufomitratus	*badius*	*rufomitratus*	*badius*	*rufomitratus*	*rufomitratus*
tephrosceles	*badius*	*rufomitratus*	*badius*	?	*tephrosceles*
gudoviusi	*badius*	syn *tephrosceles*	-----	-----	-----
oustaleti	*badius*	*rufomitratus*	*badius*	?	*oustaleti*
nigrimanus	*badius*	syn *oustaleti*	-----	-----	-----
powelli	*badius*	syn *oustaleti*	-----	-----	-----
brunneus	syn *powelli*	syn *oustaleti*	-----	-----	-----
schobotzi	syn *powelli*	syn *oustaleti*	-----	-----	-----
foai	*badius*	*rufomitratus*	*badius*	?	*foai*
lulindicus	syn *foai*	-----	-----	?	-----
graueri	*badius*	syn *foai*	-----	-----	-----
ellioti	*badius*	*rufomitratus*	*badius*	?	*ellioti*
langi	*badius*	syn. *ellioti*	-----	?	*langi*
parmentieri	undescribed	-----	-----	?	*parmentieri*
gordonorum	*badius*	*rufomitratus*	*badius*	*gordonorum*	*gordonorum*
kirkii	*badius*	*kirkii*	*kirkii*	*kirkii*	*kirkii*
Total species #	1	7	2	at least 5	16

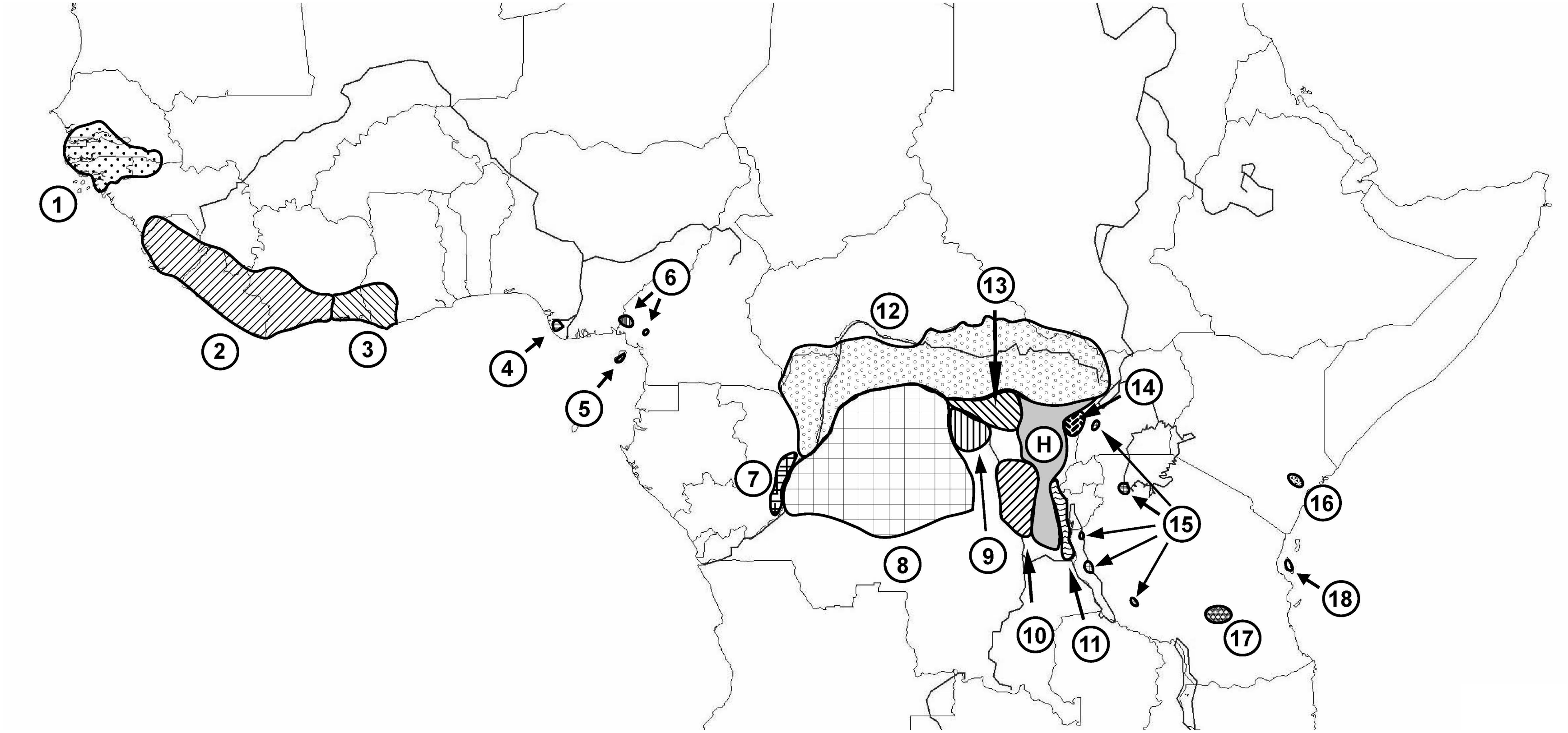

Figure 1.3: Distribution of red colobus (*Procolobus* [*Piliocolobus*]) taxa. Area marked with an H refers to a putative zone of hybridization between adjacent taxa. 1 – *temminckii*, 2 – *badius*, 3 – *waldroni*, 4 – *epieni*, 5 – *pennantii*, 6 – *preussi*, 7 – *bouvieri*, 8 – *tholloni*, 9 – *parmentieri*, 10 – *lulindicus*, 11 – *foai*, 12 – *oustaleti*, 13 – *langi*, 14 – *ellioti*, 15 – *tephrosceles*, 16 – *rufomitratus*, 17 – *gordonorum*, 18 – *kirkii*. Classification follows Grubb et al. (2003). Distributions from Colyn (1991, 1993), Oates et al. (1994), Grubb and Powell (1999), and author's own notes.

pennantii. These taxa are distributed allopatrically and possess extremely limited distributions. *Epieni* is restricted to the Niger Delta in Nigeria and is the most recently discovered red colobus form (Grubb and Powell 1999). *Preussi* is concentrated mainly in Korup National Park in Southwest Cameroon, although it extends into the adjoining Cross River National Park in Nigeria, and a population persists in Ebo forest in the Yabassi district of Cameroon (just west of the Sanaga River). *Pennantii* is restricted to the island of Bioko in Equatorial Guinea.

There is another large gap in the red colobus distribution spanning Southeast Cameroon, mainland Equatorial Guinea, and Gabon. This is an area of high primate endemism and the absence of red colobus monkeys here remains unexplained. Moving further east, *bouvieri* is found in the Republic of Congo on the west bank of the Congo River. *Oustaleti* is known from localities in close proximity to *bouvieri* and continues its distribution eastward and north of the Congo River. Although considered by most to be one wide-ranging polytypic subspecies, numerous forms have been recognized within the *oustaleti* complex. Most recently, Gautier-Hion et al. (1999) considered *oustaleti* as a complex of six subspecies (*oustaleti* sensu stricto, *powelli, brunneus, schubotzi, nigrimanus,* and an undescribed form from the Central African Republic) (Figure 1.4).

The Congo basin south of the river is predominantly occupied by the form *tholloni*. The one exception is a small area between the Lualaba and Lomami Rivers where *parmentieri* resides. The eastern Democratic Republic of Congo is where particular disagreement occurs in the recognition of red colobus forms. Traditional classifications have followed Dandelot (1968) in recognizing *ellioti* and *foai* as valid taxa, *ellioti* as a polytypic form, and possible zones of secondary intergradation between

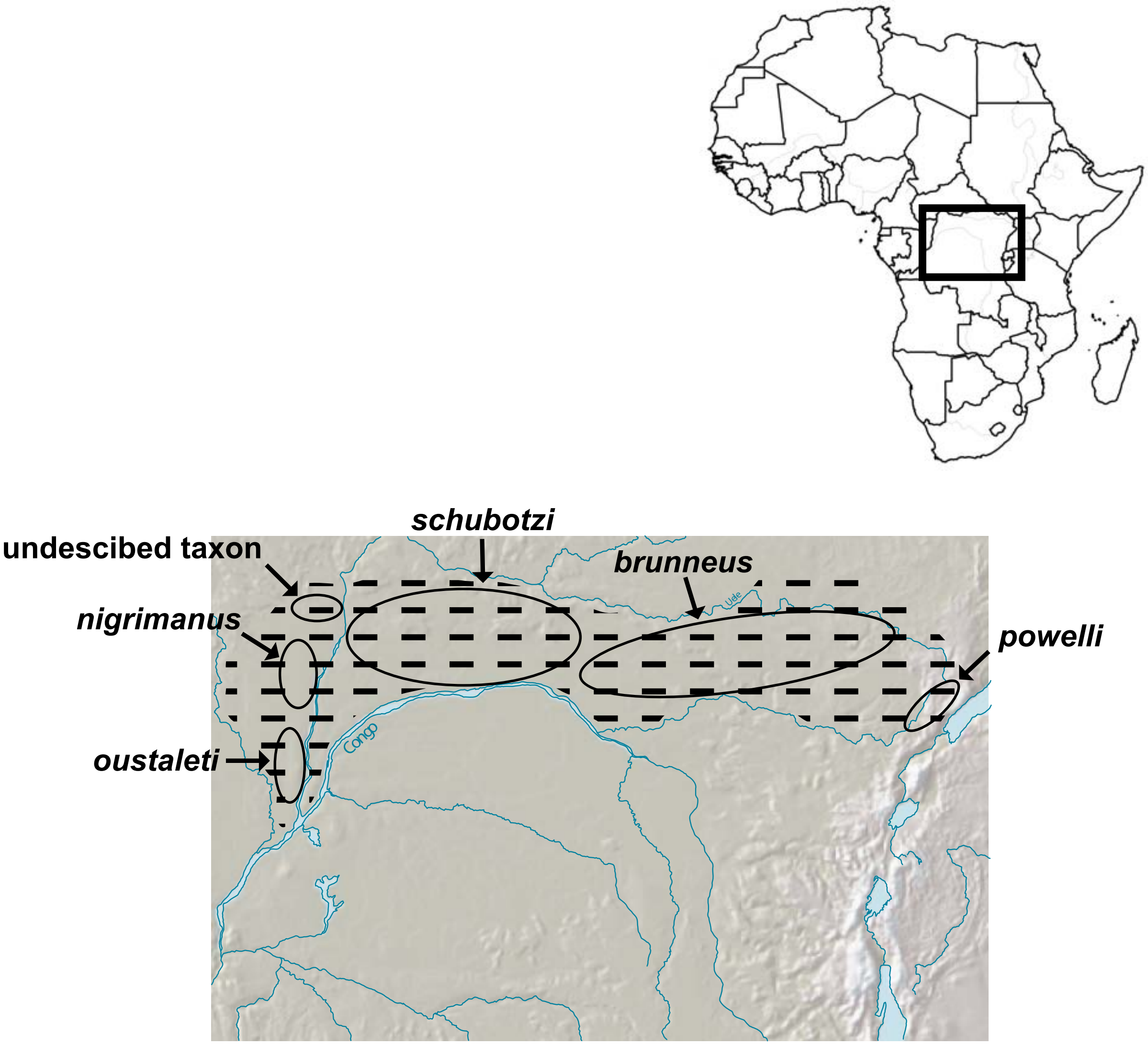

Figure 1.4: *P. b. oustaleti* sensu latu is traditionally recognised as a wide-ranging polytypic taxon distributed north of the Congo River. However, Gautier-Hion et al. (1999) recognized the presence of six taxa (*oustaleti* sensu stricto, *nigrimanus*, an undescribed taxon, *schubotzi*, *brunneus*, and *powelli*) within this complex.

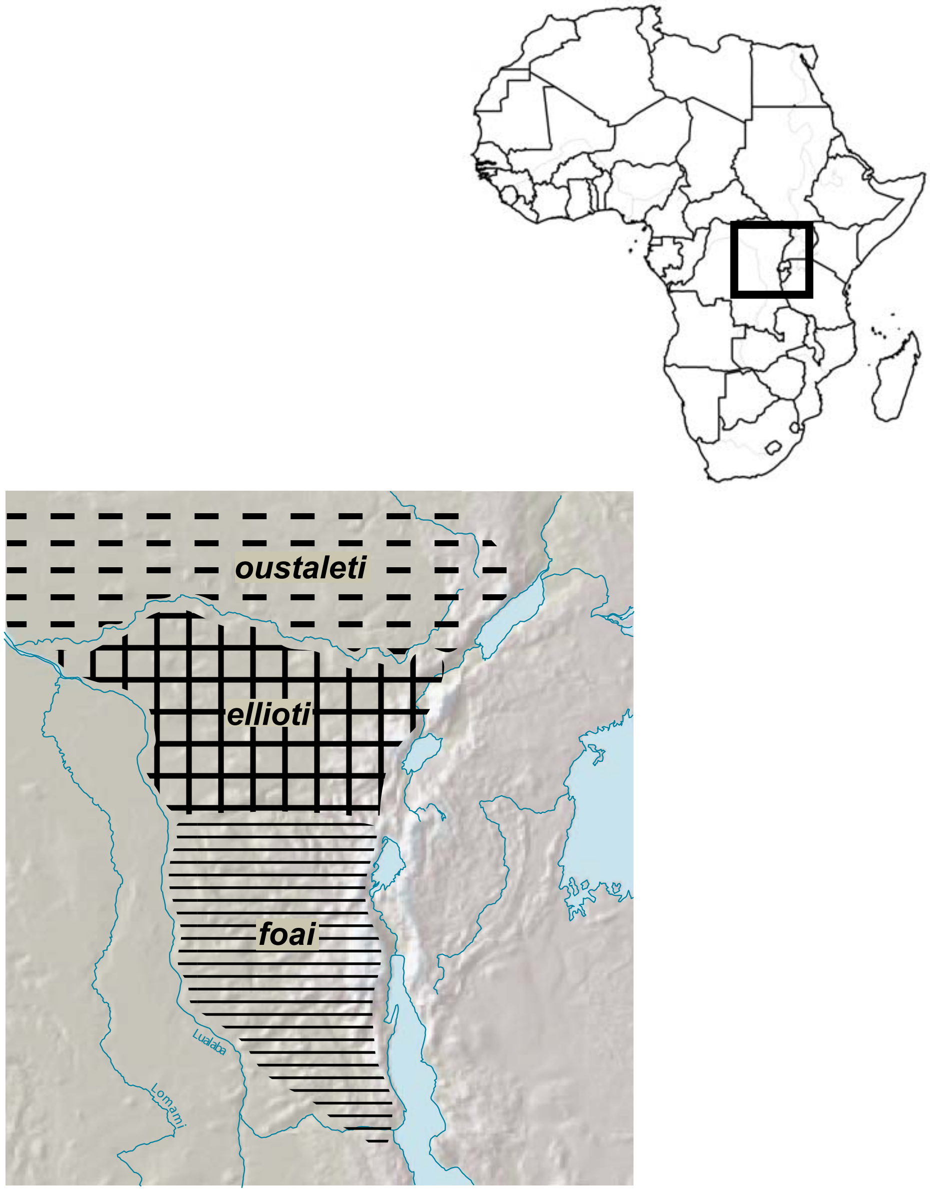

Figure 1.5: Traditional taxonomic arrangement of red colobus forms in the Eastern Democratic Republic of Congo (Dandelot 1968). Hybridization is inferred to occur between *oustaleti* and *ellioti*, and between *ellioti* and *foai*.

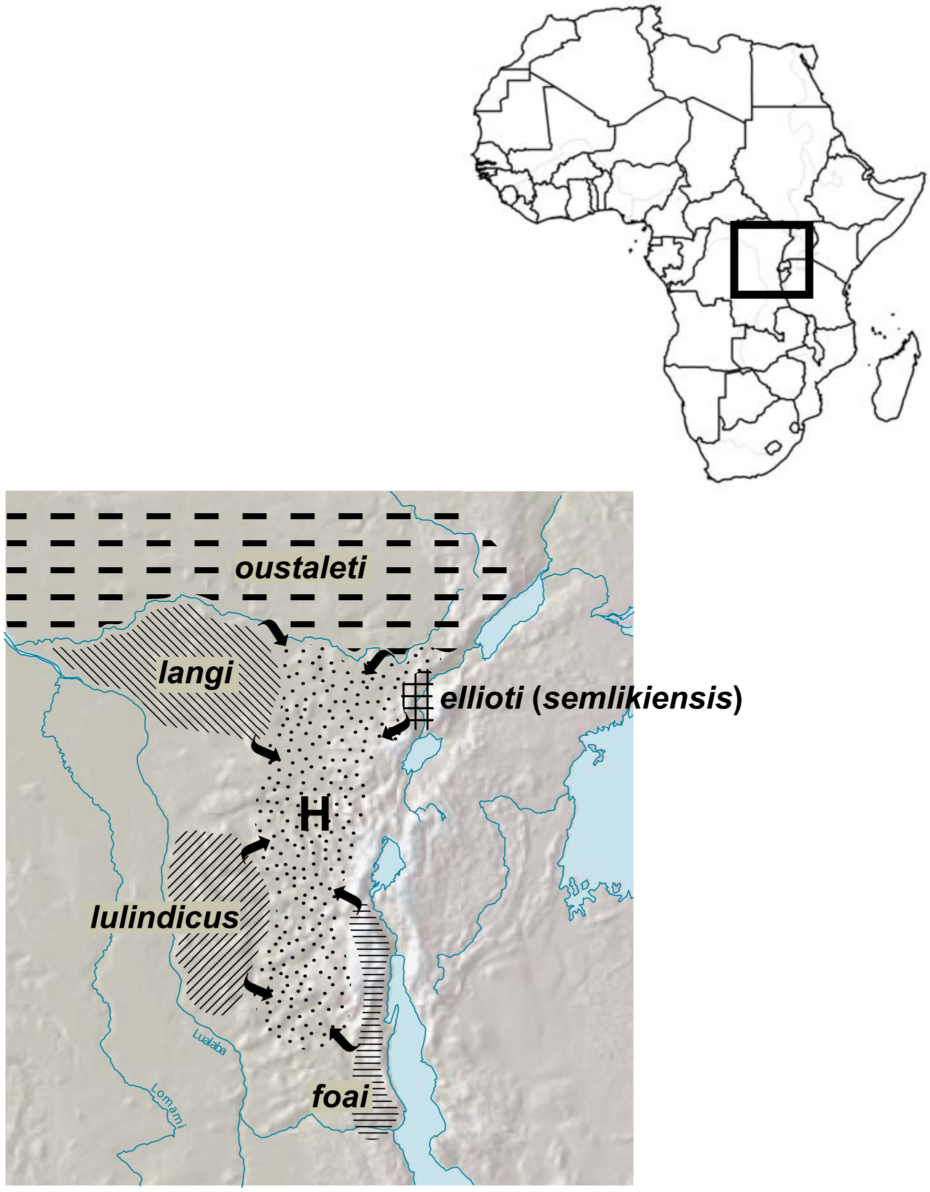

Figure 1.6: Taxonomic arrangement of red colobus forms in the Eastern Democratic Republic of Congo according to Colyn (1993). H - Area of hybridization between *foai, ellioti, lulindicus, langi,* and *oustaleti*. The form *semlikiensis* is now considered a junior synonym of *ellioti*.

the two (Figure 1.5) (Rahm 1970; Dandelot 1974; Napier 1985). However, Colyn (1993) recognized four stable coat patterns in this region and resurrected *langi* from synonymy with *ellioti,* described the new form *semlikiensis,* and resurrected *lulindicus* from synonymy with *foai* (Figure 1.6). Colyn thus believed the area between these taxa to be a zone of secondary intergradation that contained two aggregates of hybrid populations, with *ellioti* representing a hybrid population between *langi*, *semlikiensis,* and likely *oustaleti* as well. Grubb et al. (2003) are not in disagreement with this arrangement, but consider *semlikiensis* to be synonymous with *ellioti*, the latter having nominal seniority.

The remaining red colobus forms are found in East Africa. The *tephrosceles* form possesses a relatively large, albeit extremely fragmented, range from Western Uganda to Western Tanzania. It is unclear whether it shares gene flow with some of the adjacent Central African forms and/or *gordonorum*, which is found to the east in the Udzungwa Mountains. *Kirkii* is isolated on the island of Zanzibar in Tanzania, and *rufomitratus* has a limited distribution along the Tana River in Kenya.

Red colobus phylogeny

Few studies have gone into detail beyond comparison of coat patterns to establish a phylogeny for the numerous red colobus taxa. Verheyen's (1957; 1962) studies used craniometric traits and identified *kirkii* as particularly distinct, but his samples were biased towards forms in Central Africa and Zanzibar. Struhsaker (1981) used vocalizations to distinguish phylogenetic relationships. He identified various categories of red colobus monkey calls that were inferred to be functionally related to intragroup cohesion. Struhsaker then grouped taxa together based on overall similarity in these categories of calls and the shared presence or absence of categories. He found two major clades (Figure 1.7A), one of which contained the western taxa *temminckii, badius*, and *preussi*, with the former two sharing a particularly close relationship. Struhsaker's other clade contained a sister taxon relationship between *gordonorum* and *kirkii* and revealed affinities between *rufomitratus, tephrosceles,* and *tholloni*. Colyn (1993) used phenetic distances from a multivariate analysis of eight craniometric characters. His analysis produced two groups, one consisting of *tholloni, lulindicus,* and *langi*, and the other comprising *foai* sensu stricto*, oustaleti* sensu lato*, parmentieri, tephrosceles,* and *semlikiensis* (Figure 1.7B). Apart from these studies, most of the phylogenetic work done on red colobus monkeys has involved comparisons of pelage patterns, which is problematic as red colobus coat color varies at the specific, subspecific, populational, and even social group levels. This lability makes pelage patterns unreliable for reconstructing evolutionary relationships between specific and subspecific populations (Struhsaker 1981). Furthermore, Struhsaker's (1981) conclusions must be interpreted with care because they are based on the identification of discrete categories of vocalizations in a continuously graded vocal system, and neither Struhsaker nor Colyn identified plesiomorphic and apomorphic character states, thus relegating their studies to the realm of phenetics.

Grubb et al. (2003) performed the most recent assessment of African primate systematics and recognized the presence of at least three species – *P. badius, P. gordonorum,* and *P. kirkii*. Under their arrangement, *P. badius* includes the subspecies *P. b. temminckii, P. b. badius*, and *P. b. waldroni*. Meanwhile, *P. gordonorum* and *P. kirkii* are both monotypic, with previous authors noting the latter to be particularly distinct from

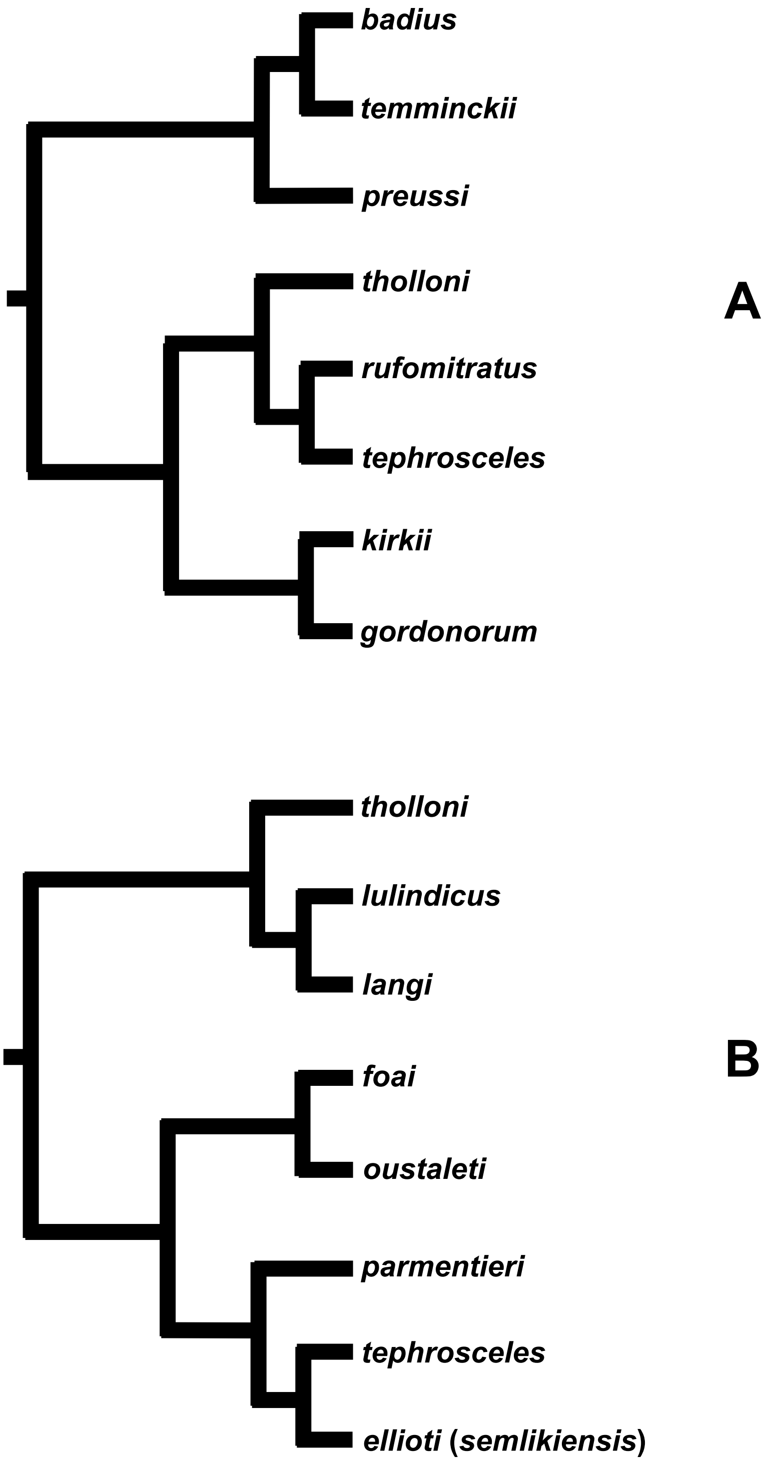

Figure 1.7: A - Relationships among various red colobus forms based on vocalization data (Struhsaker 1981). B - Relationships between various red colobus forms based on craniometric data (Colyn 1993).

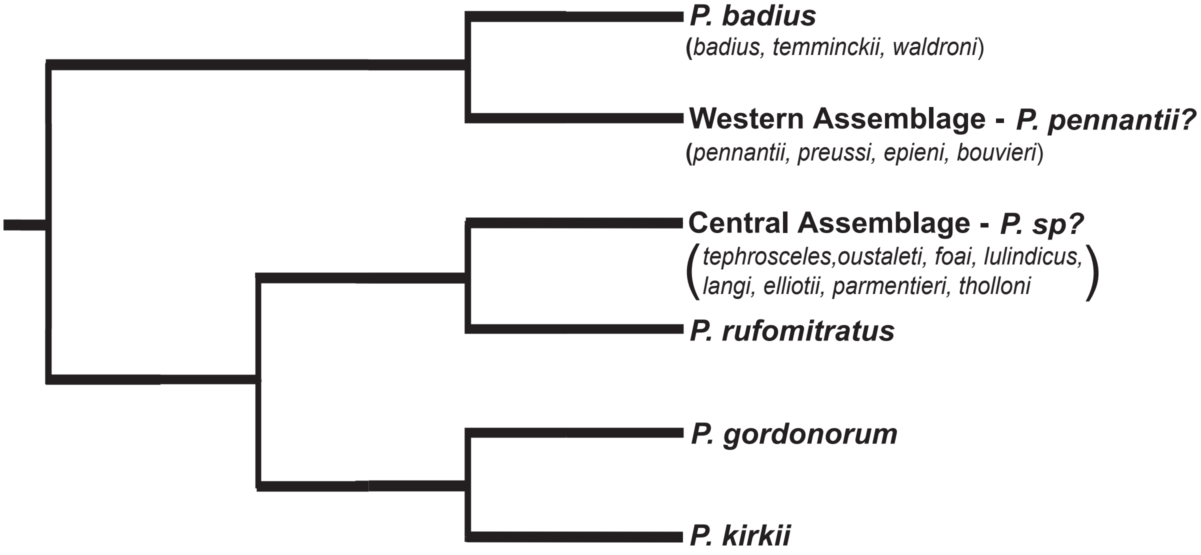

Figure 1.8: Classification and inferred relationships of red colobus taxa based on vocalizations and pelage patterns (Grubb et al. 2003). Four species of red colobus are diagnosed, and the remaining taxa are split into one of two “Assemblages,” where species names are currently unclear.

the other red colobus forms (e.g., Thorington and Groves 1970). Grubb et al. (2003) also believed *rufomitratus* could be elevated to species rank; although this population has clear affinities with *tephrosceles* and some Central African taxa in vocalizations and outward appearance, Groves (2001) considered it to be cranially distinct. The remaining red colobus forms were conservatively lumped into one of two assemblages, the Western or Central, with each containing several “good” subspecies and probably a number of species. In the Western Assemblage, which includes *epieni*, *preussi, pennantii,* and *bouvieri,* Grubb et al. provisionally recognized the presence of at least one species (*P. pennantii*). Pelage patterns group *epieni* with *pennantii,* and *preussi* is the most distinct of the group (Grubb and Powell 1999; Groves 2001). The Central Assemblage contains the forms *oustaleti, tholloni, parmentieri, langi, lulindicus, ellioti, foai,* and *tephrosceles*. However, it is unclear if this is a monophyletic group and the unresolved relationships precluded the authors from assigning any of these forms to a species name. Figure 1.8 displays phylogenetic relationships among Grubb et al’s proposed species and Assemblages as inferred from morphology, pelage, and vocalizations.

Olive colobus systematics and distribution

The olive colobus monkey is monotypic and limited to West Africa (Figure 1.9). It is found from Sierra Leone to Nigeria with a patchy distribution across the dry forest zones of the Dahomey Gap and in the vicinity of the lower Niger River in Nigeria (Oates et al. 1994; Oates 1996c).

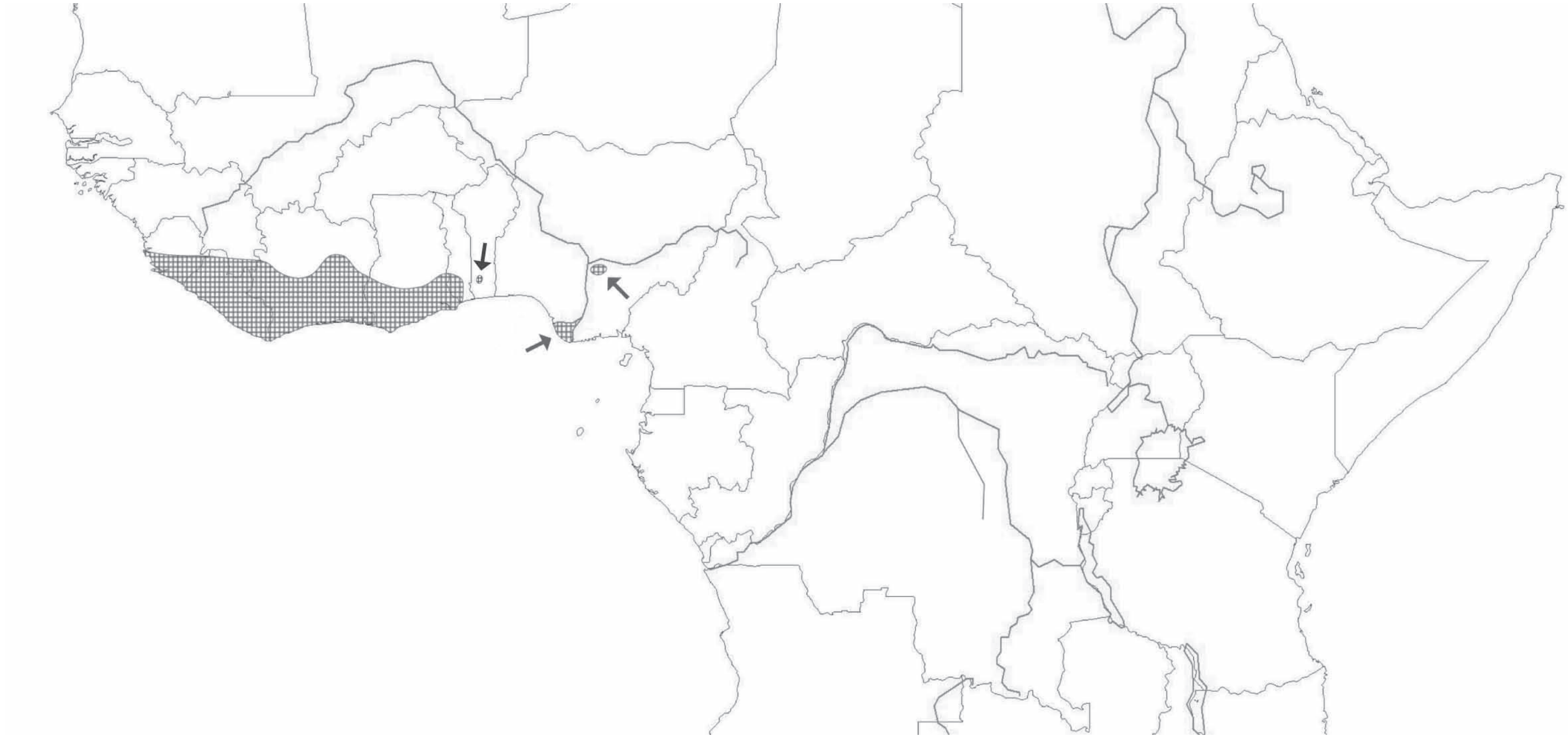

Figure 1.9: Distribution of the olive colobus monkey (*Procolobus* [*Procolobus*] *verus*). Adapted from Oates et al. (1994) with updated data from Oates (1996c).

Chapter 2: Mitochondrial relationships and divergence dates of the African colobines: Evidence of Miocene origins for the living colobus monkeys

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.

This chapter investigates the evolutionary relationships and timing of divergence events among the living colobus monkeys. Nearly 4,000 base pairs of mitochondrial DNA were extracted, amplified, and sequenced in members of all three colobus monkey groups. These data were then analyzed using likelihood methods to infer a mitochondrial gene tree and divergence dates in order to test hypotheses concerning the evolutionary history of the African colobines. The specific issues addressed in this chapter pertain to the relationships among the three colobus monkey groups and their timing of divergence from one another, as well as a preliminary assessment of the species level relationships and divergence dates within the red colobus and black-and-white colobus groups. The red colobus group, the focus of this dissertation, is given further treatment in Chapter 3.

Although African colobines are rather well represented in the fossil record, few of the fossil forms can be reliably connected to the living ones. The earliest “leaf-eating monkey” 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, which saw 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 species found in Late Pleistocene deposits (reviewed in Jablonski 2002; 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).

The timing of extant colobus monkey diversification events has also been inferred through biogeography and molecular data. Some authors (e.g., Grubb 1978; Hull 1979; Struhsaker 1981; Grubb 1982; Oates and Trocco 1983) have also suggested recent origins for these animals through the use of Pleistocene refuge theory (see Chapter 4). They infer earlier speciation events occurring 200,000-250,000 years ago and later ones as recently as 8,000 years ago. Recent analyses of mitochondrial data have suggested that the black-and-white and red colobus monkeys diverged as early as the Late Miocene (Sterner et al. 2006). However, only one individual of each was analyzed so that the placement and divergence of the olive colobus, as well as the multiple black-and-white colobus and red colobus lineages, could not be determined.

Materials and methods

Samples – All species of African colobine recognized by Grubb et al. (2003) are included in this chapter (Table 2.1). Figure 2.1 shows the various localities where samples used in this chapter were collected. 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 red colobus species level classification is so problematic, and a major aim of this dissertation is to address this problem. 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 is of mitochondrial origin. Mitochondrial DNA was chosen because of its fast rate of mutation, generally smaller effective population size than nuclear DNA, and therefore quicker 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 geographic distributions are restricted and females transfer from their natal groups. The red colobus and olive colobus 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).

Table 2.1: Individuals and taxa sampled. Individuals whose sequence data were identical to another individual have an asterisk and were not deposited into Genbank.

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	-----------
*Colobus polykomos**	Taï National Park, Côte d'Ivoire	-----------
Colobus satanas satanas	Bioko Island, Equatorial Guinea	EU580054
Colobus satanas satanas	Bioko Island, Equatorial Guinea	EU580055
*Colobus satanas satanas**	Bioko Island, Equatorial Guinea	-----------
Colobus vellerosus	Boabeng-Fiema Monkey Sanctuary, Ghana	EU580056
*Colobus vellerosus**	Boabeng-Fiema Monkey Sanctuary, Ghana	-----------
*Colobus vellerosus**	Boabeng-Fiema Monkey Sanctuary, Ghana	-----------
*Colobus vellerosus**	Boabeng-Fiema Monkey Sanctuary, Ghana	-----------
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

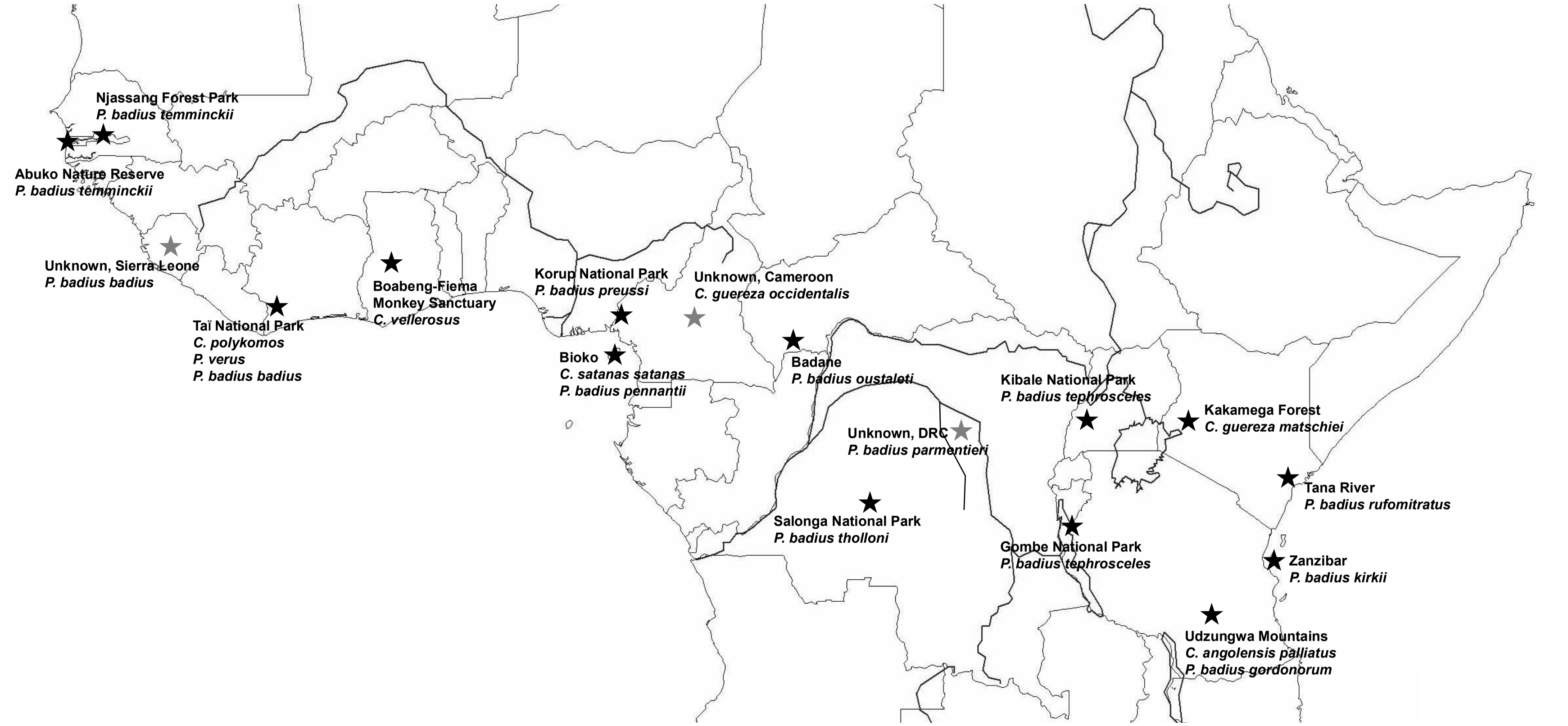

Figure 2.1: Map of localities for black-and-white (*Colobus*), olive (*Procolobus*[*Procolobus*]), and red (*Procolobus*[*Piliocolobus*]) colobus monkey samples. Beneath each locality name are the species collected there. Gray stars indicate countries where samples were collected but the exact origins are unknown.

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* below). 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 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, the DNeasy Tissue kit, and the Qiagen DNA stool kit. The Roche Expand Long Template PCR system was used to perform long-range amplifications when 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 Figure 2.2 for amplification strategy). This method increases 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 2.2) and sequencing primers were the same as those used by Sterner et al. (2006) or designed from 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) 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 2.1.

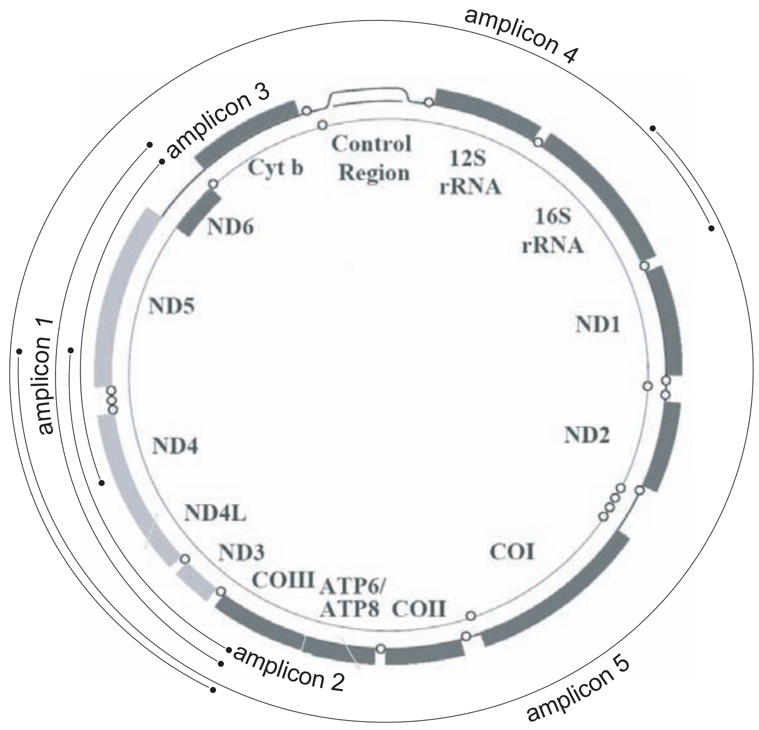

Figure 2.2: Amplification strategy. Analyzed region (ND3, ND4L, ND4, ND5) in light gray. Long range PCR was employed in an attempt to avoid amplification of nuclear pseudogenes. Amplicon 1 (primers 9210F/14103R) represents a 5,000 base pair amplification of the entire target region. For more degraded 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 2.2 for amplification primer sequences.

Table 2.2: Amplification primers

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

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 (see Table 2.3 for summary of Modeltest output). 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

Table 2.3: Summary of Modeltest output (Akaike Information Criterion).

Model selected: GTR+I+G

-lnL = 29634.4062

Base frequencies:
freqA = 0.3531
freqC = 0.3312
freqG = 0.0695
freqT = 0.2462

Substitution model:
Rate matrix
R(a) [A-C] = 0.5750
R(b) [A-G] = 28.2600
R(c) [A-T] = 0.7143
R(d) [C-G] = 1.3067
R(e) [C-T] = 15.5869
R(f) [G-T] = 1.0000

Among-site rate variation
Proportion of invariable sites (I) = 0.3771
Variable sites (G)
Gamma distribution shape parameter = 1.1039

generations and sampled every 100 generations. The burnin was determined by dividing the number of generations it took for the log likelihood of the cold chain to stabilize by the sampling frequency. The log likelihood of the cold chain had stabilized by 30,000 generations, and dividing this by the sample frequency (100) gave a burn-in 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.

Divergence date analysis – The goal of the divergence date analysis was to produce relatively conservative point estimates for the latest date by when mitochondrial lineages must have diverged. In other words, the inferred divergence dates correspond to the most recent possible separation of mitochondrial lineages given the data, but do not provide a total time frame in which a divergence occurred. This approach was taken because inferring a total time range for when lineages have separated requires the input of two values at each calibration point – one that corresponds to the most recent possible separation and another that corresponds to the oldest possible separation. Although the most recent separation can be based on the fossil record, the oldest separation is not based on actual data. I therefore prefer to infer a most recent estimate by when lineages must have diverged instead of estimating a total time range for the date of divergence.

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 *r*8*s* (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. I determined the appropriate penalty function (0.1) for the dataset by running the cross-validation check in *r8s*. 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 *tholloni*, which had two individuals retained in the analysis because they fell on two divergent branches of the tree

Penalized likelihood was chosen over the Bayesian approach of Thorne et al. (1998) and Kishino et al. (2001) because the Bayesian approach tends to generate slightly less conservative divergence dates (e.g., dates further back in time; Raaum et al. 2005; Ting unpublished data). Furthermore, the Bayesian approach is designed for the input of two dates at each calibration point (see above). If one provides only one date at each calibration point (e.g., the most recent possible split based on the fossil record), this method infers divergence dates for these data with very large confidence intervals that are uninformative (Ting, unpublished data).

Choosing calibration points for a colobine divergence date analysis is problematic. Ideally, multiple points that are bracketed and lay within the clade of interest would be 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. Regardless, calibration points need to be 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). Therefore, three calibration points outside of the colobine clade were used – *Papio-Theropithecus, Pan-Homo,* and hominoid-cercopithecoid. 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). The chimpanzee fossil record is nearly non-existent, especially further back in time, but numerous fossil specimens have been claimed as the earliest member of the hominin lineage and can be used to approximate the *Pan-Homo* split. These include *Sahelanthropus tchadensis* at 7.2 – 6.8 Ma (Lebatard et al. 2008), *Orrorin tugenensis* at 6 Ma (Pickford and Senut 2001), *Ardipithecus kadabba* at 5.8 – 4.5 Ma (Halie-Selassie 2001), and *Australopithecus anamensis* at 4.2 Ma (Leakey et al. 1998). Which of these to use, however, is contentious because there is debate as to which ones are truly hominin. At the hominoid-cercopithecoid split, the earliest fossil attributable to either of these superfamilies is the hominoid *Morotopithecus*, dated at 20.6 Ma (Gebo et al. 1997), but this is likely too young to truly represent the hominoid-cercopithecoid split. Because of the uncertainty around the *Pan-Homo* and hominoid-cercopithecoid splits, divergence date analyses were run multiple times using different dates at these calibration points. 4 Ma was always used for the *Papio-Theropithecus* split, dates between 7.2 – 4.2 Ma were used for the *Pan-Homo* split, and either 23 Ma or 20.6 Ma were used for the hominoid-cercopithecoid split. Table 2.4 shows that the inferred dates did not vary to a great degree regardless of which dates were used at the chosen calibration points. Therefore, calibration points of *Papio-Theropithecus* at 4Ma, *Pan-Homo* at 6 Ma, and hominoid-cercopithecoid at 23 Ma were chosen, the latter two based on reasoning outlined in Raaum et al. (2005). 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.

Table 2.4: Divergence dates for nodes in the African colobine tree inferred in r8s (Sanderson 2003). Inferred nodes shown are the initial African colobine split (*Colobus-Procolobus*), the olive colobus-red colobus split (*Procolobus-Piliocolobus*), the first split between modern black-and-white colobus taxa (basal modern black-and-white), and the first split between modern red colobus taxa (basal modern red). All dates in Ma. Three calibration points were used for each analysis: Hominoid-cercopithecoid / *Pan-Homo* / *Theropithecus-Papio* (indicated by # / # / #). Dates at the hominoid-cercopithecoid (23 or 20.6) and *Pan-Homo* (7.2, 6.0, 5.8 or 4.2) fixed nodes were varied to explore their effect on the inferred dates and because there is uncertainty in regard to which dates are most appropriate to use for calibration. Note that the inferred dates do not vary much regardless of what dates are used at the calibration points.

Node	23 / 6 / 4	20.6 / 6 / 4
Colobus-Procolobus	7.85	7.16
Procolobus-Piliocolobus	6.63	6.05
Basal modern black-and-white	3.65	3.33
Basal modern red	2.93	2.68

	23 / 7.2 / 4	23 / 5.8 / 4	23 / 4.2 / 4
Colobus-Procolobus	7.85	7.84	7.84
Procolobus-Piliocolobus	6.63	6.63	6.62
Basal modern black-and-white	3.65	3.65	3.65
Basal modern red	2.93	2.93	2.93

	20.6 / 7.2 / 4	20.6 / 5.8 / 4	20.6 / 4.2 / 4
Colobus-Procolobus	7.17	7.16	7.15
Procolobus-Piliocolobus	6.05	6.05	6.04
Basal modern black-and-white	3.33	3.33	3.33
Basal modern red	2.68	2.68	2.67

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 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.

The higher-level catarrhine relationships and divergence dates generated by the data (Figure 2.3, 2.4) 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). A likelihood ratio test shows that the data are not evolving at a clock-like rate ($p=0.00$; Figure 2.3). This is likely due to a slow down in rate evolution in the hominoid lineage, which is apparent when looking at the branch lengths in Figure 2.3 and has been demonstrated in past studies (e.g., Steiper et al. 2004). The data indicate that the modern African colobine radiation had 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 (Figure 2.5), *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

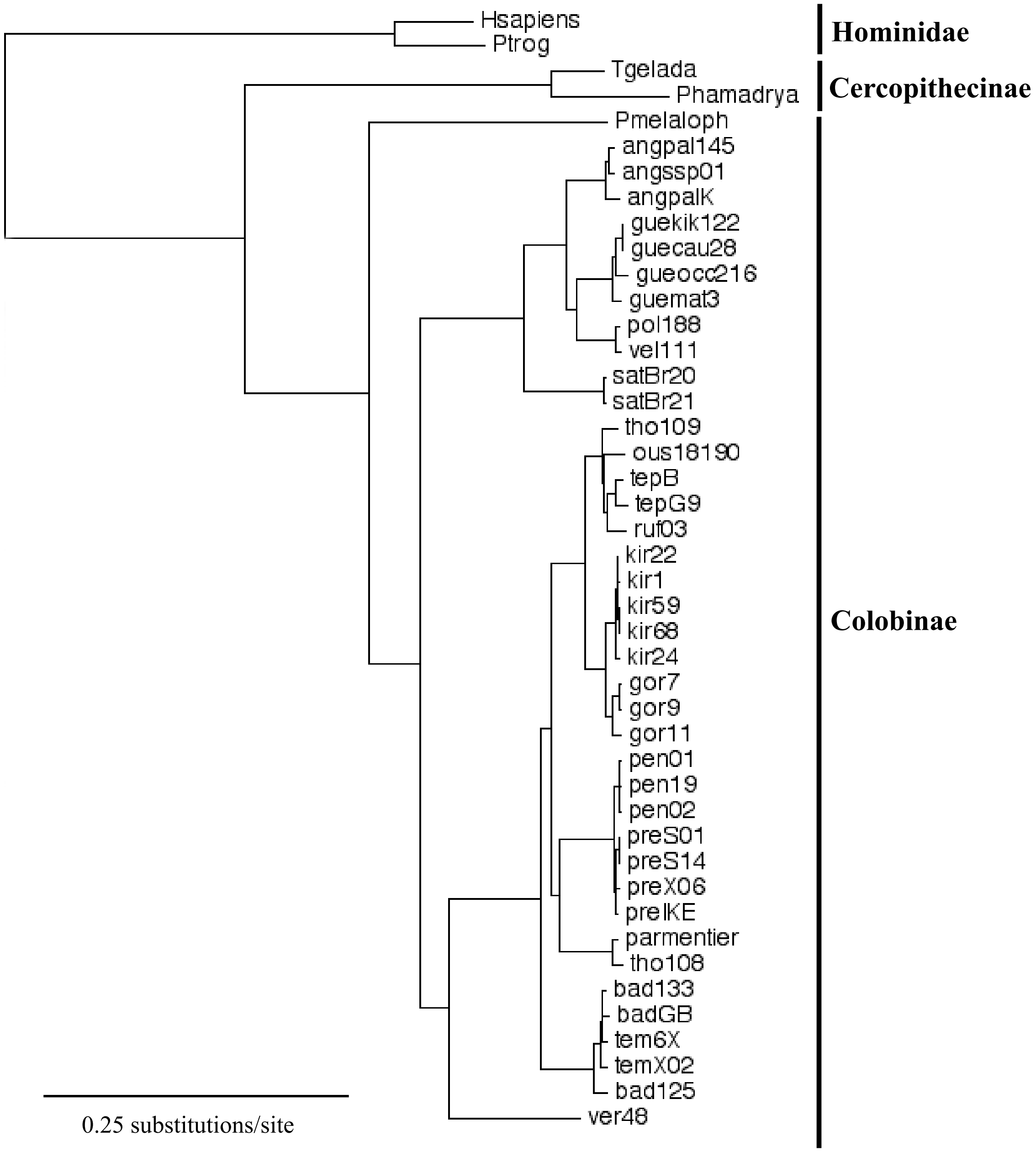

Figure 2.3: Catarhine mitochondrial likelihood tree based on NADH3, NADH4, NADH4L, and NADH5 genes (3,831 basepairs). *Cebus* was the outgroup taxon (not shown). Branch lengths drawn to scale. Notice the shorter branch lengths in the hominoid lineage. A likelihood ratio test reveals that the data are not evolving at a clock-like rate (p=0.00).

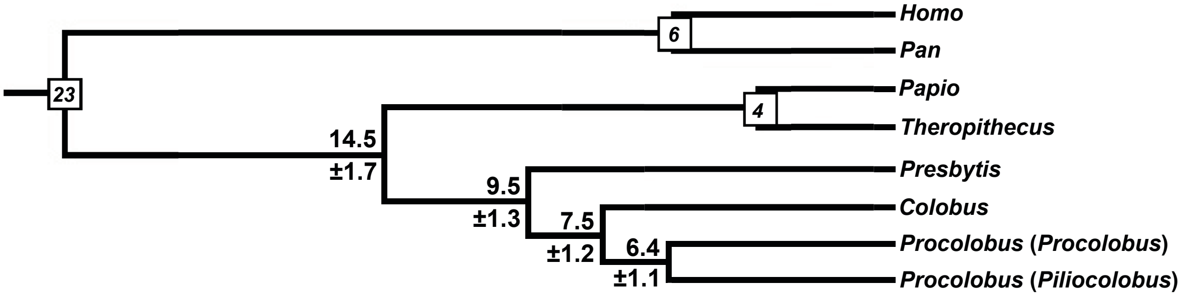

Figure 2.4: Catarrhine mitochondrial likelihood and Bayesian tree based on NADH3, NADH4, NADH4L, and NADH5 genes (3,831 base pairs). All nodes supported by bootstrap values > 85 and posterior probabilities > 0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Calibration points are boxed and italicized. *Cebus* was the outgroup taxon. Classification follows Grubb et al. (2003).

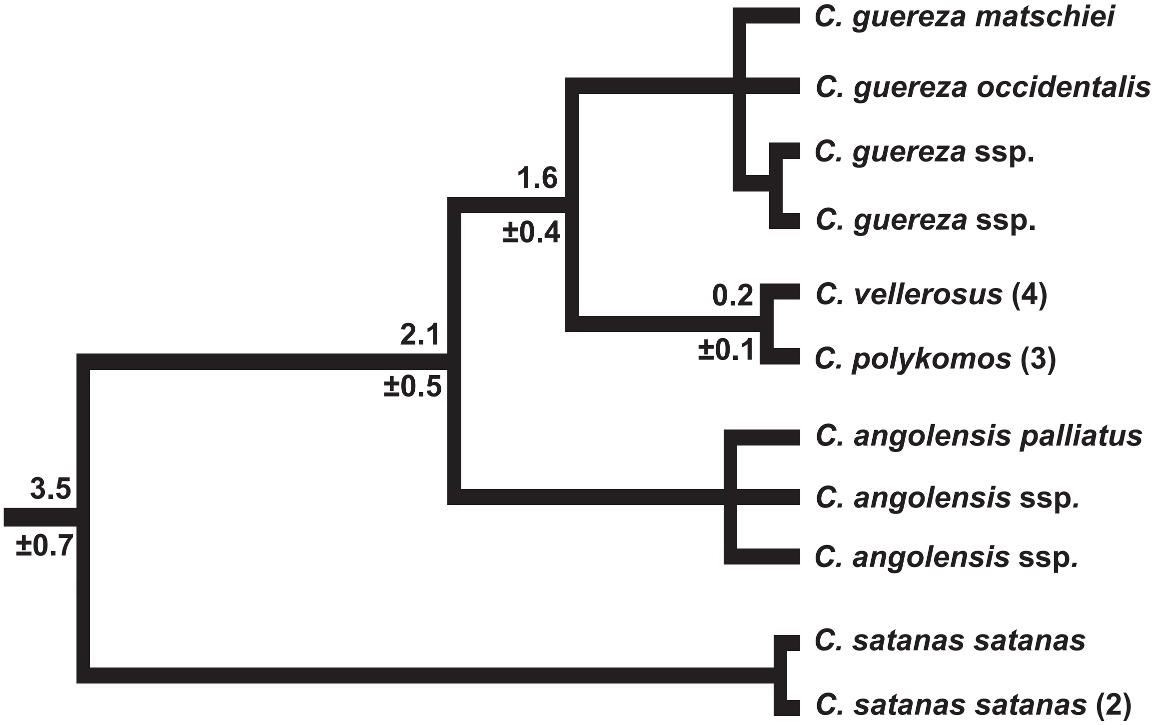

Figure 2.5: Black-and-white colobus (*Colobus*) mitochondrial likelihood and Bayesian tree. All nodes supported by bootstrap values > 85 and posterior probabilities > 0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Numbers in parentheses indicate individuals with identical sequence that were not included in the analysis. Classification follows Grubb et al. (2003).

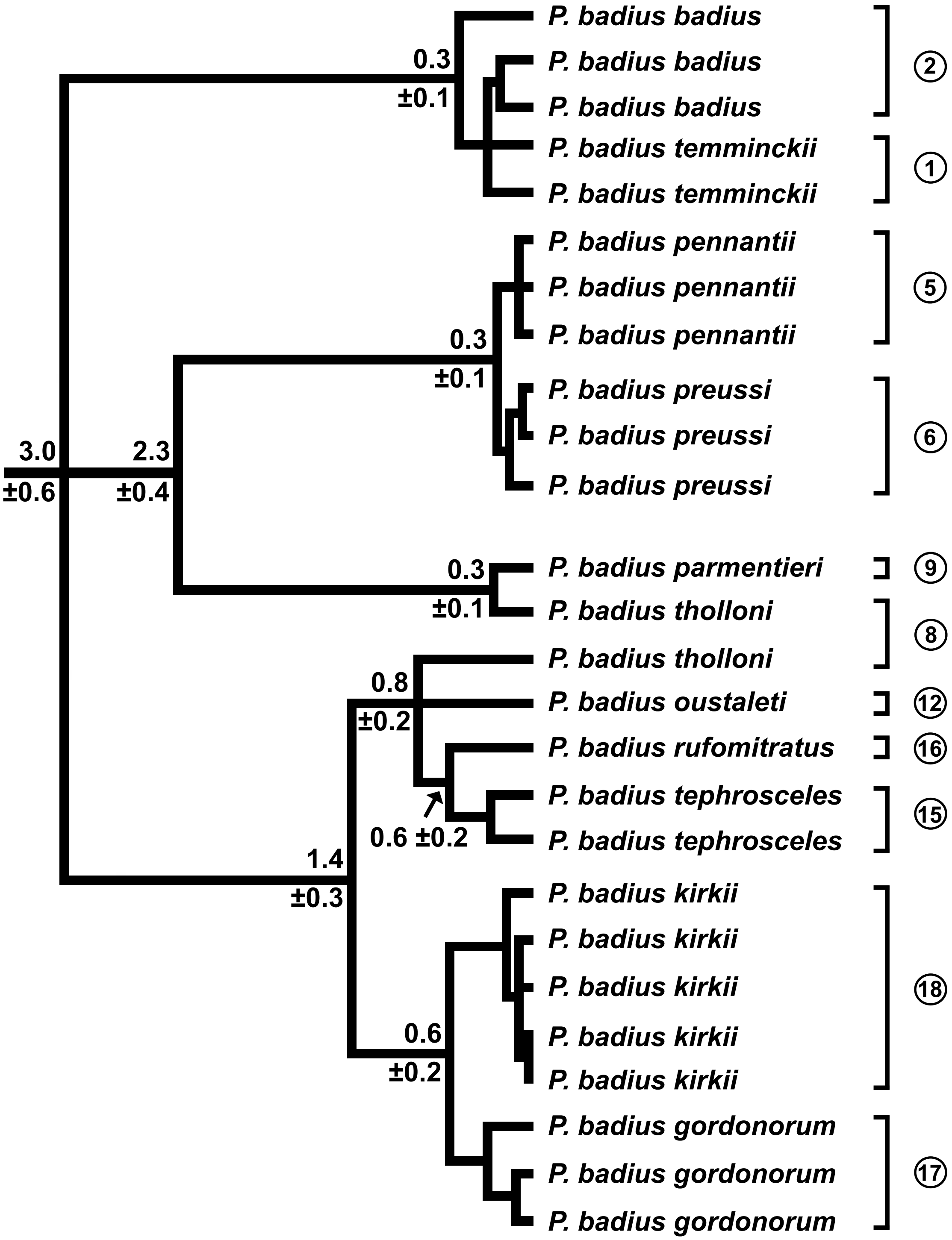

Figure 2.6: Red colobus (*Procolobus* [*Piliocolobus*]) mitochondrial likelihood and Bayesian tree. All nodes supported by bootstrap values > 85 and posterior probabilities > 0.90. Divergence date estimates (Ma) from penalized likelihood shown with two standard deviations. Numbers correspond to range distributions from Figure 1.3. Classification follows Oates et al. (1994).

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 (Figure 2.6) there exist some mitochondrially paraphyletic or polyphyletic groupings. For example, the subspecies *badius* is mitochondrially paraphyletic as allelic lineages of *temminckii* are phylogenetically nested among those of *badius*. Meanwhile, *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 *badius* and *temminckii*. Another contains the Western Equatorial subspecies (*pennantii*, *preussi*) and two individuals from taxa that reside in the Congo basin (*tholloni* and *parmentieri*). A second individual of one of these Congo basin forms (*tholloni*) appears in the third clade in a group that contains *oustaleti*, *rufomitratus,* and *tephrosceles*. The third clade also contains a sister taxon relationship between *kirkii* and *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 *pennantii* and *preussi* separating from *tholloni* and *parmentieri* by 2.3 Ma, and *gordonorum* and *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) (Figure 2.7). 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). This may indicate convergence of vocalization character states. However, because mitochondrial DNA and male loud calls are presumably inherited through different systems of genetic transmission, these conflicting signals could also 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. Another explanation of the incongruence is ancestral hybridization between *C. guereza* and *C. polykomos*. *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.

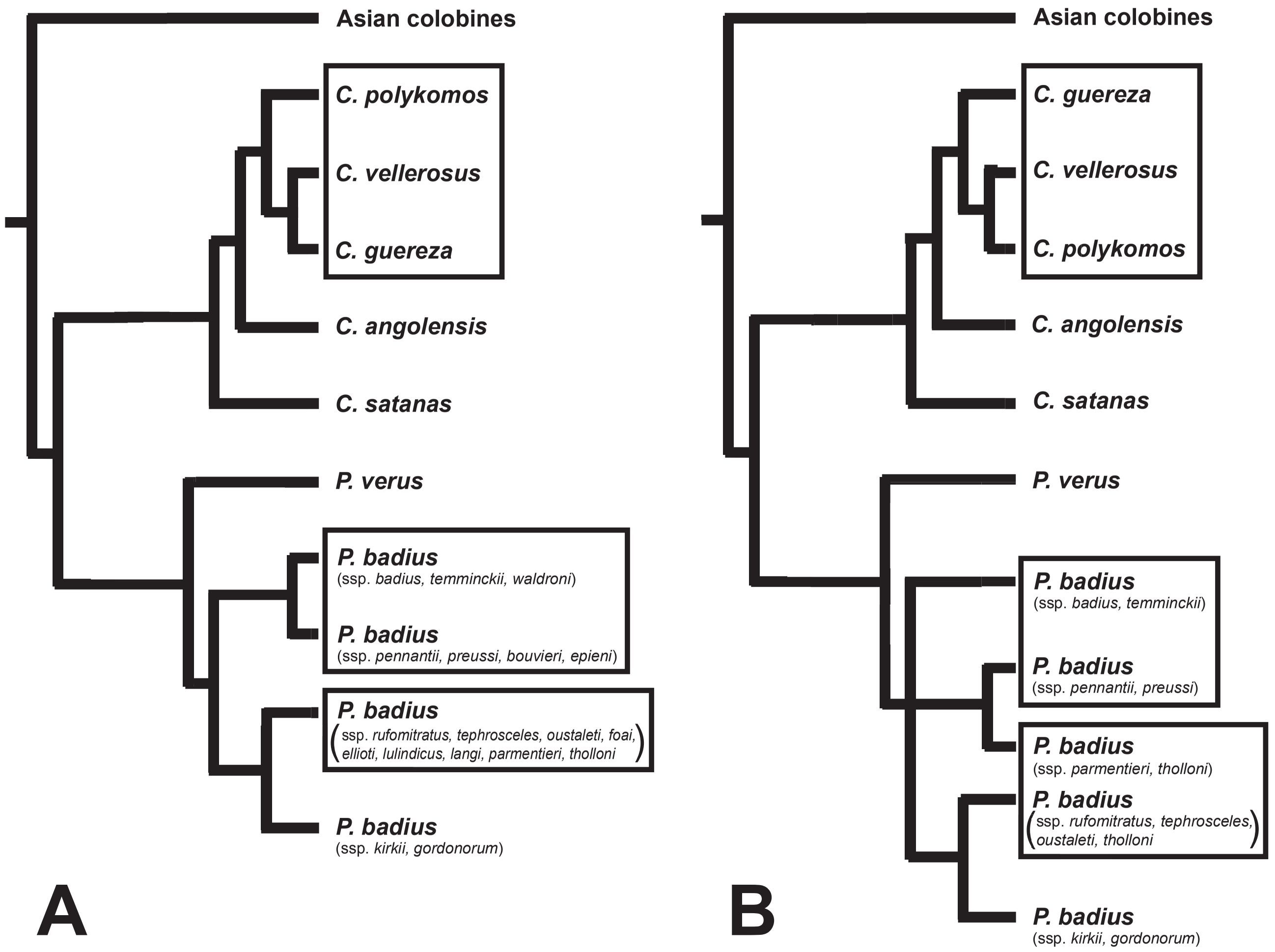

Figure 2.7: Cladograms showing incongruent relationships between current phylogenetic hypotheses and mitochondrial relationships among the living African colobines. A – Phylogenetic hypotheses based on morphology, pelage, and vocalizations (Struhsaker 1981, Oates and Trocco 1983, Groves et al. 1993, 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.

Additional sampling is required as the individuals for some 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 (Figure 2.7). Within the red colobus group, congruence between the mitochondrial relationships and those based on pelage and vocalization data are most evident in the grouping of *gordonorum* with *kirkii*, *badius* with *temminckii*, and *pennantii* with *preussi*. The paraphyly seen in *badius* with respect to *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 (Figure 2.7). The *pennantii* and *preussi* pair does not seem to have any particular affinity with the *badius* and *temminckii* pair; rather they seem to be more closely related to some, if not all, of the taxa found further east. That *parmentieri* groups away from most of the Central African forms is unexpected as this form shares with them many similarities in pelage (Colyn and Verheyen 1987;

Groves 2001). It is also *very* unexpected to find individuals of *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 *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. Chapter 3 presents a further sampling of red colobus forms and more thoroughly explains these complex patterns.

<u>Divergence dates and the modern African colobine radiation</u> – 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 the modern lineages coexisted with the Plio-Pleistocene diversification of now extinct African colobines (Figure 2.8), 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 relatively small specimens that date to the Late Miocene whose fragmentary preservation precludes proper diagnosis; these represent a major component 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 from where no colobine fossil deposits are known. 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 (Hlusko 2006).

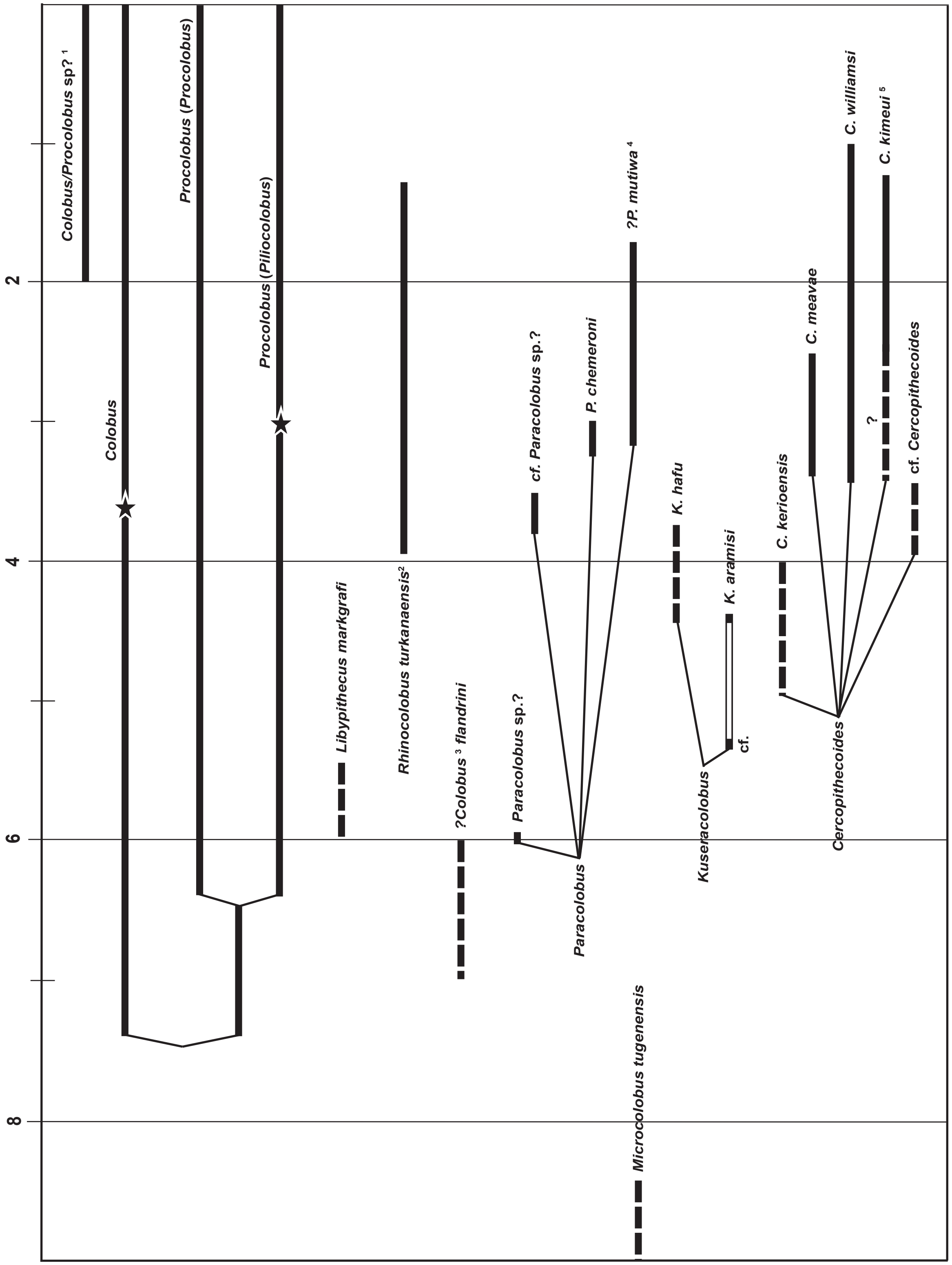
2
4
6
8
Colobus/Procolobus sp.? 1
Colobus
Procolobus (Procolobus)
Procolobus (Piliocolobus)
Libypithecus markgrafi
Rhinocolobus turkanaensis2
?Colobus 3 flandrini
Paracolobus sp.?
cf. Paracolobus sp.?
P. chemeroni
?P. mutiwa 4
Paracolobus
Microcolobus tugenensis
K. hafu
K. aramisi
cf.
Kuseracolobus
C. kerioensis
C. meavae
C. williamsi
C. kimeui 5
?
cf. Cercopithecoides
Cercopithecoides

Figure 2.8: 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), Harrison and Harris (1996), Frost (2001), Ting (2001), Deino and Hill (2002), Frost and Delson (2002), Jablonski (2002), Frost et al. (2003), Leakey et al. (2003), Hlusko (2006), Frost and Alemseged 2007, Frost et al. (2007), Hlusko (2007), and Leakey (2007).

Chapter 3: Phylogenetic relationships among the red colobus monkeys

Introduction

This chapter is a detailed investigation into the phylogenetic relationships among the red colobus forms. It differs from the previous chapter in that it focuses solely on the red colobus monkeys. Patterns of evolution within the red colobus group were investigated using a fragment of the target region from the Chapter 2 analysis. This allowed for the inclusion of individuals sampled from degraded biomaterials (e.g., field and museum specimens) that preclude the amplification of long stretches of DNA, thus providing better taxonomic and geographic sampling of red colobus forms.

Materials and Methods

<u>Samples</u> – To assess the phylogenetic relationships within the red colobus group, 14 red colobus monkey individuals were added to the Chapter 2 sample (Table 3.1). The biomaterials from these individuals are skins and tooth roots from museum and field specimens collected between 15 and 100 years ago. Inclusion of these samples allowed the addition of four taxa (*waldroni, epieni, langi, lulindicus*) and gave better geographic sampling throughout the red colobus range. Figure 3.1 shows a map of localities for all red colobus samples used.

<u>Molecular Marker</u> – Long stretches of DNA (e.g., thousands of base pairs) are logistically difficult to obtain from biomaterials such as museum specimens. The degraded state of DNA in such samples allows only a few hundred base pairs to be

Table 3.1: Individuals and taxa sampled. Those in bold font are from degraded biomaterials (field and museum specimens) and represent new data analyzed in this chapter. AMNH = American Museum of Natural History, Department of Mammalogy. Z = Personal collection of Marc Colyn. Sequences without Genbank numbers (xxxxxxxx) have not yet been submitted.

Outgroups	**ID**	**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	**ID**	**Locality**	**Genbank#**
C. guereza ssp.	122	unknown, zoo specimen, ssp *kikuyuensis*?	EU580050
C. guereza ssp.	28	unknown, zoo specimen, ssp. *caudatus?*	EU580049
C. guereza matschiei	3	Kakamega Forest, Kenya	EU580051
C. guereza occidentalis	216	Unknown locality, Cameroon	EU580052
C. angolensis palliatus	K	Udzungwa Mountains, Tanzania	EU580048
C. angolensis ssp.	145	wild born zoo specimen, ssp. *palliatus?*	EU580047
C. angolensis spp.	01	unknown, zoo specimen	EU580046
C. satanas satanas	Br20	Bioko Island, Equatorial Guinea	EU580054
C. satanas satanas	Br21	Bioko Island, Equatorial Guinea	EU580055
C. vellerosus	111	Boabeng-Fiema Monkey Sanctuary, Ghana	EU580056
C. polykomos	188	Taï National Park, Côte d'Ivoire	EU580053

Procolobus	**ID**	**Locality**	**Genbank#**
P. verus	48	Taï National Park, Côte d'Ivoire	EU580082
P. badius waldroni	**----**	**Ehy Forest, Côte d'Ivoire**	**xxxxxxxx**
P. badius badius	----	Unknown locality, Sierra Leone	DQ355301
P. badius badius	125	Taï National Park, Côte d'Ivoire	EU580057
P. badius badius	133	Taï National Park, Côte d'Ivoire	EU580058
P. badius temminckii	X02	Abuko Nature Reserve, The Gambia	EU580076
P. badius temminckii	6X	Njassang Forest Park, The Gambia	EU58007
P. badius epieni	**----**	**Niger Delta, Nigeria**	**xxxxxxxx**
P. badius pennantii	01	Bioko Island, Equatorial Guinea	EU580069

P. badius pennantii	02	Bioko Island, Equatorial Guinea	EU580070
P. badius pennantii	19	Bioko Island, Equatorial Guinea	EU580071
P. badius preussi	IKE	Korup National Park (North), Cameroon	EU580072
P. badius preussi	X06	Korup National Park (South), Cameroon	EU580073
P. badius preussi	S01	Korup National Park (South), Cameroon	EU580074
P. badius preussi	**S14**	**Korup National Park (South), Cameroon**	**xxxxxxxx**
P. badius rufomitratus	03	Tana River, Kenya	EU580075
P. badius parmentieri	Z2676	D.R. Congo	EU580068
P. badius parmentieri	**Z5022**	**Mabobi, D.R. Congo**	**xxxxxxxx**
P. badius lulindicus	**Z5244**	**Kalima, D.R. Congo**	**xxxxxxxx**
P. badius lulindicus	**Z5245**	**Kalima, D.R. Congo**	**xxxxxxxx**
P. badius lulindicus	**Z5246**	**Kipakata, D.R. Congo**	**xxxxxxxx**
P. badius langi	**Z1863**	**Batiakuya, D.R. Congo**	**xxxxxxxx**
P. badius langi	**Z3022**	**Batiakuya, D.R. Congo**	**xxxxxxxx**
P. badius oustaleti	**Z4025**	**Bomahe, D.R. Congo**	**xxxxxxxx**
P. badius oustaleti	**Z4027**	**Bomahe, D.R. Congo**	**xxxxxxxx**
P. badius oustaleti	**Z3900**	**Nia-Nia, D.R. Congo**	**xxxxxxxx**
P. badius oustaleti	**AMNH52278**	**Akenge, D.R. Congo**	**xxxxxxxx**
P. badius oustaleti	R12190	Badane, Central African Republic	EU580067
P. badius tholloni	**Z5388**	**Simba, D.R. Congo**	**xxxxxxxx**
P. badius tholloni	108	Salonga National Park, D.R. Congo	EU580080
P. badius tholloni	109	Salonga National Park, D.R. Congo	EU580081
P. badius tephrosceles	KB	Kibale National Park, Uganda	EU580078
P. badius tephrosceles	G9	Gombe National Park, Tanzania	EU580079
P. badius gordonorum	7	Ndundulu, Udzungwa Mountains, Tanzania	EU580060
P. badius gordonorum	9	Matundu, Udzungwa Mountains, Tanzania	EU580059
P. badius gordonorum	11	Mwanihana, Udzungwa Mountains, Tanzania	EU580061
P. badius kirkii	1	Zanzibar Island, Tanzania	EU580062
P. badius kirkii	59	Zanzibar Island, Tanzania	EU580063
P. badius kirkii	68	Zanzibar Island, Tanzania	EU580064
P. badius kirkii	24	Zanzibar Island, Tanzania	EU580065
P. badius kirkii	22	Zanzibar Island, Tanzania	EU580066

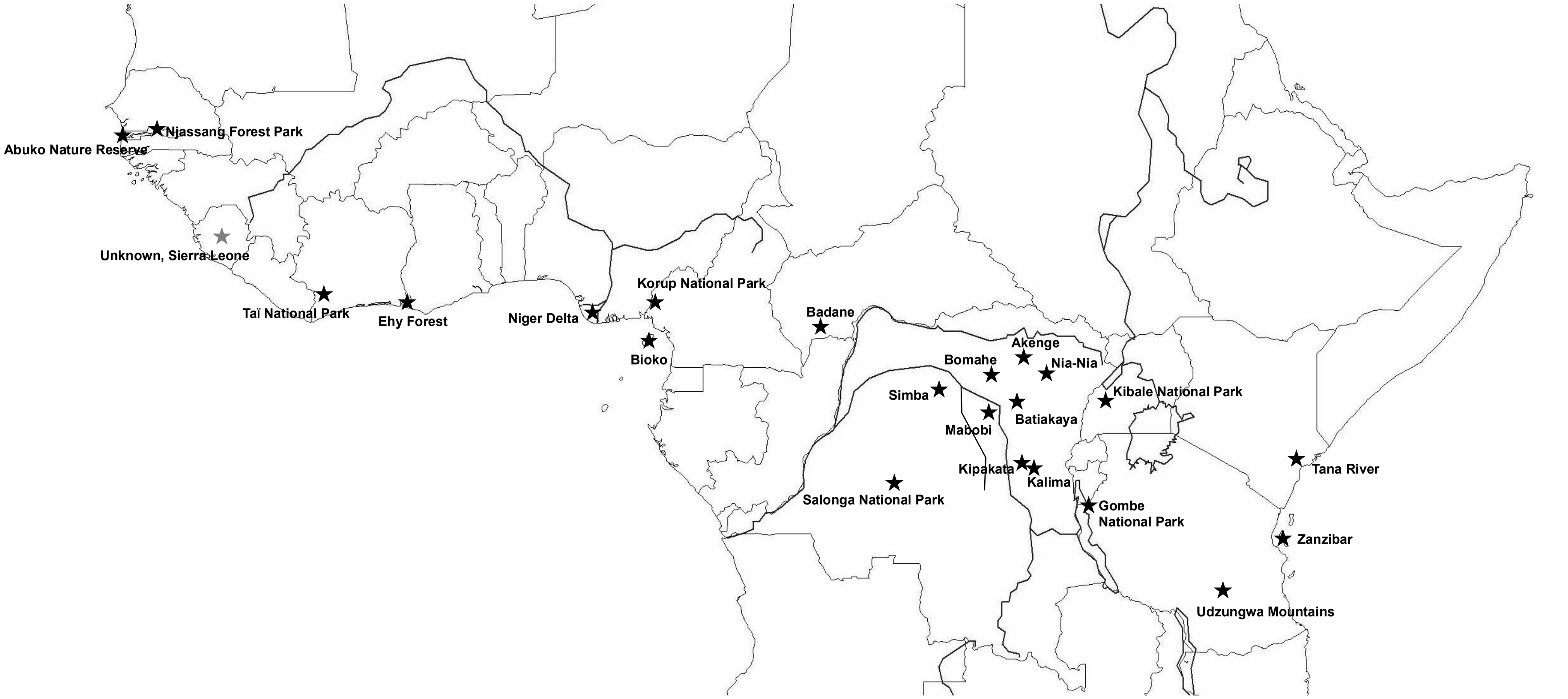

Figure 3.1: Locality data for red colobus specimens

amplified at once. Thus, acquiring the full Chapter 2 marker (3,871 base pairs) in the 14 newly added red colobus individuals was not possible. However, a subset of this marker can be obtained in these samples by amplifying several small overlapping fragments and assembling them into one piece.

The marker used here incorporates the last 897 base pairs of the NADH4 gene, which was inferred to be the most phylogenetically informative stretch of DNA from the target used in Chapter 2. This was done through a resampling process that parsed the Chapter 2 dataset into numerous smaller fragments. These fragments were subsequently analyzed to see which produced the red colobus topology closest to that inferred from the complete 3,871 base pair alignment.

Extraction, amplification, and sequencing of museum and field data – DNA extractions and PCR setup were conducted in a clean room that has positive airflow and has never been exposed to high quality DNA or PCR product. The room was wiped down with bleach and irradiated with ultraviolet light for 30 minutes before and after each use. Total genomic DNA was extracted from bone powder drilled from tooth roots using the GENECLEAN Kit for Ancient DNA. Total genomic DNA was isolated from skin samples using the Qiagen QIAamp DNA Micro Kit following the manufacturer's protocols for tissue samples. Blank extractions were processed with each round to control for contamination, and final extracted volumes went through a 1:10 dilution. PCR was performed with the Qiagen HotStar HiFidelity Polymerase Kit using red colobus specific primers designed from the data generated in Chapter 2 (Table 3.2). The amplification strategy (Figure 3.2) targeted multiple overlapping 150-400 base pair fragments, and each

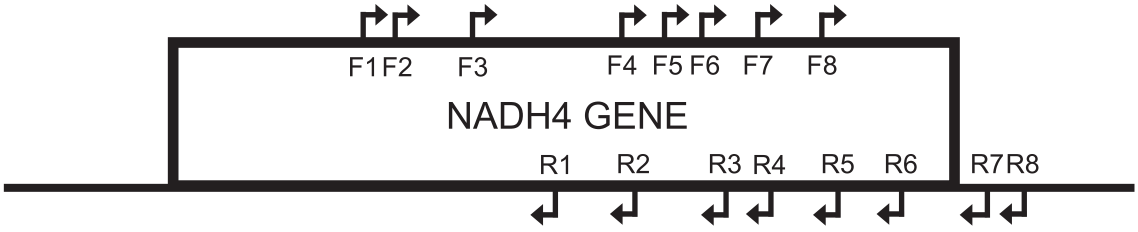

Figure 3.2: Amplification strategy for the last 897 base pairs of the mitochondrial NADH4 gene. 150-400 basepair fragments were amplified using various combinations of the above forward (F1-F8) and reverse (R1-R8) primers. Any given part of the target region was amplified using at least two different primer pairs.

Table 3.2: Primers used. See Figure 3.2 for amplification strategy.

F1	10616F	AATCAAGCAGAACGCCTCAACGC
F2	10629F	GGCAATCAAGCAGAACGC
F3	10760F	CTAACA ACCACCTGAGCACACAGC
F4	RCFE1	TCCTCCGTAAGCCACATAGCCCTA
F5	11022F	GCTCAATCTGCCTCCGACAAACAG
F6	11100F	GTAGCATCCCTCATTCAAACCCC
F7	11239F	TCAAAYTACGAACGAACCCACAGC
F8	11315F	CCCACTATCAACTTAGTAGGAGAACTACTGG
R1	10970R	ATTCCGTAAGAGGGTCAAGGATTAGGG
R2	11105R	GGATGCTACAATTACTAGG
R3	11120R	GGTTTGAATGAGGGATGCTACAATTACTAGGG
R4	11239R	TGGAARTAGAGTTTGAAGTCCTCGGG
R5	11318R	AGTTCTCCTACTAAGTTGATAGTGGGAGG
R6	11463R	GATRTGGTGTGTAAATATTCCTCGTTGGG
R7	11549R	GGATAGTAGAATAATGGGGGAAAGGTG
R8	11662R	GAAGCATGGATTAGCAGTCCTTGC

one was amplified with two different primer sets. This was done to verify the production of identical sequence from different primer pairs, which decreases the chance of incorporating nuclear pseudogenes into the dataset. PCR cleanup and cycle sequencing followed protocols described in Chapter 2.

Phylogenetic analysis - The analyzed dataset was assembled in ClustalW (Chenna et al. 2003) by aligning the 14 newly generated red colobus sequences with those from Chapter 2. Since the new sequences are shorter than the original marker, the dataset was cropped to include only the last 897 base pairs of the NADH4 gene so that there were no missing data. The sequences were also translated in MacClade 4.08 (Maddison and Maddison 2005) in order to test for the presence of frameshift mutations or stop codons, which would indicate the presence of nuclear pseudogenes. Maximum likelihood in PAUP 4.0b10 (Swofford 2002) and Bayesian methods in Mr. Bayes 3.1 (Ronquist and Huelsenbeck 2003) were used to infer red colobus mitochondrial gene trees. For the maximum likelihood (ML) analysis, 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 data were best fit by the Tamura-Nei (TrN) model with invariant sites (I) and a gamma distribution (G) of site-specific rates. The Modeltest output is summarized in Table 3.3.

A constraint tree was used to aid in the resolution of short internodes. This tree was constructed in MacClade 4.08 using the inferred topology from Chapter 2 (Figure 2.4), which is a better approximation of the true mitochondrial tree because it incorporates a larger amount of sequence data. The constraint was imported into PAUP as a backbone, which effectively forced the search to find the most likely positions of the 14 new individuals on the Chapter 2 topology. 100 bootstrap replicates were performed under a heuristic search with the taxon addition set to random, the maximum number of retained trees set to increase automatically, 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 over four chains sampled every 100 generations. The first 25 percent of the samples (2,500) were discarded as burnin. Nodes that were supported by a bootstrap value lower than 85 and a posterior probability value lower than 0.90 were manually collapsed.

Table 3.3: Summary of Modeltest output (Akiake Information Criterion).

Model selected: TrN+I+G
-lnL = 7324.9170

Base frequencies:
freqA = 0.3563
freqC = 0.3322
freqG = 0.0715
freqT = 0.2401

Substitution model:
Rate matrix
R(a) [A-C] = 1.0000
R(b) [A-G] = 47.6880
R(c) [A-T] = 1.0000
R(d) [C-G] = 1.0000
R(e) [C-T] = 22.1083
R(f) [G-T] = 1.0000

Among-site rate variation
Proportion of invariable sites (I) = 0.3864
Variable sites (G)
Gamma distribution shape parameter = 0.9133

Divergence date analysis – Divergence dates were not calculated for this dataset because of the relatively short marker (897 base pairs). Chapter 2 used a much longer marker (3,831 base pairs) and thus provides more accurate estimates than this chapter could in regard to dates of divergence among red colobus mitochondrial lineages. I therefore refer to the Chapter 2 dates in discussing certain nodes of the red colobus tree in both Chapters 3 and 4.

Results

The dataset contained no evidence of nuclear pseudogenes when translated in MacClade 4.08. The ML and Bayesian analyses yielded congruent mitochondrial gene tree topologies, although with differing amounts of resolution. Figure 3.3 displays branches from the inferred red colobus topology that found high support in both analyses (bootstrap ≥ 85, posterior probability ≥ 0.90). At the base of this tree is a polytomy with five branches that had diverged from one another 3 Ma (see Chapter 2). For ease of reference, four of these branches are here assigned as phylogroups A through D while a fifth branch spits into three phylogroups (E through F) that had diverged from one another by 1.4 Ma (see Chapter 2). Phylogroups A and B contain the western red colobus forms (*waldroni, badius, temminckii*) and were united by a branch that was collapsed due to moderate support (bootstrap = 66; posterior probability = 0.89). Phylogroup C has the Western Equatorial forms *preussi* and *pennantii*. The red colobus forms found in phylogroups D and E are the same as those in Grubb et al.'s (2003) "Central Assemblage" (*tholloni, parmentieri, oustaleti, langi, lulindicus, tephrosceles, rufomitratus*). Phylogroup F contains the Tanzanian forms *kirkii* (Zanzibar) and *gordonorum* (Udzungwa Mts.), and phylogroup G contains *epieni* from the Niger Delta (Nigeria).

Because of the use of the constraint tree, nearly all of the relationships found in Chapter 2 were well supported in this analysis. One of the few that found weak support

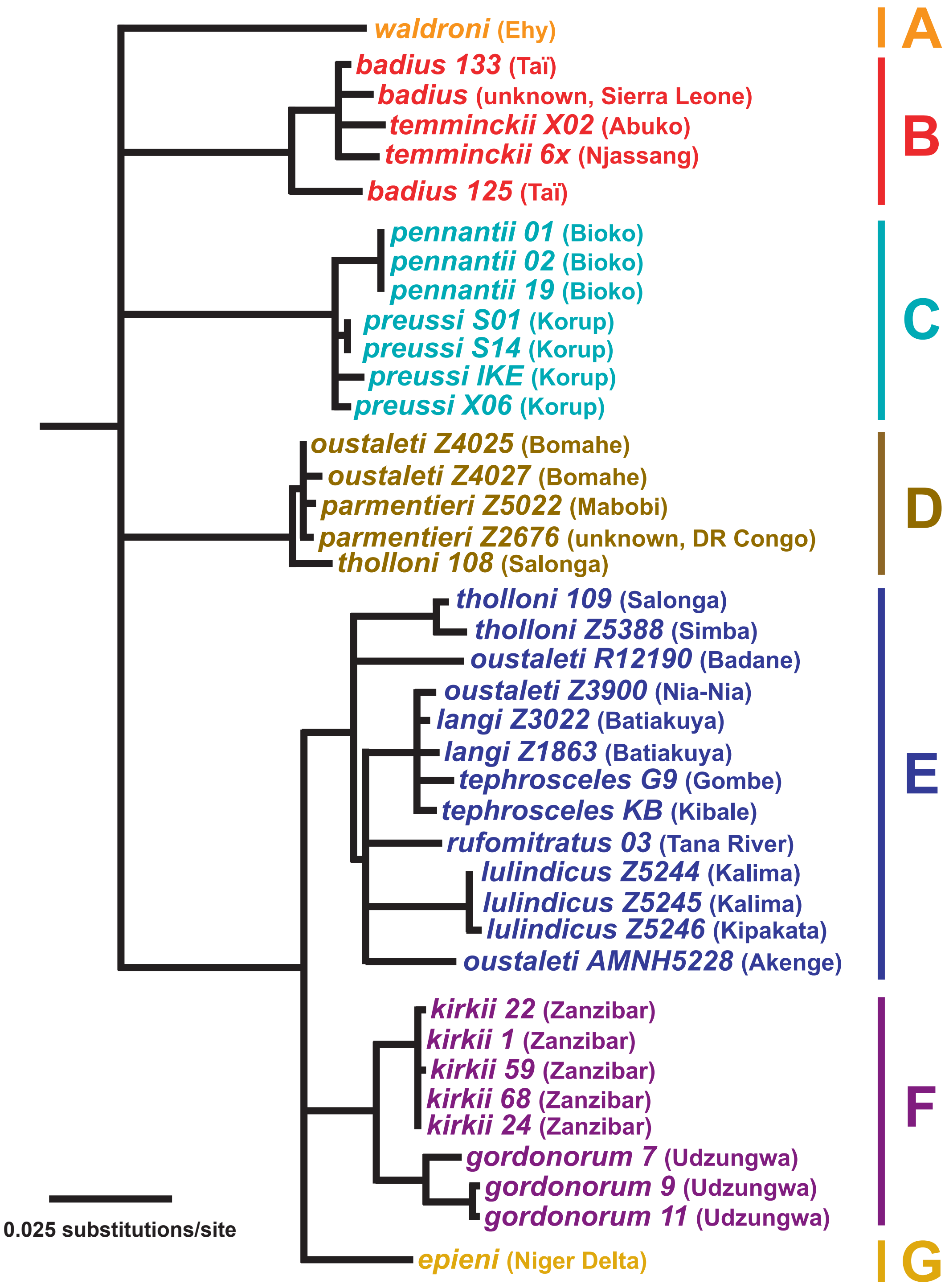

Figure 3.3: Red colobus monkey mitochondrial tree inferred from maximum likelihood (ML) and Bayesian analyses using the last 897 base pairs of the NADH4 gene. Branch lengths drawn to proportion (from ML analysis) and only those with high bootstrap and posterior probability support are displayed (≥ 85 and .90, respectively). Outgroup taxa not shown. Letters correspond to assigned phylogroups referred to in the text. Taxon names followed by individual ID, and locality data in parantheses.

was the relationship between phylogroup C and phylogroup D, which had high posterior probability but a low bootstrap value (posterior probability = 0.93, bootstrap = 74). Also, the branch uniting *preussi* into a monophyletic group was not well supported in the Bayesian analysis, but it had a bootstrap value of 100 in the likelihood analysis. Sampling more mitochondrial data would likely lead to increased support of these relationships and other short internodes such as the branch linking *waldroni* (phylogroup A) to the other western red colobus forms (phylogroup B; *badius* and *temminckii*), which is strongly supported by both pelage and biogeography (Booth 1958a, b; Oates 1988; Grubb et al. 2003).

Although red colobus forms such as the Niger Delta red colobus (*epieni*; phylogroup G) and Miss Waldron's red colobus (*waldroni*; phylogroup A) were only represented by one individual, these taxa have relatively restricted distributions and show female transfer social systems. It is therefore likely that mitochondrial lineages in these populations have sorted and lack ancestral polymorphisms. Supporting this notion is the lack of divergent lineages within the red colobus forms *pennantii, kirkii,* and *gordonorum*, which were better sampled, have restricted ranges, and show mitochondrial alleles that have sorted into one lineage. Furthermore, in the case of *epieni*, although only one individual yielded data complete enough to use in this analysis, fragmentary sequences from other samples were nearly identical to this individual.

The two most complex patterns in the results pertain to phylogroups D and E, which reinforces the Chapter 2 notion that the evolution of red colobus monkeys in Central Africa has been very complex. The first is the failure of the phylogroup D and E taxa to form a monophyletic clade despite their inferred relatedness through vocalizations and pelage patterns (Struhsaker 1981; Colyn 1991). Lumping these taxa together (by combining phylogroups D and E) thus creates a mitochondrially paraphyletic group. The second pattern is the polyphyly seen *within* certain taxa. Specifically, both *tholloni* and *oustaleti* have individuals that fall in both phylogroups D and E, thus contributing to the aforementioned paraphyly. In addition, none of the four *oustaleti* individuals sampled group together, which is not necessarily surprising given the polytypic nature and large distribution of this taxon. This either supports Gautier-Hion et al.'s (1999) proposition that *oustaleti* sensu lato consists of numerous taxa, or indicates past introgression of mitochondrial lineages between neighboring red colobus forms. Regardless, at least some of the relationships uncovered here are likely unique to mitochondrial lineages and are not following the organismal phylogeny of these taxa. For example, individuals of *tholloni* sampled from the same area of Salonga National Park on the same day (samples 108 and 109) show two divergent mitochondrial lineages – one falling in phylogroup D and the other in phylogroup E. It is highly improbable that this represents relationships across their nuclear genomes, and it raises questions concerning how well the mitochondrial lineages are tracing the organismal phylogeny in the phylogroup D and E taxa.

Discussion

The only red colobus forms not sampled in this chapter are *ellioti* (syn. with *semlikiensis*), *foai* (sensu stricto), and *bouvieri*. The former two readily hybridize with and may be senior synonyms of taxa included in this study (*langi* and *lulindicus*, respectively; Colyn 1991; Colyn 1993; Groves 2007), which leaves *bouvieri* as the only potentially distinct red colobus form not assessed here. The addition of 14 new individuals to the Chapter 2 dataset offers further insight into the phylogeny and evolution of the red colobus group, which began to diversify by 3 Ma (see Chapter 2). In particular, patterns of evolution in the Central and East African red colobus forms are revealed, as are the phylogenetic affinities of the Niger Delta red colobus (*epieni*) and Miss Waldron's red colobus (*waldroni*).

The most complex patterns found in this chapter are the paraphyly and polyphyly in the phylogroup D and E taxa and are likely related to the complex forest history of the Congo Basin. One possible explanation of these patterns could be that some of the taxa in phylogroup D (*tholloni*, *oustaleti*, or *parmentieri*) are truly unrelated to other forms inhabiting the Congo Basin. The presence of some taxa in clade D might be due to mitochondrial introgression between phylogroups D and E and would explain the polyphyly seen in *tholloni* and *oustaleti* (Figure 3.4A). In fact, interbreeding between the numerous Central African forms may explain why the relationships in phylogroups D and E do not support those inferred by Colyn (1993) (Figure 1.7B), as hybridization in a female transfer species such as red colobus monkeys would erase geographic and taxonomic structure in a mitochondrial tree.

An alternative hypothesis that can explain the patterns of paraphyly and polyphyly is incomplete sorting and the presence of ancestral mitochondrial lineages (polymorphisms) within phylogroups D and E. Fluctuating climates throughout the Pleistocene continually altered the hydrology and vegetation of the Congo Basin. This likely created some periods where different red colobus populations became panmictic,

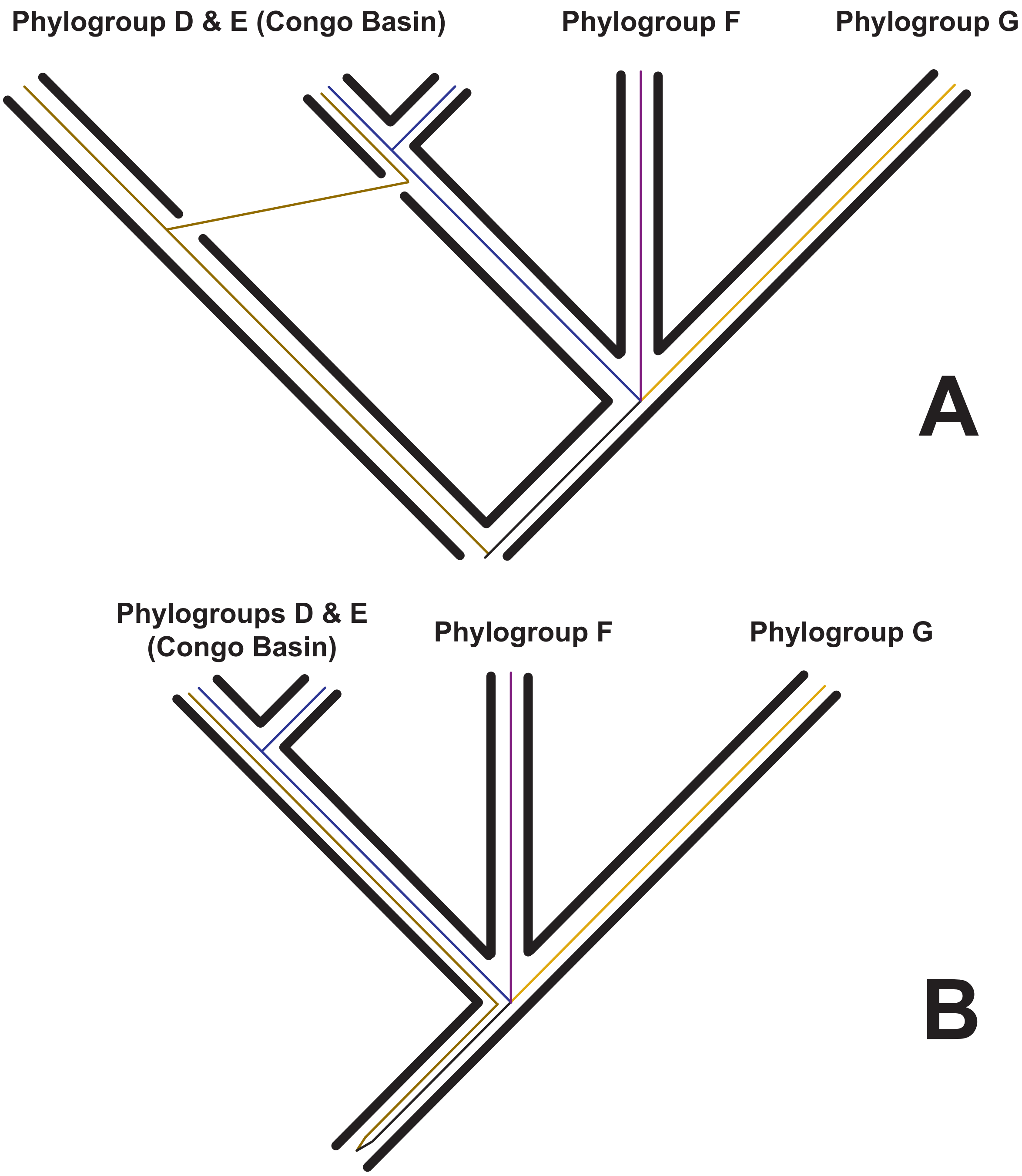

Figure 3.4: Two hypotheses that explain the patterns among mitochondrial lineages (phylogroups D, E, F, and G) in red colobus monkeys. Thick black lines reperesent the organismal phylogeny. Thin colored lines represent mitochondrial lineages. A - Phylogroup D represents a divergent branch of the organismal phylogeny. This makes the Congo Basin forms truly paraphyletic, and introgression from one branch of the organismal tree to another explains the mitochondrial polyphyly seen in certain Congo Basin taxa (shown here by introgression of D, but it could have happened either or both ways). B - Incomplete lineage sorting. Phylogroup D represents an ancestral lineage that is retained in the same branch of the organismal phylogeny as phylogroup E, thus explaining the polyphyly seen in certain Congo Basin forms.

and others where populations were isolated in refuges. These processes would have made the fixation of a single mitochondrial lineage difficult and would have also facilitated occasional hybridization. Phylogroup D thus might represent an ancestral mitochondrial lineage whose traces are retained in living populations of *tholloni*, *oustaleti*, and *parmentieri*, but it has either been lost or never occurred in phylogroups F and G (Figure 3.4B). If incomplete lineage sorting is to explain the mitochondrial patterns documented here, processes such as selection might have played a role in the coalescence of some nuclear genes, which would explain why nuclear genes that influence pelage patterns have sorted before mitochondrial ones.

These hypotheses explaining the mitochondrial paraphyly and polyphyly in the phylogroup D and E red colobus taxa can be tested by conducting a phylogeographic study of the Congo Basin forms using nuclear loci and expanding the geographic scope of the sampling. Such a study would indicate to what extent the mitochondrial relationships demonstrated here are tracing the population history of these animals and in which direction mitochondrial introgression may have occurred. Nuclear gene trees that group these taxa together in a monophyletic group would support a hypothesis of incomplete sorting and the presence of ancestral mitochondrial lineages, while nuclear gene trees that place taxa in the phylogenetic position of phylogroup D would support the hypothesis of true paraphyly among the Central African forms. Collection of more quickly evolving markers, such as microsatellite data, would also allow for the use of statistical phylogeographic and population genetic methods (reviewed in Excoffier and Heckel 2006) to infer the presence and direction of hybridization (see also papers in Molecular Ecology 2004, issue 13:4).

The Niger Delta red colobus has been thought to have close affinities with the forms to its immediate east (phylogroup C), particularly *pennantii* on the island of Bioko (Equatorial Guinea) because these taxa share many similarities in pelage pattern (Grubb and Powell 1999). However, vocalizations unite this animal with forms found further east (T.T. Struhsaker pers. comm.). The mitochondrial data support the vocal evidence and place *epieni* in a clade with East and Central African taxa that had begun to diversify by 1.4 Ma (see Chapter 2). Included in this clade are *kirkii* and *gordonorum* in Tanzania, *rufomitratus* in Kenya, *tephrosceles* in Uganda and Tanzania, and various populations found in the Congo Basin (phylogroups E, F, and G; Figure 3.3). Because vocalizations are presumably under the influence of nuclear DNA, this relationship is supported by genes from two different genetic systems (nuclear + mitochondrial). If this congruence is representative of true population history, the disjunct distribution of this clade (phylogroups E, F,G), and the presence of another clade (phylogroup C) in the intervening area, needs biogeographic explanation (see Chapter 4).

The results here (Figure 3.3) suggest that the mitochondrial lineage found in *waldroni* split from the other western red colobus lineages shortly after the initial diversification of all red colobus forms. Although it is possible that this is an ancestral polymorphism, the retention of such a lineage in a population with a relatively restricted range and where females disperse is unexpected. Since *waldroni* is the most distinct of the western red colobus forms (Groves 2007), and at least one classification regards it as a “potential” full species (Dandelot 1971), an alternative hypothesis is that this lineage represents a derived haplotype historically unique to *waldroni.* Even if non-exclusive molecular lineages between *waldroni* and *badius* are found, they could be due to hybridization through a narrow contact zone reported by Booth (1954), which may have been facilitated by increasing human disturbance (e.g., hunting and logging). Analysis of more western red colobus individuals is necessary to elucidate the evolution of this clade. However, further sampling of *waldroni* will have to be done through museum specimens as it may be extinct in the wild (Oates et al. 2000; McGraw 2005).

Chapter 4: Implications of red colobus monkey phylogeny for the biogeographic history of the African rainforest

Introduction

Biogeography seeks to understand the processes that create geographic patterns of species distribution. Many species show disjunctions within their own range, or between themselves and closely related taxa. These same disjunctions are often shared by many different organisms and correspond to ecological and historical features of the region. Studying the nature of these patterns can aid in understanding the evolutionary history of both the taxon and geographic area under study, as well as the overall process of speciation.

Numerous researchers have used species distributions and paleoecological data to infer biogeographic models of evolution for African rainforest taxa (e.g., Moreau 1969; Kingdon 1971; Laurent 1973; Hamilton 1976; Mayr and O'Hara 1986; Maley 1996). These models focus heavily upon the importance of vicariance events facilitated by rainforest expansion and contraction (refuge theory; see below), as well as processes that form rivers, rifts, mountains, and other potential barriers to gene flow (Haffer 1982). Molecular phylogenetic and phylogeographic studies have provided powerful ways of testing and refining these models, and much has been learned from such research regarding primates (e.g., Goldberg and Ruvolo 1997; Gagneux et al. 2001; Erikkson et al. 2004; Thalmann et al. 2004; Gonder et al. 2006). The phylogenetic relationships among the red colobus monkeys can thus offer valuable information on the evolution of other African rainforest taxa as well as the biogeographic history of their shared ecosystem.

This chapter discusses what the red colobus monkey phylogenetic relationships inferred in Chapter 3 reveal about the history of the African rainforest.

References to the various ecological biomes of tropical Africa are made throughout this chapter (e.g., savanna, rainforest, dry forest), especially in discussion of the background literature. However, describing these biomes is very difficult because different authors define them in different ways. This makes it impossible to provide a single definition for a given biome that truly reflects what each author had in mind when referencing that biome. Instead of attempting to figure out exactly how every author was defining each of these biomes (which is nearly impossible), I have listed some of the biomes in Table 4.1 with some defining characteristics from Deshmukh (1986). Although these may not correspond exactly to how every author defines these terms, it provides a general description of these biomes that are important in conceptualizing the history of the African rainforest.

Modern distribution and features of the African rainforest

Tropical Africa has a high mean elevation but relatively few mountain ranges. Plateaus over 1000m, however, are common, with some in Ethiopia reaching altitudes over 2000m. There are also isolated mountains rising above 5000m in East Africa, and in western Africa Mt. Cameroon and Mt. Nimba (Guinean highlands) are approximately this height (Livingstone 1975). Areas of high rainfall often coincide with topographic relief as parts of the East African rift, the south side of Mt. Cameroon, and the Guinean highlands are all particularly wet (Anhuf et al. 2006). African rainfall comes from monsoonal winds that originate in the southern Atlantic and Indian Oceans, with the former being

Table 4.1: Brief characteristics of the various ecological biomes discussed in regard to African forest history and biogeography. From Deshmukh (1986).

Forest	Contains a closed canopy in the tree stratum with a sparse herbaceous and grass layer.
Lowland forest	Approximately 0 – 1000 m above sea level, trees taller than 30 m, buttresses commonly found on trees, predominant leaf size is large, conifers rare, woody climbing plants common.
Submontane forest	Approximately 1000 – 2200 m above sea level, trees 15-35 m tall, buttresses rarely found on trees, predominant leaf size is large, conifers common, nonwoody climbing plants common.
Montane forest	Approximately 2200-4000 m above sea level, trees shorter than 13 m, buttresses on trees absent, predominant leaf size is small, conifers common, climbing plants rare.
Rainforest	More than 1000 mm rainfall per year.
Wet forest	Evergreen rainforest with no regular annual dry season, average monthly rainfall greater than potential evapotranspiration throughout the year.
Moist forest	Rainforest with several consecutive months where rainfall is greater than potential evapotranspiration throughout the year and a brief annual dry season lasting 3-5 months. Consists mostly of evergreen trees, but some large deciduous trees are present.
Dry forest	Deciduous/semi-deciduous forest with a long annual dry season that lasts more than 6 months.
Gallery forest	Strips of evergreen forest alongside rivers in dry forest or savannas.
Woodland	Contains a dominant but incomplete tree canopy, often found as a transition between forest and savanna zones.
Savanna	Broken tree or shrub canopy with an herbaceous layer dominated by grasses.
Scrub	Semi-desert with scattered grasses, often found as a transition between savanna and desert regions.
Desert	Scattered herb and shrub canopy interspersed with bare soil.

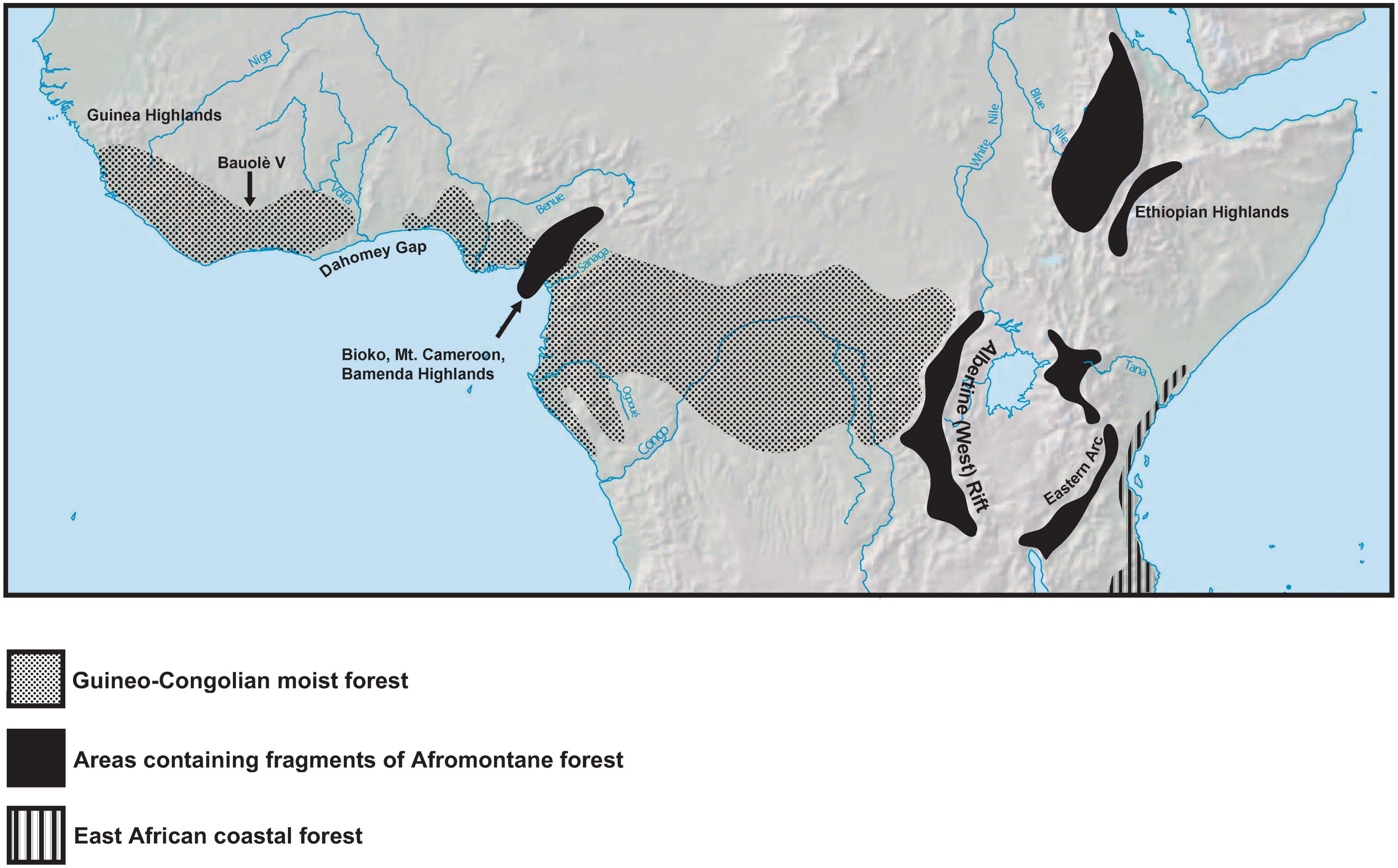

Figure 4.1: Current distribution and features of the African rainforest. Adapted from Maley (1996).

particularly important to West and Central Africa and the latter important to East Africa (Kingdon 1989; O'Brien and Peters 1999; Anhuf et al. 2006). Figure 4.1 displays features of modern African rainforest vegetation. The lowland forest belt of West and Central Africa is commonly referred to as the Guineo-Congolian moist forest block and runs from the Sierra Leone/Guinea border in the west to the Rift Valley in the east. This lowland evergreen and semi-evergreen forest merges with montane forest in the highlands of Cameroon (e.g., Mt. Cameroon, Bamenda highlands) and the Albertine Rift (e.g., Virunga, Ruwenzori ranges). The main discontinuity in the lowland forest is known as the Dahomey Gap – a 320 km wide region that today consists of savanna, coastal scrub, grassland, farmland, and patches of dry forest between the Volta River in Ghana and the Ouémé River near the western border of Nigeria (Booth 1958a; Moreau 1969; Matsuda Goodwin 2007).

The forests of East Africa are different from the Guineo-Congolian block due to a drier climate and greater topological relief. This region is characterized by montane forest in the mountains, gallery forest adjacent to rivers, wooded savannas in the lowlands, and coastal forest near the Indian Ocean (Hamilton 1988). East Africa in general is unusually dry and possesses vegetation approaching desert types at latitudes within the “humid tropics” (Livingstone 1975). Although much of the diversity and distribution of African vegetation can be attributed to long-term processes, human disturbance has had an enormous influence (Hamilton 1976; Livingstone 1982).

Climate change

Global climates oscillate between warm and cold temperatures in a cyclical manner. These are often called Milankovitch cycles and are caused by oscillations of the Earth's orbit around the sun, tilt of axis relative to the sun, and wobble around the tilt angle. Complete cycles of these fluctuations occur in approximately 100,000, 40,000, and 23,000 year intervals, respectively (Cox and Moore 2000). In addition to these cyclical fluctuations, the global climate has also seen an overall trend of cooling throughout the Cenozoic. Two sharp declines in particular have occurred that correspond to the initial appearance of polar ice caps. The first was between 15.6 – 12.5 Ma when Antarctica moved into its current position and the southern ice cap formed. The second was between 2.95 – 2 .52 Ma when continental drift prevented currents of warm water from reaching the Arctic Ocean, thus causing it to freeze (Maley 1996; Denton 1999). With the presence of two polar ice caps the ability of the earth to reflect heat increased, which caused its retention of energy to decrease and temperatures to cool (Hamilton 1988; Cox and Moore 2000).

Prior to the sharp decline in global temperature in the Late Pliocene, a 23-19 ky Milankovitch cycle was predominant. After 2.8 Ma, the 41 ky cycle is most noticeable and increases in amplitude and periodicity. At 800 ka, the dominant cycle shifts to 100 ky intervals and becomes even more pronounced (Mayr 1996; Denton 1999; deMenocal 2004). Changes in polar ice sheet volume are correlated with these cycles and have affected the climate of tropical Africa. As ice sheet expansion occurs, monsoonal winds are deprived of atmospheric moisture and tropical rainfall in Africa is reduced. Global warming and glacier retreat allow monsoonal winds to absorb water, which in turn causes an increase in rainfall. Thus, Milankovitch and glacial cycles have modulated the climate in tropical Africa, with increases in the amplitude and periodicity of these cycles affecting temperature and humidity (Hamilton 1988; Kingdon 1989; Livingstone 1993).

Refuge theory

Mid 20th century investigations into the geographic ranges of plants, birds, amphibians, reptiles, and mammals found that groups of unrelated organisms often share distributional boundaries and areas of endemism (e.g., Booth 1958a; Moreau 1963, 1969; Kingdon 1971; Laurent 1973; Hamilton 1976). Also occurring in the mid 20th century was an increase in knowledge concerning how vegetation has changed throughout the Pleistocene. Congruence between distributional patterns of biodiversity and paleoecological reconstructions led to the development of refuge theory, which explains current species distributions as products of repeated range expansions and contractions during times of cyclical climatic change (Haffer 1982).

According to refuge theory, periods of favorable climate allowed for the geographic advance of particular ecosystems. As global climates changed, these became fragmented and eventually disappeared, existing only in isolated areas where local conditions remained stable. During this time, conspecific populations found in different "refugia" could potentially differentiate from one another and develop reproductive isolating mechanisms. These in turn would prevent or reduce gene flow between these populations if contact was re-established during subsequent periods of favorable climate and ecosystem advance (Haffer 1982; Mayr and O'Hara 1986). Combining this process with others that fragment populations (e.g., rifting of continental plates, uplift of mountains, formation of rivers) produces a model for allopatric speciation, thus providing an explanation for how diversity is generated (Haffer 1982). Since these processes act on ecosystems that unite organisms into a synergistic community, they are expected to affect numerous taxa in similar ways. This could explain the congruence seen today between the centers of endemism for a wide variety of taxa. If two centers contain numerous closely related taxa, then the centers themselves can also be inferred to possess a close historical relationship. Furthermore, since refuges were focal areas for the differentiation of ancestral populations, they are expected to contain high levels of biodiversity and are thus inferred to coincide with modern centers of endemism (Laurent 1973; Hamilton 1976; Hafer 1982; Maley 1996). For African rainforest taxa these will be localized areas that currently receive a very high rainfall and where paleoecological data support the persistence of wet conditions throughout the past (Anhuf et al. 2006). Figure 4.2 displays putative refuge areas for rainforest taxa during glacial periods.

Testing the refuge theory model is difficult because there is a lack of concrete expected outcomes. Proposed evidence for this model includes a decline of species diversity in a step-like fashion as one moves away from these “refuges,” and the presence of zones of secondary contact between closely related taxa between refuges (Hafer 1982; Mayr and O’Hara 1986). However, forest breaks, expansions, and contractions likely occurred repeatedly and in a consistent manner so that multiple dispersal events were superimposed throughout time (Maley 1996). Because of this process it is likely that most precise signatures of ancient radiations have been erased (Kingdon 1971; Hamilton 1988; Hafer 1982). Smaller patches of vegetation, especially along rivers and coastlines, could have persisted and sustained certain populations or promoted movement between core areas (Hamilton 1988; Livingstone 1993), and since different organisms have their own individual histories, environmental tolerances, and dispersal capabilities, exceptions from the documented patterns are expected (Oates 1988). Also, repeated expansion and contraction of biomes played but one part in the modern distribution of biodiversity as other ecological and historical processes have had major roles as well (Mayr and O'Hara 1986). These complexities must be kept in mind when interpreting historical biogeographic data.

Biogeographic implications of the red colobus phylogenetic relationships

The phylogenetic relationships among the red colobus monkeys inferred in Chapter 3 (Figure 3.3) can provide insight into the biogeographic history of the African rainforest. For the sake of simplicity, I have organized the following discussion by areas of African rainforest endemism (Figure 4.2). Whether these areas truly represent past "refuges" is debatable, but the goal is not to explicitly test the refuge model of repeated cycles of rainforest expansion and contraction. Instead, patterns of relationships between red colobus forms are used to simply infer past connections between forested regions that are currently disjunct. This being said, the Chapter 2 divergence dates show that the red colobus radiation had started by the Mid Pliocene and continued throughout the Pleistocene. Therefore, this radiation at least coincided in part with the cooling global climates and fluctuating temperatures that occurred throughout the (Mid) Pliocene and Pleistocene, and it was likely affected by the periods of rainforest contraction and expansion important to the refuge theory model.

Upper Guinea –The Upper Guinea forest block is separated from the rest of the Guineo-Congolian forest by the Dahomey Gap. The ability of the Dahomey Gap to impede gene flow of rainforest adapted organisms is unclear as it was likely forested at some point during the Late Pleistocene (Hamilton 1976, Livingstone 1975, Livingstone 1993, Maley 1996) and seems to have caused varying levels of differentiation (Booth 1958a, b; Moreau 1969; Oates 1988). Red colobus monkeys exist immediately west of the Gap and east of the gap in the Niger Delta (they are absent in Western Nigeria). The mitochondrial lineages of these populations share a common ancestor at the base of the red colobus tree, which suggests that this area, either the Dahomey Gap or western Nigeria (or both), has been a biogeographic barrier for these animals since the beginning of the modern red colobus radiation.

Within the Upper Guinea forest block are two inferred rainforest refuges (Figure 4.2). The fauna and flora from these refuges currently meet in Côte d'Ivore but are separated in the vicinity of the Baoulé V – a modern latitudinal lowering of the rainforest that happens to coincide with the course of the Bandama River and likely reached the coast during glacial periods (Moreau 1969; Anhuf et al. 2006). Several primates, tree frogs, and birds differentiate there (including *P. badius badius* from *P. badius waldroni*), although not typically at the (biological) species level. Thus, the organisms in these refuges are thought to be closely related (Booth 1958a, b; Moreau 1969; Oates 1988). However, the results here do not fully support this notion. The *badius* and *waldroni* red colobus forms possess mitochondrial lineages that bear no particular relation to one another and share a common ancestor near or at the base of the red colobus tree. Although it is unclear if the lineage found in *waldroni* is ancestral or derived, the fact that

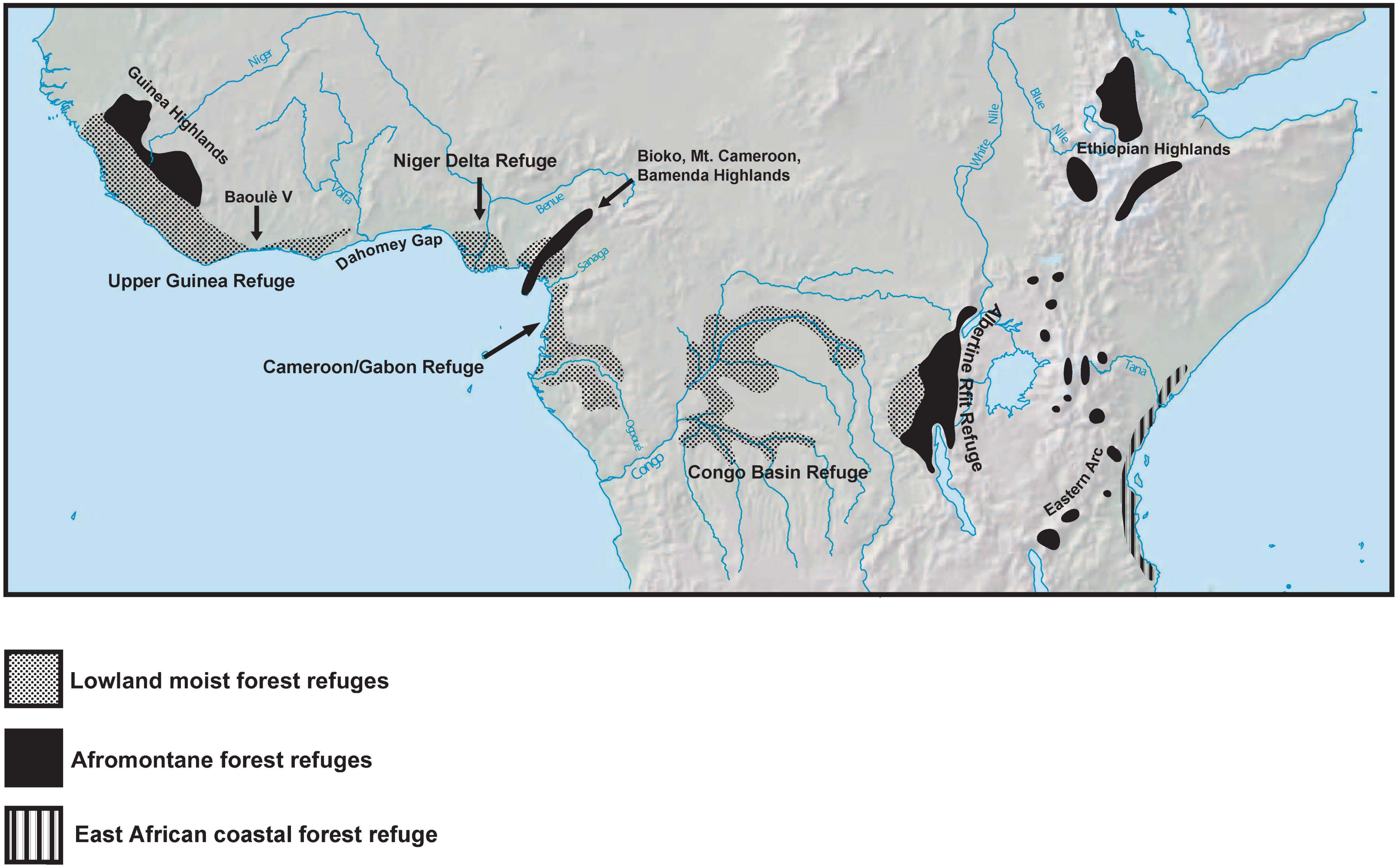

Figure 4.2: Inferred presence of past forest refuges for the last glacial maximum. Complied from Laurent (1973), Kingdon (1971), Hamilton (1976), Grubb (1982), Colyn (1988), Prigogine (1988), Maley (1996), and Anhuf et al. (2006).

it is found nowhere else in the western red colobus range suggests that either the Baoulé V or Bandama River, or both, may have been a formidable biogeographic barrier to at least some taxa.

Niger Delta – This area possesses several endemics and likely harbored a minor rainforest refuge in the past (Laurent 1973; Grubb 1982; Prigogine 1988). Isolated populations of more widely spread taxa are present as well (reviewed in Powell and Grubb 2002), with some animals representing the eastern most populations of West African taxa, and others representing the western most populations of Central African taxa. The Niger Delta red colobus (*epieni*) seems to fit in this latter category, as its closest relatives revealed by mitochondrial DNA and vocalizations are found east of the Sanaga River (Cameroon). However, this phylogenetic relationship makes it difficult to account for the geographic position of *pennantii* and *preussi* (phylogroup C) in between *epieni* and its sister taxa. One biogeographic explanation could be that past range expansions of these clades did not overlap with one another. This could have happened if an ancestral East and Central African red colobus population was connected to the Niger Delta through a more northern distribution while the ancestors of *pennantii* and *preussi* were restricted to a more southern range. A northern route could have existed above the Bamenda highlands in Cameroon, which is similar to a forested dispersal route discussed by Grubb (1982) and could have followed an ancient aquatic system that connected the Nile and Niger river fauna (Kingdon 1989). Alternatively, it is possible that *epieni* may represent a relic red colobus population from a larger polymorphic ancestral one that ranged from the Niger Delta to East Africa, and whose mitochondrial lineages have differentially sorted into the relationships for *epieni* uncovered in Chapter 3. However, if *epieni* is truly unrelated to the East and Central African forms, then the vocal similarities it shares with those taxa must be primitive retentions or convergent. Surveys of nuclear markers and biogeographic studies of the other Niger Delta fauna will aid in discerning between these two hypotheses.

Cameroon and Gabon – One of the largest centers of endemism for African rainforest taxa occurs in the Cameroon and Gabon region (Hamilton 1976; Maley 1996). However, with the exception of *pennantii* on Bioko Island and *preussi* in Cameroon (west of the Sanaga River), red colobus monkeys are largely absent from this area. It is possible that *pennantii* and *preussi* are remnants from an ancestral population that occupied this region. Red colobus monkeys may now be missing from Gabon, mainland Equatorial Guinea, and Cameroon (east of the Sanaga River) due to competition, hunting, or unsuitable habitat.

Albertine Rift and Congo Basin– These represent two major centers of biodiversity that may be composed of several areas of endemism (Colyn 1988; Colyn 1991; Colyn et al. 1991; Colyn 1993). They are considered together here because the red colobus monkeys from these regions fall in one of two clades (with some polyphyletic taxa that appear in both) that show no geographic structure (phylogroups D and E; Fig 3.3). This suggests that these two areas of biodiversity were connected at times throughout the Pleistocene. Furthermore, it appears that the rivers within the Congo Basin were not always formidable barriers to gene flow. Phylogroup D contains individuals from the taxa *tholloni, parmentieri,* and *oustaleti*. Situated in between the localities for these individuals (Salonga National Park, Mabobi, Bomahe, respectively; Figure 3.1) are three rivers inferred to be major biogeographic barriers – the Lomami, Lualaba, and

Congo Rivers (Colyn 1991). The recent diversification within phylogroup D suggests that there was past gene flow between these three areas, and the Chapter 2 divergence dates indicate that they were connected as recently as 300 ka. Contact may have occurred across these rivers during glacials when water flow was reduced, or during wet periods when rainforests possibly expanded around their headwaters. Reasons for the strong differentiation of the chimpanzee across the Lualaba and Congo Rivers (into *Pan troglodytes* and *Pan paniscus*) still need explanation.

East Africa – The red colobus mitochondrial data support Kingdon's (1971; 1981) hypothesis of two dispersal routes radiating out of the Albertine Rift to colonize the forests of East Africa. The *gordonorum* (Udzungwa Mountains) and *kirkii* (Zanzibar Island) forms followed the southern corridor that reached the coast through the Eastern Arc Mountains. Like the other endemics along this path, these taxa are particularly distinct from forms in the Congo Basin and Albertine Rift, and the Chapter 2 divergence dates support a relatively old separation from these areas (at least 1.4 Ma). Although Kingdon believed the Tana River in Kenya was colonized through this route as well, it seems that *rufomitratus* followed the other corridor through the northern highlands and mountains of Uganda, Ethiopia, and Kenya to reach the Kenyan coast (Tana River). The *rufomitratus* form is nested within phylogroup E and thus very closely related to the Congo Basin and Albertine Rift forms, which supports Kingdon's notion that the northern route remained connected to those regions for a much longer period than the southern one.

Chapter 5: Molecular diversity and classification of the red colobus monkeys

Introduction

This chapter is an assessment of red colobus monkey molecular diversity and focuses specifically on quantifying how divergent the different red colobus lineages are from one another. The results are used to help build a classification for these animals that reflects their phylogenetic diversity. The specific issues addressed in this chapter pertain to the genus level classification of the red colobus group and the diagnosis of red colobus species.

It has been a matter of debate as to whether the red colobus group should be ranked as a subgenus of *Procolobus* or as a full genus (*Piliocolobus*). Part of the problem is the subjective manner in which taxonomic categories above the species level are defined, which in turn leads to classifications that are inconsistent in the way they distribute biological variation. In order to explore this issue, part of a published colobine dataset is reanalyzed with the inclusion of the olive colobus, and maximum likelihood branch lengths are used to compare how divergent the African colobine groups are from one another compared to the Asian colobine genera.

Within the red colobus group, there is still no consensus on how many species should be recognized. Between 1 and 16 have been described, but it is unclear what taxonomic arrangement best reflects their phylogenetic diversity because no study has objectively and quantitatively assessed variation throughout the group. Another major problem is a lack of universal agreement upon which species concept should be used when recognizing species. The methods used in this study diagnose units that are most comparable to biological species. This approach is taken for the following reasons. 1) The phylogenetic species concept (PSC) places an emphasis on diagnosability, which should not be the most important attribute of a species if species are to be considered significant participants in the process of evolution. 2) Although the BSC has difficulty in diagnosing species when populations are allopatric, the PSC has just as much difficulty in diagnosing species when different forms are sympatric and interbreed. Since phylogenetic species are defined as populations with shared fixed characters, their diagnosis requires knowledge of what constitutes a population. If two populations diagnosed as different phylogenetic species interbreed extensively, can they not be considered one large population, in which case the presence of more than one species can no longer be recognized? This problem is exemplified by the hybrid swarm of red colobus monkeys in the Eastern Democratic Republic of Congo, where Groves (2007) has had difficulty in applying the PSC. 3) The PSC does not provide information concerning divergent lineages and evolutionary relationships within a group. 4) Although other species concepts based on phylogenetic systematics, such as the genealogical species concept (Donoghue 1985; Baum and Donoghue 1995), do provide information on relationships, they require analysis of multiple nuclear DNA markers. Unfortunately, the biomaterials available for red colobus monkeys preclude the collection of such data.

At the genus level, this chapter compares maximum likelihood branch lengths that separate well-defined Asian colobine genera to those between the red colobus and olive colobus to infer whether or not the red colobus is divergent enough to merit genus rank. At the species level, maximum likelihood branch lengths are compared between well-defined black-and-white colobus species to those within the red colobus clade to infer which red colobus mitochondrial lineages might be divergent enough to possess features indicative of speciation. The use of these lineages for the diagnosis of red colobus species is then assessed and combined with the pelage, morphology, and vocalization evidence to provide a classification based on an integration of various types of data.

Materials and Methods

Samples – Mitochondrial data from nearly all commonly recognized colobine genera (*Colobus, Procolobus* [*Procolobus*], *Procolobus* [*Piliocolobus*], *Presbytis, Semnopithecus, Trachypithecus, Nasalis, Pygathrix, Rhinopithecus*) and various outgroup taxa (*Cebus albifrons, Homo sapiens, Pan troglodytes, Papio hamadryas* [sensu lato], *Macaca sylvanus*) were used to assess the genus level classification of the red colobus group (Table 5.1). These data were downloaded as part of a published alignment (Sterner et al. 2006) with the exception of the olive colobus data, which were obtained from Chapter 2.

Table 5.1: Individuals sampled for genus level analysis (dataset 1).

Taxon	**Genbank Number**
Cebus albifrons	NC002763
Homo sapiens	NC001807
Pan troglodytes	NC001643
Papio hamadryas	NC001992
Macaca sylvanus	NC002764
Nasalis larvatus	DQ355298
Rhinopithecus roxellana	DQ355300
Pygathrix nemaeus	DQ355302
Presbytis melalophos	DQ355299
Trachypithecus obscurus	AY863425
Semnopithecus entellus	DQ355297
Colobus guereza	AY863427
Procolobus verus	EU580082
Procolobus badius	DQ355301

The Chapter 3 dataset was used to assess the diversity within the red colobus group. This incorporates 58 individuals and includes samples from all commonly recognized species of black-and-white-colobus (*Colobus guereza, C. polykomos, C. angolensis, C. satanas, C. vellerosus*), the olive colobus (*Procolobus* [*Procolobus*] *verus*), and nearly all recognized forms of red colobus (*Procolobus* [*Piliocolobus*]) (Table 3.1) (Grubb et al. 2003; Groves 2007).

Molecular Marker – When assessing diversity among DNA sequences, longer stretches of data are more accurate because smaller ones are more likely affected by stochastic events that are unrepresentative of the whole molecule (e.g., mutations that are randomly clumped together). In order to maximize the longest stretches of DNA available while incorporating the appropriate taxonomic samples, different datasets were used for the genus level and species level analyses. The dataset for the genus level analysis incorporates the same 3,871 base pair marker used in Chapter 2 (NADH3, NADH4L, NADH4, and NADH5 genes). Meanwhile, the species level analysis uses the shorter 897 base pair maker (part of the NADH4 gene) from Chapter 3 to maximize the number of red colobus forms included.

Phylogenetic analysis

(*Dataset1, genus level analysis*) – This dataset was downloaded from the supplementary material of Sterner et al. (2006), which is an alignment of the protein-coding regions of the mitochondrial genome in eight colobine genera and various outgroup taxa. It was then cropped to incorporate only the NADH3, NADH4L, NADH4, and NADH5 genes and aligned with the *Procolobus verus* data from Chapter 2 by eye. A maximum likelihood heuristic search was employed in PAUP 4.0b10 (Swofford 2002) to obtain likelihood branch lengths. 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 data were best fit by the general time reversible (GTR) model with invariant sites (I) and a gamma distribution (G) of site-specific rates. The branch lengths were subsequently used to calculate an average distance (substitutions/site) from each well-supported node (as inferred in Sterner et al. 2006 and Ting et al. 2008) to its terminal tips. This distance for the node uniting *Procolobus* and *Piliocolobus* was then compared to those that unite the Asian colobine sister genera in order to determine how divergent the olive colobus and red colobus are from one another relative to the other colobines.

(*Dataset2, species level analysis*) – As in the genus level analysis, a distance based approach was used in the species level analysis to compare levels of divergence between colobine taxa. Use of distance methods for establishing species boundaries has been criticized for the arbitrary way in which they define species limits, because the majority of evidence finds no universal distance value that is indicative of speciation (Johns and Avise 1998). This finding is not necessarily surprising as distance measures (e.g., pairwise distances, branch lengths) are functions of rate variation and time, both of which may only be loosely associated with the process of speciation. Other factors considered to be important in the process of speciation are life history traits (e.g., generation time), ancestral population size, and selection. Some have suggested that closely related taxa have a better chance of possessing similar amounts of intraspecific and interspecific variation among mitochondrial lineages (Baker and Bradley 2006). This is likely due to similarities in variables such as life history traits and rate variation in phylogenetically constrained groups. Thus, using a well-defined species level standard from a sister group is a way of controlling for certain confounding variables when seeking a distance value to represent species boundaries. Although it is noted that other variables may render distance standards imperfect, the long-standing problem of how to classify the red colobus monkeys requires some attempt at a consistent, objective, and quantitative method for species diagnosis, even if it has minor flaws. It must be kept in mind that all methods of species diagnosis require certain amounts of interpretation (Sites and Marshall 2004), and all classifications should be considered hypotheses to be tested.

A maximum likelihood heuristic search was used to infer a mitochondrial gene tree and obtain likelihood branch lengths. 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 data were best fit by the Tamura-Nei (TrN) model with invariant sites (I) and a gamma distribution (G) of site-specific rates. The maximum likelihood analysis was conducted with a backbone constraint tree (inferred in Chapter 2) to aid in the resolution of short internodes (methods described in Chapter 3). The search returned six most likely trees with the only differences between them being the interrelationships among three samples (*tephrosceles* KB, *langi* Z3022, *oustaleti* Z3900). The tree that collapsed these individuals into an unresolved trichotomy was chosen for analysis. Average likelihood branch lengths were calculated from each well-supported node (inferred from the Chapter 3 analysis) in the African colobine clade to its terminal tips. This average distance for the node uniting a back-and-white colobus species (*C. guereza*) with its sister taxa (*C. polykomos* and *C. vellerosus*) was used as a standard to define divergent mitochondrial lineages within the red colobus group. Black-and-white colobus monkeys were chosen for comparison because they are the most closely related polytypic group to the red colobus monkeys. The species *C. guereza* was chosen because it is distinct and derived in its craniofacial morphology, pelage patterns, and vocalizations, and its status as a species is uncontroversial as it is historically sympatric with and reproductively isolated from two other black-and-white colobus forms (*C. angolensis* and *C. satanas*) (Delson 1975; Hull 1979; Oates and Trocco 1983; Oates et al. 1994)

Results

Dataset1(genus level analysis) – The mitochondrial tree inferred from the genus level analysis has reciprocal African and Asian colobine clades (Figure 5.1). Within the African clade, the olive colobus (*Procolobus* [*Procolobus*]) and red colobus (*Procolobus* [*Piliocolobus*]) are sisters to the exclusion of the black-and-white colobus (*Colobus*). In the Asian clade, there is a monophyletic odd-nosed group with a sister relationship between *Pygathrix* and *Rhinopithecus* to the exclusion of *Nasalis*. There is also a *Presbytis* and *Semnopithecus* group, while *Trachypithecus* occupies a position that makes this genus a sister to all other Asian colobines. However, neither the relationship between *Pygathrix* and *Rhinopithecus* nor those among *Presbytis*, *Semnopithecus*, and *Trachypithecus* are well-supported by analyses of the protein-coding regions of the mitochondrial genome and of an X-chromosomal intergenic region (Sterner et al. 2006;

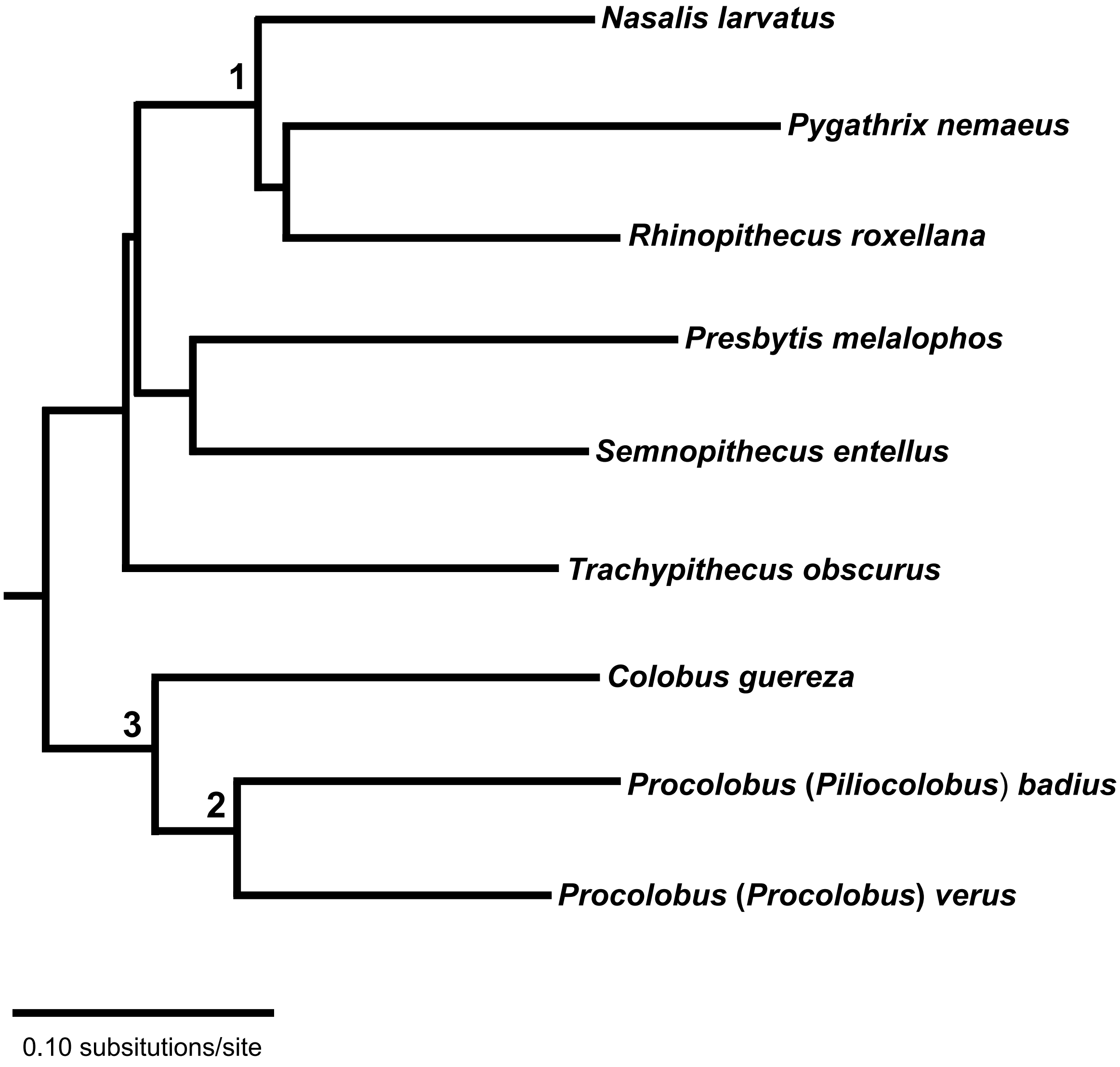

Figure 5.1: Colobine mitochondrial tree inferred from a maximum likelihood heuristic search using the NADH3, NADH4L, NADH4, and NADH5 genes. Branch lengths shown to proportion. Outgroup taxa not shown.The only well-supported relationship (based on Sterner et al. 2006, Ting et al. 2008) within the Asian colobine clade is that which unites the odd-nosed taxa (node number 1). The average likelihood branch length from this node to its terminal tips was compared to that which unites the olive colobus and red colobus (node number 2) to provide an estimate of how divergent the olive colobus and red colobus are from one another relative to other colobine genera. Node 1 = 0.1519; Node 2 = 0.1296; Node 3 = 0.1580. See Table 4.1 for more information concerning the distance measures, and see the text for details.

Ting et al. 2008). This leaves only one well-supported node within the Asian colobine clade – that which unites the odd-nosed colobines (*Nasalis, Pygathrix, Rhinopithecus*) into a monophyletic group. This node is a particularly good comparison for the olive colobus and red colobus because there is universal agreement that the variation within the odd-nosed colobines exceeds that which can be grouped into one genus (Groves 1970; Delson 1975; Brandon-Jones 1984; Brandon-Jones et al. 2004). Average likelihood branch lengths (substitutions/site) from these nodes to their terminal tips (Table 5.2) are as follows: node (1) uniting odd-nosed group - 0.1519; node (2) uniting the olive colobus and red colobus - 0.1296; node (3) uniting *Colobus* and *Procolobus* - 0.1580.

The average distance from the node uniting the odd-nosed colobines (node 1) to its terminal tips was equivalent to the average distance separating *Colobus* from *Procolobus* (node 3), which supports the presence of more than one genus among the African colobines (Figure 5.1, Table 5.2). The distance from node 1 was also on average greater than the distance separating the olive colobus and red colobus (node 2), but their ranges showed considerable overlap. Primarily driving the high average distance between the odd-nosed colobines was the *Pygathrix* distance (0.1932), and driving the low average distance between the olive colobus and red colobus was the distance of the olive colobus (0.1125). Meanwhile, the distance of the red colobus from node 2 (0.1467) exceeded the *Nasalis* and *Rhinopithecus* distances to node 1 (0.1259 and 0.1365, respectively). This analysis therefore provides little additional evidence to help resolve the most appropriate genus level arrangement for the red colobus group.

These results may be related to small samples sizes ($n < 3$) or the stochastic nature of rate variation (and thus branch lengths) when analyzing relatively small fragments of

DNA. More data need to be collected to boost sample sizes and incorporate longer stretches of DNA, including DNA from the nuclear genome. In the absence of further evidence it is best to defer to the morphological hypothesis of *Piliocolobus* ranked as a subgenus of *Procolobus* (Kuhn 1967).

Table 5.2: Average likelihood branch lengths from nodes labeled in Figure 5.1 to their terminal tips. Notice the average distance between the red and olive colobus (node 2) is lower than that between the odd-nosed colobines (node 1), but their ranges show considerable overlap. See text for details.

Node	Average	Std Dev	Range
1	0.1519	0.036	0.1259 – 0.1932
2	0.1296	0.024	0.1125 – 0.1467
3	0.1580	0.019	0.1462 – 0.1804

Table 5.3: Average likelihood branch lengths from the nodes labeled in Figure 5.2 to their terminal tips. The distance from Node 1 (separating well-defined black-and-white colobus species) was used as a standard to delimit divergent lineages within the red colobus group. Nodes in bold text represent ones in the red colobus clade that show distances greater than or equal to Node 1. See text for details.

Node	Average	Std Dev	Range
1	0.0337	0.0038	0.0278 – 0.0376
2	0.0510	0.0070	0.0369 – 0.0587
3	0.0773	0.0065	0.0640 – 0.0847
4	0.0153	0.0027	0.0116 – 0.0183
5	0.0050	0.0039	0.0011 – 0.0091
6	0.0076	0.0008	0.0066 – 0.0088
7	0.0212	0.0044	0.0158 – 0.0285
8	0.0121	0.0055	0.0078 – 0.0198
9	**0.0307**	**0.0056**	**0.0227 – 0.0406**
10	**0.0697**	**0.0075**	**0.0578 – 0.0837**

Dataset2 (species level analysis) - The African colobine mitochondrial tree inferred from the maximum likelihood heuristic search is displayed in Figure 5.2. The node that unites *Colobus guereza* with its sister-species (node 1) has an average distance of 0.0337 substitutions/site from its terminal tips (Table 5.3, Figure 5.2). Divergent red colobus mitochondrial lineages are defined here as those that are united at nodes whose average distance to the tips of their clades are equal to or greater than the average distance that separates black-and-white colobus species (node 1: 0.0337).

The node at the base of the red colobus tree (node 10) showed an average distance from its tips that was clearly greater than that for node 1 (0.0697 vs. 0.0337). If only well-resolved nodes are taken into consideration, node 10 represents a basal red colobus polytomy with five branches (see chapter 3, figure 3.3), which can each be considered divergent lineages according to the above definition. In addition, the average distance from Node 9 to its tips (0.0307) approaches the node 1 distance, and a two-tailed student's T-test shows that these values are not significantly different from one another (p=.25). The three branches splitting from node 9 are therefore also considered to be red colobus mitochondrial lineages that are as divergent from one another as black-and-white colobus species. There are thus seven divergent mitochondrial lineages within the red colobus clade, labeled A-G (Figure 5.2).

Although phylogroups A-G can be considered as divergent from one another as the black-and-white colobus species are from one another, it is clear that these seven lineages cannot be simply considered different species, particularly because certain individuals of the same taxon appear in both phylogroups D and E (polyphyly). These patterns are not inferred to be representative of the population history of these animals,

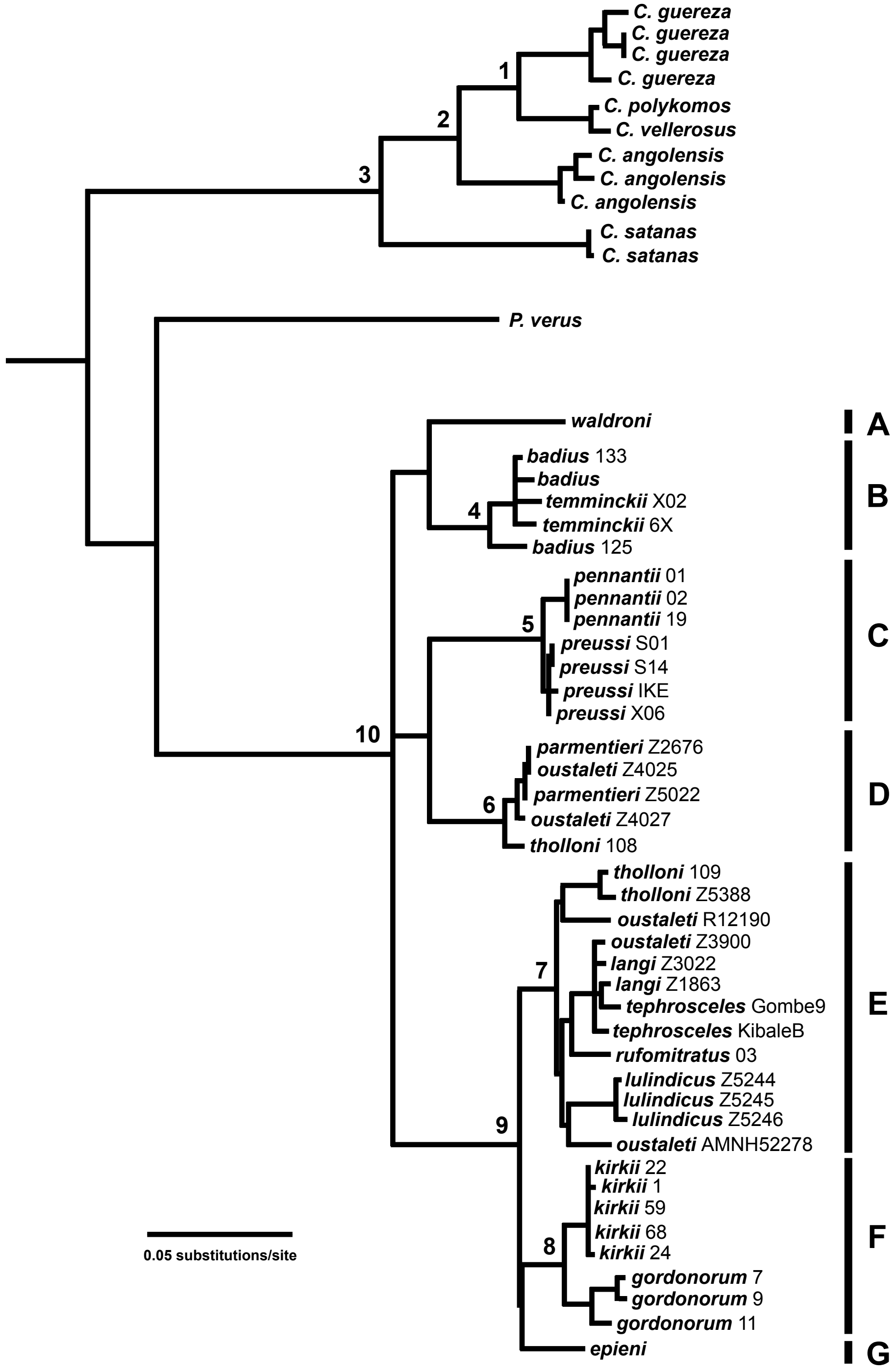

Figure 5.2: African colobine mitochondrial tree inferred from a maximum likelihood heuristic search using the last 897 base pairs of the NADH4 gene. Outgroup taxa not shown. Branch lengths shown to proportion. Select well-supported nodes (inferred from bootstrap and Bayesian analysis, see Figure 3.3) within the black-and-white colobus clade and the red colobus clade are numbered. Average likelihood branch lengths were calculated from these nodes to the tips of the tree. Distances within the red colobus group were compared to those within the black-and-white colobus group to identify divergent lineages among the red colobus monkeys (Phylogroups A-G). See Table 4.3 for more information concerning the distance measures. See text for details.

and they are likely due to incomplete lineage sorting or past hybridization among the Central African forms (phylogroups D and E; as discussed Chapter 3). Since the position of phylogroup E is supported by pelage and vocalization data (Struhsaker 1981), the position of phylogroup D likely represents a mitochondrial artifact, and phylogroups D and E should not be diagnosed into two different species. Instead, they can be classified as one species – *P. rufomitratus* Peters, 1879*,* which is the first name given to any member of the group.

Two other lineages that should probably not be separated into different species given the available evidence are phylogroups A (*waldroni*) and B (*badius* and *temminckii*). These three taxa are inferred to be very closely related based on pelage and biogeography, and separating *waldroni* based on the results here would be premature since only one individual was used in the analysis. However, this taxon can justifiably be elevated to species rank if further sampling finds that the mitochondrial lineage from phylogroup A is derived and unique to *waldroni*, which would support Dandelot's (1971) proposal of species status for this taxon. The earliest described species name for the taxa in phylogroups A and B is *P. badius* Kerr, 1792.

The remaining phylogroups (C, F, G) each need to be recognized as different species. After poorly supported branches are collapsed (see chapter 3, Figure 3.3), they all diverge from either nodes 9 or 10. Furthermore, a relatively long and well-supported branch unites each of them, and they all form tightly knit monophyletic groups. Lastly, because the relationships among phylogroups C, F, and G are relatively unresolved, placing them into the same species as either phylogroups A and B or phylogroups D and E might create paraphyletic species. Phylogroup C would be classified as *P. pennantii*, F would be *P. kirkii*, and E would be *P. epieni*. This brings the number of red colobus species recognized here to five (*P. rufomitratus, P. badius, P. pennantii, P. kirkii, P. epieni*).

The relationships revealed among the black-and-white colobus species and the inferred red colobus species suggest the presence of hierarchical patterns of species divergence. This leaves room for one to identify species groups (i.e., groups of closely related species) within both the black-and-white colobus and red colobus. For example, among the red colobus monkeys, phylogroups E, F and G form a tight-knit cluster united by a branch length comparable to that of phylogroups B and C. This suggests that the species *P. rufomitratus* (sensu this study), *P. kirkii*, and *P. epieni* are much more closely related to one another than they are to any other species. In the same vein, the *Colobus* species at node 1 also form a tight cluster with a hierarchical pattern out to nodes 2 and 3. One could argue that this hierarchical pattern in both genera might reflect real phylogenetic history that should be recognized in a classification or merely variation in the rate and pattern of species diversification. In the former case, one could, for example, refer to *P. rufomitratus, P. kirkii*, and *P. epieni* as the *P. kirkii* species group (based on the nomenclatural seniority of *P. kirkii*). This is a question beyond the scope of this project but one worth revisiting at a later time.

Discussion

<u>Genus level classification of the red colobus group</u> – The three African colobine groups have been classified in a variety of ways at the genus level. They have all been placed in the genus *Colobus* (Groves 1970), or as different subgenera in *Colobus* (Delson 1975).

The olive colobus and red colobus have been separated from the black-and-white and placed in the genus *Procolobus*, with each retaining subgenus status (*Procolobus* and *Piliocolobus*, respectively; Kuhn 1967). The three groups have also each been recognized at the genus level (Groves 2001). The results of this study support the presence of at least two genera among the African colobines – *Colobus* and *Procolobus*. However, it is remains unclear whether *Piliocolobus* should be separated from *Procolobus* as a distinct genus. Since the olive colobus and red colobus do not seem to differ from one another morphologically at the genus level, and the molecular evidence shows that they form a closely knit group united by a relatively long branch length (Figure 4.2), it is best to consider them as a single genus with two subgenera. The only strong argument that can currently be made for raising *Piliocolobus* to genus rank is the use of a 6-4 Ma divergence time standard for genera (Goodman et al. 1998; Groves 2001), in which case *Piliocolobus* would be considered a genus based on its divergence from *Procolobus* at least 6 Ma (see Chapter 2). However, the use of a universal time standard for the delineation of taxonomic ranks remains controversial, and the choice of time standards remains entirely subjective.

<u>*Species level classification of the red colobus group*</u> –One of the aims of this study was to compare the phylogenetic diversity within the red colobus group to that of the closely related black-and-white colobus group, where species are much better defined, in order to gain insight into how red colobus monkeys should be classified at the species level. Doing so produced a classification that recognizes five red colobus species (*P. rufomitratus, P. badius, P. pennantii, P. kirkii, P. epieni*). Although the diagnosis of these

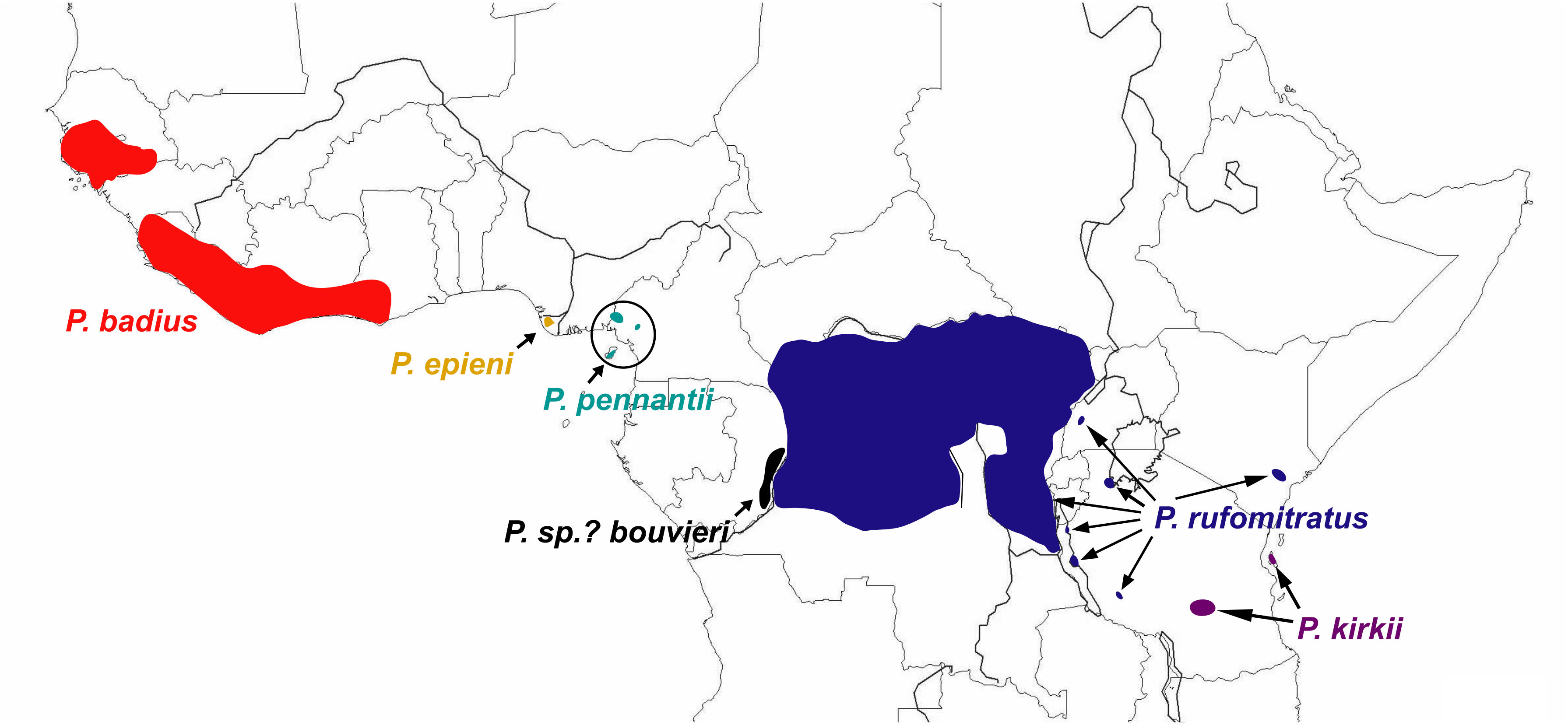

Figure 5.3: Distribution of inferred red colobus species across Africa. Adapted from Oates et al. (1994) and Colyn (1993). Distributions are circled and/or colored according to the proposed species level classification (see Table 5.4 and text for details). The relationships and classification of Bouvier's red colobus (*Procolobus sp.? bouvieri*) were not assessed here.

Table 5.4: Taxonomic arrangements for the red colobus monkeys. Red colobus forms are listed on the far left followed by each author's species level diagnosis. Total number of species for each classification at bottom.

Taxon	**Napier 1985**	**Groves 2001**	**Groves 2007**	**Grubb et al. in press**	**This study**
temminckii	***badius***	***badius***	***badius***	***badius***	***badius***
badius	***badius***	***badius***	***badius***	***badius***	***badius***
waldroni	***badius***	***badius***	***waldroni***	***badius***	***badius***
epieni	**undescribed**	***pennantii***	***epieni***	***pennantii***	***epieni***
preussi	***badius***	***preussi***	***preussi***	***pennantii***	***pennantii***
pennantii	***badius***	***pennantii***	***pennantii***	***pennantii***	***pennantii***
bouvieri	***badius***	***pennantii***	***bouvieri***	***pennantii***	**?**
tholloni	***badius***	***tholloni***	***tholloni***	***rufomitratus***	***rufomitratus***
rufomitratus	***badius***	***rufomitratus***	***rufomitratus***	***rufomitratus***	***rufomitratus***
tephrosceles	***badius***	***tephrosceles***	***tephrosceles***	***rufomitratus***	***rufomitratus***
oustaleti	***badius***	***foai***	***oustaleti***	***rufomitratus***	***rufomitratus***
foai	***badius***	***foai***	***foai***	***rufomitratus***	***rufomitratus***
lulindicus	**syn *foai***	**syn *foai***	**syn *foai***	***rufomitratus***	***rufomitratus***
***ellioti* (*semlikiensis*)**	***badius***	***foai***	***semlikiensis***	***rufomitratus***	***rufomitratus***
langi	**syn. *ellioti***	**syn. *ellioti***	***langi***	***rufomitratus***	***rufomitratus***
parmentieri	**undescribed**	***foai***	***parmentieri***	***rufomitratus***	***rufomitratus***
gordonorum	***badius***	***gordonorum***	***gordonorum***	***gordonorum***	***kirkii***
kirkii	***kirkii***	***kirkii***	***kirkii***	***kirkii***	***kirkii***
Total species #	**2**	**9**	**16**	**5**	**5**

species does not exactly conform to commonly used criteria for species recognition in sexually reproducing organisms (e.g., reproductive isolation, mate recognition, shared adaptive zone, shared fixed characters, monophyly of multiple genetic markers, etc.), these species have been separated for a long enough period of time for reproductive isolating mechanisms to arise between populations in a closely related group (black-and-white colobus). This is not to say that these inferred red colobus species are reproductively isolated from one another, but they are diagnosed in a quantitative and objective manner and provide a species level classification that reflects phylogenetic diversity within this group.

Figure 5.3 shows the distribution of the inferred red colobus monkey species, while Table 5.4 shows how this taxonomic arrangement compares to recently proposed classifications. The traditional one species classification (Schwartz 1928; Oates et al. 1994) and Groves's (2007) 16 species arrangement are at opposite extremes of the lumping and splitting continuum. They both recognize nearly all forms at the same rank with the former listing all red colobus taxa as subspecies, and the latter raising nearly all of them to species rank. While they are not necessarily phylogenetically inconsistent with the results here, neither provides more information than the other as they both fail to reflect phylogenetic diversity and identify distinct lineages in the middle of the "lumping" and "splitting" continuum. Napier's (1985) recognition of *kirkii* as the most distinct of the red colobus forms is not consistent with the data here, nor is Groves's (2001) nine species arrangement. In fact, most of the taxa Groves (2001) recognized at the species level (e.g., *tephrosceles, preussi, gordonorum, kirkii,* and *tholloni*) do not belong to distinct lineages of their own and are very closely related to other populations.

Grubb et al. (2003) provide a result that is in some ways similar to the classification produced here, although they failed to assign species names to the forms in the Congo Basin. Grubb et al. (2003) also tentatively rank *epieni* as a subspecies of *P. pennantii,* which is not consistent with the phylogenetic evidence, and they separate *gordonorum* and *kirkii* at the species level. Although *kirkii* has long been considered the most distinct of the red colobus taxa, the major craniometric study identifying *kirkii* as different (Verheyen 1962) contained a limited sample of red colobus forms and did not include *gordonorum*. Also, although the two occupy different ecological niches, with *gordonorum* reaching submontane elevations and *kirkii* restricted to lowland habitats, it has been shown that *gordonorum* is actually more abundant at lower elevations (Marshall et al. 2005). At least one classification (Grubb 1991, updated in Grubb et al. 2003) recognized the close relationship between these two taxa by ranking them both in *P. kirkii*, which is followed here. Lastly, since *bouvieri* was not sampled in this study its phylogenetic position and taxonomic placement among the other red colobus forms cannot be determined. It is thus tentatively placed under *P. pennantii* following Groves (2001) and Grubb et al. (2003). Table 5.5 shows the classification of red colobus monkeys resulting from this study.

Table 5.5: Classification of red colobus monkeys resulting from this study.

***Procolobus* de Rochebrune, 1887**	Olive colobus, red colobus
Subgenus *Piliocolobus* de Rochebrune, 1887	Red colobus
Procolobus badius (Kerr, 1792)	Western red colobus
P. b. badius (Kerr, 1792)	Upper Guinea red colobus
P. b. temminckii (Kuhl, 1820)	Temminck's red colobus
P. b. waldroni (Hayman, 1936)	Miss Waldron's red colobus
Procolobus pennantii (Waterhouse, 1838)	Pennant's red colobus
P. p. pennantii (Waterhouse, 1838)	Bioko red colobus
P. p. bouvieri (Rochebrune, 1887)	Bouvier's red colobus
P. p. preussi (Matschie, 1900)	Preuss's red colobus
Procolobus kirkii (Gray, 1868)	Kirk's red colobus
P. k. kirkii (Gray, 1868)	Zanzibar red colobus
P. k. gordonorum (Matschie, 1900)	Udzungwa red colobus
Procolobus rufomitratus (Peters, 1879)	Rufous crowned red colobus
P. r. rufomitratus (Peters, 1879)	Tana River red colobus
P. r. tholloni (Milne-Edwards, 1886)	Tshuapa red colobus
P. r. oustaleti (Trouessart, 1906)	Oustalet's red colobus
[synonyms: *P. r. brunneus* (Lönnberg, 1919); *P. r. powelli* (Matschie, 1913); *P. r. nigrimanus* (Trouessart, 1906); *P. r. schubotzi* (Matschie, 1914)]	
P. r. parmentieri (Colyn and Verheyen, 1987a,b)	Lomami River red colobus
P. r. tephrosceles (Elliot, 1907)	Ashy red colobus
[synonym: *P. r. gudoviusi* (Matschie, 1914)]	
P. r. foai (de Pousargues, 1899)	Kivu red colobus
[synonym: *P. r. graueri* (Dollman, 1909)]	
P. r. ellioti (Dollman, 1909)	Semliki red colobus
[synonym: *P. r. semlikiensis* (Colyn, 1991)]	
P. r. lulindicus (Matschie, 1914)	Lulindi red colobus
P. r. langi (Allen, 1925)	Kisangani red colobus
Procolobus epieni (Grubb and Powell, 1999)	Niger Delta red colobus

Chapter 6: Systematics and the designation of red colobus monkey conservation priorities

Introduction

Red colobus monkeys currently rank among the most endangered primates in Africa, but their unresolved relationships and unstable species level classification have hampered efforts to protect them (Oates 1996a; Grubb et al. 2003; Struhsaker 2005). This chapter focuses on the importance of systematics to conservation planning. It provides an overview of the threats to the various red colobus populations and discusses the implications of applying the classification produced in Chapter 5 to the designation of red colobus monkey conservation priorities.

Systematics, conservation planning, and red colobus monkeys

Conservationists must prioritize their efforts in such a way as to minimize the loss of biodiversity while maximizing the use of limited conservation resources. Two of the major approaches to identifying conservation priorities are species-based methods and area-based methods.

<u>Species-based methods</u> – Conservation has traditionally adopted a species-based approach, which aims to protect taxonomically distinct populations at risk of extinction. Today, this strategy relies heavily on the World Conservation Union's (IUCN) Red List of threatened species to establish which populations are judged to be most at risk of extinction. The Red List assesses the conservation status of the world's plants and animals by assigning species (and subspecies) to one of several risk categories using

criteria based on geographic distribution, population size and trends, habitat and ecology, and threats (Rodrigues et al. 2006). It thus attempts to provide an objective and transparent assessment of species extinction risk (although see Oates 2006). Governments, NGOs, and scientific institutions often use the Red List to prioritize conservation activities so that limited resources are not wasted and are most appropriately used to accomplish conservation objectives. There is often a direct connection between the Red List and international and national legislation (e.g., Convention on International Trade in Endangered Species, US Endangered Species Act), and more funding tends to be directed towards species in higher threat categories (Possingham et al. 2002). Although the Red List provides an initial guide to species-based conservation, it should not simply be used as a ranked list of priorities since factors such as feasibility and cost of implementing effective protection strategies also need be taken into account (Oates 1996a; Possingham et al. 2002; Rodrigues et al. 2006).

Area-based methods – One common area-based approach towards identifying conservation priorities focuses on geographic areas that contain high levels of species endemism and/or richness. This method relies on biogeographic analyses and species lists to identify what taxa are present in a given region. In general, an area that contains a high number of endemic species is judged to warrant high conservation priority and has a greater chance of being designated for protection. However, levels of species endemism and richness can change depending on what species classification is used. This has been well demonstrated in the birds of both Mexico (Peterson and Navarro-Siguenza 1999) and the Philippines (Peterson 2006), where a simple change in species concept can alter priority areas for conservation.

Systematics and conservation – Both species-based and area-based methods use species as a fundamental unit in identifying conservation priorities, and for this reason several authors have called for the disciplines of conservation and systematics to work more closely together (e.g., Oates 1994; Dubois 2003; Agapow et al. 2004). Effective conservation strategies cannot be established without well-resolved classifications and species definitions that reflect biodiversity (or the underlying genetic variation responsible for biodiversity). Without such systematic information, prioritizing distinct lineages for protection becomes impossible and the assignment of protected areas based on the presence of species diversity can be compromised by mistaken assessments of species richness and endemism (Rojas 1992; Agapow et al. 2004).

If the goal of conservation is to preserve patterns of biodiversity, greater priority needs to go towards divergent lineages if they are at high risk of becoming extinct. Otherwise, extinctions may more likely lead to losses in evolutionary history. Prioritization of divergent lineages can be accomplished through a species definition that reflects these lineages, so that protecting species when doing conservation would effectively protect divergent lineages. However, some commonly used species definitions fail to accomplish this. The phylogenetic species concept (PSC) in particular is commonly used for the designation of conservation priorities but its failure to identify divergent lineages can potentially divert limited funds and resources towards species that are not as distinct as others. Furthermore, employment of the PSC is the likely cause of recent increases in numbers of recognized species, and thus is probably significantly contributing to apparent increases in numbers of threatened species that are recognized (Isaac et al. 2004). The constant appearance of new threatened "species" may eventually result in a loss of scientific credibility among conservation biologists and ultimately an apathetic public, whose support is one of the primary generators of conservation funds (Agapow et al. 2004). Although currently in fashion, the use of the PSC might create a backlash in conservation, with the extinction of distinct lineages as an ultimate consequence. For these reasons, a species definition that identifies divergent lineages is more useful for the designation of conservation priorities than a species definition such as the PSC.

Red colobus monkey conservation priorities

Threats to red colobus monkeys – Red colobus monkeys rank among the most threatened primates in Africa (Oates 1996a, Struhsaker 2005). As with most primates, the two greatest threats to the survival of red colobus monkeys are habitat alteration by humans and human hunting. Because both of these factors are anthropogenic, their significance varies according to human population density and cultural practices. Hunting poses a more serious problem in the moist forest zones of Central and West Africa than in East Africa, where humans do not commonly consume primate meat. In parts of East Africa and West Africa, high human population density has led to major habitat loss or fragmentation (Oates 1996b). Although complete habitat loss is the most serious threat to the long-term survival of these animals, the effects of human hunting can be devastating to red colobus monkey populations. Compared to other African primates, red colobus monkeys are particularly susceptible to hunting because they are relatively large-bodied, slow moving, congregate in large groups, have poor behavioral strategies to avoid human hunters, and tend to be very vocal (Struhsaker 1975; Oates 1996b; Struhsaker 2005).

Table 6.1: Major threats to the various red colobus forms, summarized from Struhsaker (2005) and references therein. Assessment of extinction risk is from the 2007 IUCN Red List. Red colobus forms *oustaleti, tholloni, parmentieri, ellioti, foai, lulindicus,* and *langi* are not shown because they were assessed as either Data Deficient or Not Evaluated. CR – Critically Endangered. EN – Endangered.

Taxon	**Assessment**	**Primary Threats**
temminckii	**EN**	Habitat loss, increasingly fragmented and dry ecosystem.
badius	**EN**	Hunting, habitat loss, exists in an area of civil unrest. The few remaining secure populations require continued protection.
waldroni	**CR**	Hunting, habitat loss. Largely eliminated across its entire range in Eastern Côte d'Ivoire and Ghana. Has not been seen by scientists since 1978. Surveys between 1993 and 1997 found no evidence of surviving individuals. Small populations may exist but likely not viable. Possibly extinct.
epieni	**EN**	Hunting, habitat loss, exists in an area of civil unrest, does not exist in any protected area, extremely restricted distribution.
preussi	**EN**	Hunting, restricted distribution. Primarily restricted to Korup National Park and adjacent area (SW Cameroon). Populations also exist in Yabassi District, just west of the Sanaga River.
pennantii	**EN**	Hunting, habitat loss, extremely restricted distribution (limited to the southern one third of Bioko Island).
bouvieri	**CR**	Hunting, restricted distribution, no scientifically documented records in several decades and a poorly known taxon. Possibly extinct.
tephrosceles	**EN**	Habitat loss, restricted distribution, fragmented into several small and isolated populations along the Albertine rift region in Uganda and Tanzania. Has experienced unexplained population declines since the 1970's.
rufomitratus	**CR**	Habitat loss, alterations to the flow of the Tana River due to up river dams. Restricted distribution, small population size ($\leq$ 800). Less than half of the population exists inside the Tana River Primate National Reserve, and the habitat outside the reserve is crucial to the survival of this animal.
gordonorum	**EN**	Habitat loss, fragmentation, restricted distribution. They are reasonably secure in Udzungwa Mountain National Park, but only half of the population occurs there. Population estimates range from 10,000-15,000 individuals but are unreliable. The largest interbreeding population is less than 2,000 individuals.
kirkii	**EN**	Habitat loss, fragmentation, population compression, restricted distribution, small population size ($\leq$ 2000). Less than half the population resides in small Jozani-Chwaka Bay National Park, where the animals are reasonably secure.

Also, red colobus monkeys do not survive for long in captivity, so breeding programs do not exist. Table 6.1 summarizes the threats to the different red colobus forms. Struhsaker (2005) provides a detailed description of threats to most of the populations.

Past and current problems in designation of conservation priorities – Red colobus monkeys exemplify how an unresolved species level classification can hamper both species-based and area-based conservation efforts. Until recently, despite the precarious status of many of the red colobus forms (see Table 6.1) and the widespread agreement that the variation in this group exceeds that which is typically seen in one primate species, none of the individual forms were listed as endangered species because they were all conservatively grouped into *Procolobus badius* (sensu lato); thus, red colobus monkeys were considered a Lower Risk species (Baillie and Groombridge 1996). This assessment assigned low conservation priority to the red colobus monkeys as a group and may have undermined some efforts to protect red colobus populations, some of which are particularly distinct, may be considered to represent full species, and are at high risk of extinction, thus meriting a high conservation priority. Furthermore, recognizing a single species of red colobus monkey makes this a widely distributed species, and geographic areas that contain endemic and distinct red colobus forms may not be recognized as important areas for protection.

The most recent Red List (IUCN 2007) follows Grubb et al. (2003) in recognizing five species of red colobus. However, this is problematic because Grubb et al. did not assign a species name to numerous forms in Central Africa, and the Red List has difficulties in dealing with unnamed taxa. It therefore lumped these forms into *P.*

badius, which does not reflect phylogenetic relationships within the red colobus group, confuses efforts to prioritize distinct red colobus lineages, and could have important implications for how resources are allocated. A classification used by the IUCN that accurately reflects the evolutionary history and genetic variation of the red colobus monkeys is thus imperative to the designation of rational conservation priorities for both this group and the tropical forests of Africa that these monkeys inhabit.

Applying a new classification – Among the numerous red colobus classifications that have been proposed, I believe the arrangement I laid out in Chapter 5 best reflects the molecular variation and phylogenetic diversity within this group. Applied to conservation, it has advantages over the one species arrangement (Oates et al. 1994), which underestimates the diversity of the group and assigns low conservation priority to some forms that may deserve high conservation priority based on taxonomic distinctiveness and risk of extinction. The Chapter 5 arrangement also has advantages over the classification into 16 phylogenetic species (Groves 2007). If a loss in evolutionary history is to be prevented, greater priority needs to be assigned towards more divergent lineages. The PSC fails to identify these lineages and therefore might divert limited conservation funds away from distinct taxa in desperate need of protection. Although numerous classifications have attempted to diagnose more than one red colobus species (Napier 1985; Kingdon 1997; Groves 2001), the one supported in this dissertation is most useful because it reflects evolutionary history and was produced in an objective manner using quantitative criteria applied equally across the whole group. Employment of this classification in the IUCN Red List would assign conservation priorities in a way that would minimize the loss of evolutionary history if priorities were acted upon.

Because the species in this classification were diagnosed based on phylogenetic distinctiveness, all recognized species represent divergent lineages, and attention would be directed to those with restricted and/or fragmented distributions and projected population declines.

Chapter 5 identified seven red colobus mitochondrial lineages that are as divergent from one another as those found between well-agreed black-and-white colobus species. Two of these lineages were inferred as possible mitochondrial artifacts, however, which means the seven lineages can sensibly be grouped into five different species (*P. badius, P. pennantii, P. epieni, P. rufomitratus, P. kirkii*). This classification highlights the red colobus diversity present in western Africa (Figure 6.1D). While previous classifications identify East and Central Africa as having numerous distinct forms (e.g., Groves 2001, 2007), this arrangement suggests that the red colobus monkeys in western Africa are equally, if not more, speciose. This also demonstrates how a change in classification and species concept can alter priority areas for conservation. Application of the PSC (Figure 6.1 C) would assign high conservation priority to Central Africa, as the Congo Basin contains the highest levels of red colobus monkey species endemism. However, focusing conservation efforts in this region would favor the protection of numerous closely-related taxa and lead to the potential neglect of several highly divergent red colobus forms in East and West Africa. This is a direct outcome of the PSC's failure to identify distinct lineages.

The following summarizes how adoption of the Chapter 5 classification alters the conservation priorities for red colobus monkeys. Following each species name in

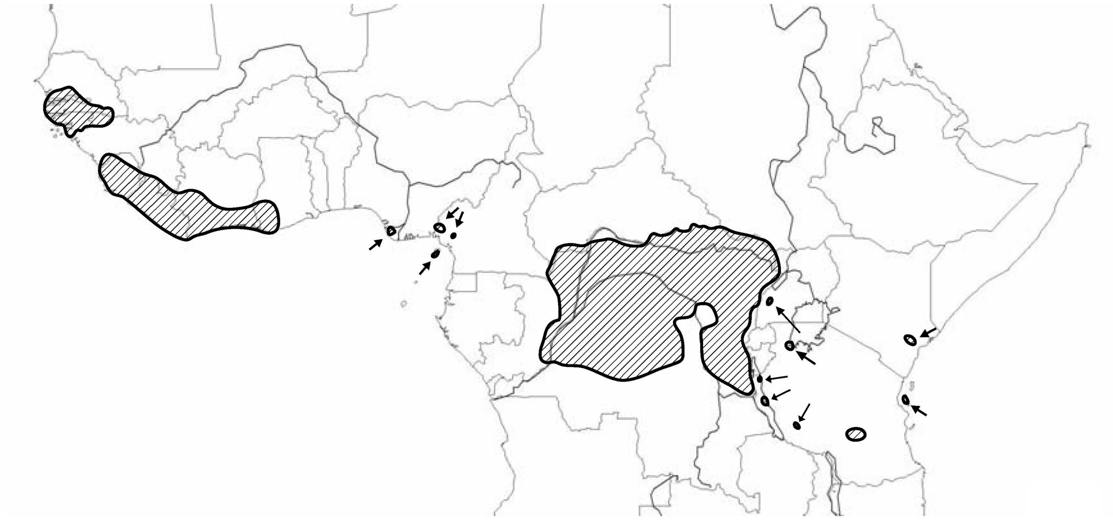

Figure 6.1 A: One species arrangement of the red colobus monkeys (*Procolobus* [*Piliocolobus*] *badius*) following Oates et al. (1994). Notice the lack of red colobus species endemism in any particular area of tropical Africa due to the widespread distribution of the species.

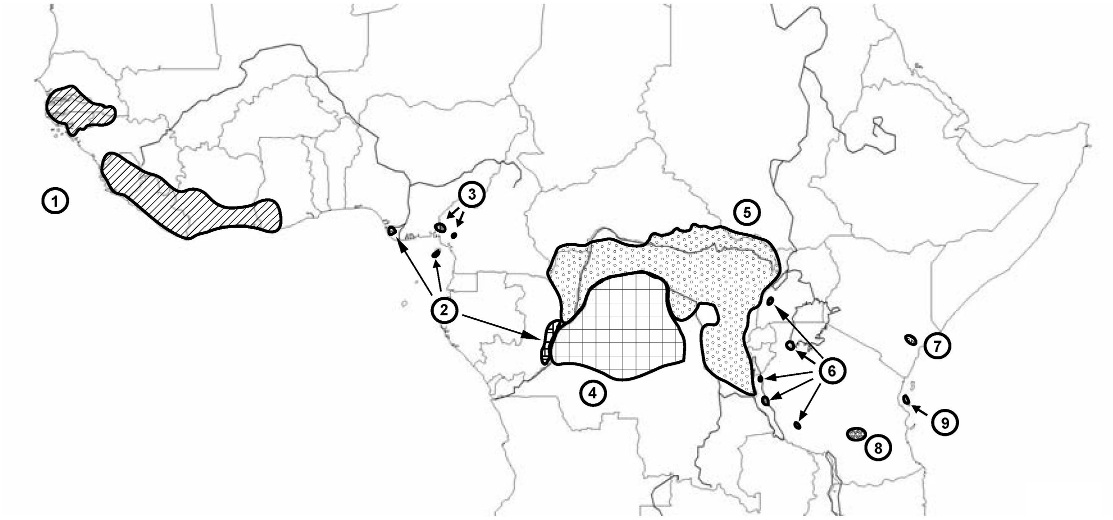

Figure 6.1 B: Nine species arrangement of the red colobus monkeys (*Procolobus* [*Piliocolobus*]) following Groves (2001). 1 - *P. badius*. 2 - *P. pennantii*. 3 - *P. preussi*. 4 - *P. tholloni*. 5 - *P. foai*. 6 - *P. tephrosceles*. 7 - *P. rufomitratus*. 8 - *P. kirkii*. 9 - *P. gordonorum*. Notice how this classification recognizes more species in East and Central Africa than western Africa.

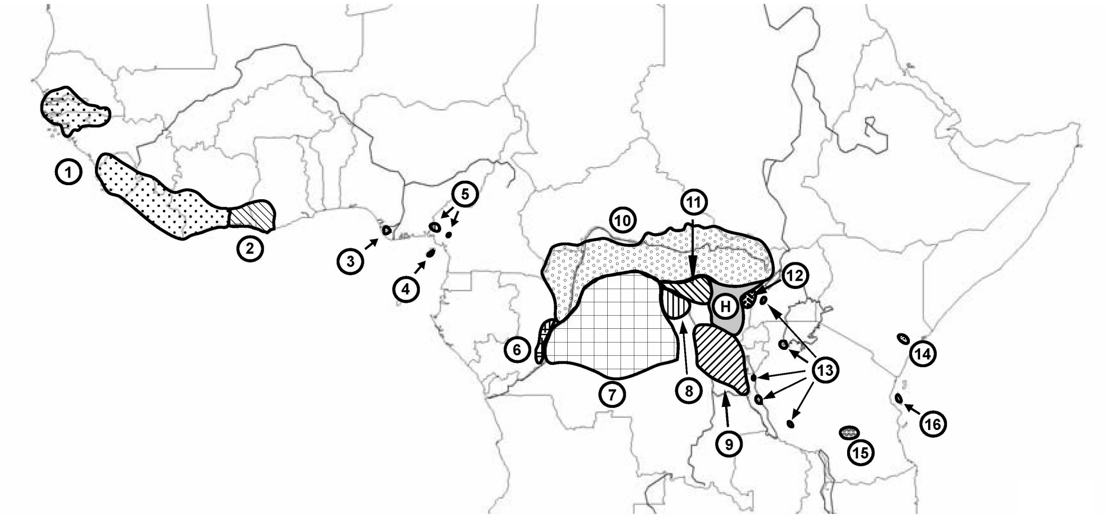

Figure 6.1 C: 16 species arrangement of the red colobus monkeys (*Procolobus* [*Piliocolobus*]) following Groves (2007). 1 - *P. badius*. 2 - *P. waldroni*. 3 - *P. epieni*. 4 - *P. pennantii*. 5 - *P. preussi*. 6 - *P. bouvieri*. 7 - *P. tholloni*. 8 - *P. parmentieri*. 9 - *P. foai*. 10 - *P. oustaleti*. 11 - *P. langi*. 12 - *P. semlikiensis*. 13 - *P. tephrosceles*. 14 - *P. rufomitratus*. 15 - *P. gordonorum*. 16 - *P. kirkii*. H - putative area of hybridization between adjacent species. Notice how this classification identifies Central Africa as the highest area of red colobus monkey species diversity.

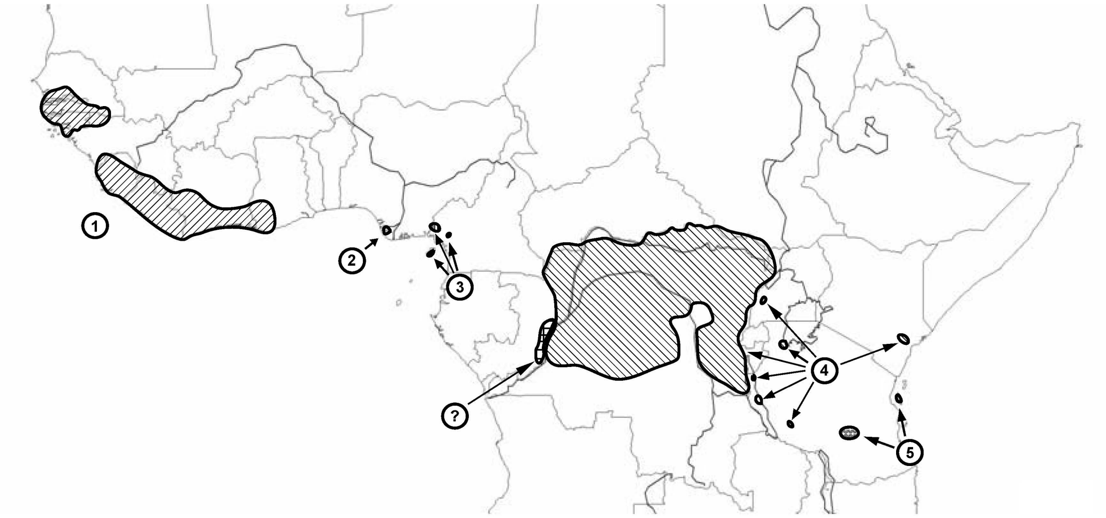

Figure 6.1 D: Five species arrangement of the red colobus monkeys (*Procolobus* [*Piliocolobus*]) following the Chapter 5 classification. 1 - *P. badius*. 2 - *P. epieni*. 3 - *P. pennantii*. 4 - *P. rufomitratus*. 5 - *P. kirkii*. ? - *P. sp.? bouvieri*. Notice how this arrangement recognizes distinct lineages outside of Central Africa, particularly in western Africa.

parentheses is how the species would currently be assessed against the IUCN criteria (provided by M. Hoffmann, Global Mammal Assessment, pers. comm.).

P. epieni (Critically Endangered) – Under the Chapter 5 classification, the Niger Delta red colobus is the only monotypic species, and thus it is the most (phylogenetically) distinct of the red colobus forms. This species was only recently discovered (Grubb and Powell 1999), it exists in an extremely precarious state because of an extremely limited distribution, and it is threatened by habitat destruction and hunting. *P. epieni* also exists in an area of civil unrest, and its range does not overlap with any protected area (Grubb and Powell 1999; Werre 2000). Its threatened status and phylogenetic distinctiveness warrants it high conservation priority, and surveys are urgently needed to determine how many individuals remain. However, the civil unrest in the Niger Delta makes it difficult, in practice, to implement any kind of conservation strategy for this species at this present time.

P. pennantii (Critically Endangered) – The most recent IUCN Red List (2007) followed Grubb et al. (2003) in lumping *pennantii, preussi, epieni* and *bouvieri* in this species. However, the classification adopted here removes *epieni* from *P. pennantii*, and the phylogenetic and taxonomic positions of *bouvieri* were not assessed. Thus, according to the Chapter 5 classification this species contains only two subspecies (*P. p. pennantii* and *P. p. preussi*), both of which would now be assessed as Critically Endangered (M. Hoffmann, pers. comm.). They are threatened by small population size, extremely restricted geographic distributions, and human hunting pressure. *P. pennantii* thus remains near the top of red colobus monkey conservation priorities, and the possible inclusion of *bouvieri* (Critically Endangered; see below) to this species only emphasizes the precarious state of this lineage. Better protection and more effective management are needed to prevent the entire loss of this species and divergent lineage.

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P. kirkii (Endangered) – The *gordonorum* and *kirkii* forms were each assessed as full species by the most recent Red List (IUCN 2007). The Chapter 5 classification lumps them into one species – *P. kirkii*. This action effectively lowers the conservation priority of these taxa relative to some other red colobus forms (*P. epieni* and *P. pennantii* in particular), but the threatened status of both *P. k. kirkii* and *P. k. gordonorum* still warrants high priority for this species as a whole. Both subspecies are endangered with restricted and fragmented distributions. Although some populations are secure in national parks, the majority of this species requires further protection to prevent future declines.

P. badius (Endangered) – The Chapter 5 species level classification of the three western red colobus forms into *P. badius* (*P. b. temminckii, P. b. badius* and *P. b. waldroni*) is similar to the classification used in the IUCN Red List (2007). However, the Red List also lumped into *P. badius* numerous other forms to which Grubb et al. (2003) did not assign a species name. There is no phylogenetic basis for this action, and it makes *P. badius* appear to have many more subspecies and thus less distinct. None of the three *P. badius* forms recognized in Chapter 5 are particularly secure (see Table 6.1). Furthermore, the molecular data indicate that *P. b. waldroni* may merit species status, and thus this taxon warrants the very highest of conservation attention if it is not already extinct.

P. rufomitratus (Least Concern) – The 2007 IUCN Red List recognized *P. rufomitratus* as a monotypic species (Tana River red colobus). Since the *rufomitratus* form is among the most endangered of all primates with fewer than 800 individuals remaining and no secure populations (Karere et al. 2004), this taxonomic arrangement warranted this species very high conservation priority. However, the Chapter 5 classification lumps numerous forms into *P. rufomitratus* based on close phylogenetic relationships. Thus the Tana River red colobus is not as phylogenetically distinct as previously assessed.

Of the five species recognized in Chapter 5, *P. rufomitratus* is actually the most common as many of its subspecies occupy a large range throughout areas of low human population density in the Congo Basin. Although it would likely be assessed as Least Concern against the IUCN criteria, some forms are highly threatened. In particular, *P. r. rufomitratus* and *P. r. tephrosceles* possess extremely limited and fragmented distributions and have undergone population size declines since the 1970's. Although they have numerous closely related forms, these two subspecies, especially *P. r. rufomitratus*, should not be neglected and require continued conservation attention to prevent their extinction (Struhsaker 2005). Furthermore, very little data exist for most other forms. In general, many of them face human hunting pressure, exist in areas of civil unrest, and find little legal protection. Among them, *P. r. tholloni* is thought to be particularly vulnerable to hunting pressure.

P. sp.? bouvieri (Critically Endangered) – The taxonomic status of Bouvier's red colobus was not assessed in Chapter 4, and it may very well represent a distinct lineage. Very little is known about this animal. It has a restricted distribution in the Republic of Congo and there have been no scientifically-documented records in several decades. It is possible that this taxon is already extinct (Oates 1994, 1996a). Surveys for Bouvier's red colobus must be conducted urgently to discover any surviving populations, although its presence in a large marshy region makes potential survey and conservation action logistically difficult (Oates 1996a). Because the Red List cannot assess unnamed taxa, it is best to assign the *P. pennantii* species name to this taxon (following Grubb et al. [2003]) until more data are collected. Although this adds a population to this species, the Critically Endangered status of all three subspecies (*pennantii, preussi, bouvieri*) still warrants high conservation priority for *P. pennantii*.

Chapter 7: Summary and Conclusions

This dissertation addressed the systematics and evolution of the living African colobines, which are comprised of the black-and-white, olive, and red colobus groups. It focused specifically on the red colobus monkeys, whose allopatric distribution and complex patterns of variation have led to a poorly resolved phylogeny and classification. Past attempts to elucidate the evolution of the extant African colobines have relied solely on craniometrics, pelage patterns, and vocalizations. Molecular methods were used here for the first time to test hypotheses concerning the timing of divergence events, phylogenetic relationships, and classification of these animals. A mitochondrial marker was used because it has a higher probability of tracing the species phylogeny compared to nuclear genes, and also because the use of degraded biomaterials precluded the amplification of long stretches of nuclear DNA. The results yielded new insight into the evolutionary history of the colobus monkeys, and they have implications for the biogeography of the African rainforest and the designation of conservation priorities for the endangered red colobus group. The following is a summary of the findings of the dissertation organized around the three main questions proposed in Chapter 1:

1) *What are the higher-level relationships and divergence dates among the living African colobines?*
2) *What are the evolutionary relationships within the red colobus group, and what do they reveal about the biogeographic history of the African rainforest?*

3) *What red colobus classification is most consistent with their phylogeny and molecular diversity, and how does it affect the designation of red colobus monkey conservation priorities?*

1. What are the higher-level relationships and divergence dates among the living African colobines?

This question was addressed in Chapter 2, which found a sister taxon relationship between the olive colobus and red colobus. These results can be combined with those from other studies that used mitochondrial DNA (e.g., Sterner et al. 2006; Whittaker et al. 2006) to support a hypothesis of reciprocal monophyly among the Asian and African colobines. Within the African clade, the mitochondrial relationships among the black-and-white colobus species were congruent with those inferred from male loud calls except for the position of *C. vellerosus*, whose evolutionary origins require further investigation. Among the red colobus monkeys, the mitochondrial relationships failed to support several relationships inferred from vocalizations and pelage patterns, especially in regard to the forms in West and Central Africa. This issue is further addressed by the second main question proposed in this dissertation (see below).

Despite the choice of relatively conservative calibration points (using earlier ones or a range of dates at each point would only serve to push the inferred dates further back in time), the mitochondrial divergence dates inferred here show that the modern African colobine radiation began much earlier than previously thought. The black-and-white colobus group had diverged from the olive and red colobus groups by 7.5 Ma, and the olive colobus had diverged from the red colobus by 6.4 Ma. Furthermore, both the modern black-and-white colobus and red colobus diversifications had begun by the Mid Pliocene (3.5 Ma and 3.0 Ma, respectively), with several of the species lineages in each group diverging by the Late Pliocene and Early Pleistocene. These lineages thus co-existed with the large-bodied African Plio-Pleistocene fossil colobines and were likely part of the same adaptive radiation. Furthermore, since the living colobus monkeys are all predominantly arboreal, it is likely that their basal members were also arboreal, which supports the notion that at least some of the Late Miocene colobines were adapted to life in the trees.

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. However, the extant guenon and African ape radiations show similar temporal depths based on molecular data and are likewise rare or absent in fossil deposits. Most living members of these clades (African colobine, guenon, African ape) are adapted to a moist forest 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 as fossils. These forest regions 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.

2. What are the evolutionary relationships within the red colobus group, and what do they reveal about the biogeographic history of the African rainforest?

This question was addressed in Chapters 3 and 4. Chapter 3 presented a detailed investigation into the phylogenetic relationships of the red colobus group and included all commonly recognized forms with the exception of Bouvier's red colobus (*bouvieri*). A number of mitochondrial relationships supported those inferred from the vocalization data, the pelage data, or both. In these cases, it is more likely than not that a true organimsal relationship was uncovered due to phylogenetic congruence between signals from independent data sets. However, relationships supported by only vocalizations, pelage, or mitochondrial DNA must be interpreted with care. Red colobus vocalizations are graded so that discrete characters are difficult to identify, useful phylogenetic characters in red colobus pelage are hard to establish because red colobus pelage patterns can be quite variable (even within a social group), and analysis of mitochondrial DNA only provides one gene tree estimate of phylogenetic relationships. Furthermore, analyses of vocalization and pelage data have been conducted using a phenetic approach with little consideration for primitive and derived character states.

At the base of the red colobus mitochondrial tree was a polytomy, but taxa from certain geographically proximate regions clustered together. For example, close relationships were found between the forms *badius* and *temminckii* in West Africa, *pennantii* and *preussi* in the Gulf of Guinea (Cameroon and Bioko Island), and *kirkii* and *gordonorum* in Tanzania (Udzungwa Mountains and Zanzibar Island). These relationships, as well as one between *rufomitratus, tephrosceles,* and the numerous Congo Basin forms, were congruent with those inferred from the pelage and vocalization data. Also supporting the vocalization data was a mitochondrial relationship between the Niger Delta red colobus (*epieni*) and the Central and East African taxa.

Several unexpected and complex evolutionary patterns were also present in the mitochondrial tree, including paraphyly of the Central African forms and polyphyly of *tholloni* and *oustaleti*. It is likely that at least some of these complex mitochondrial patterns are not tracing the population history of these taxa, and a phylogeographic study is needed to infer more accurately the presence of incomplete lineage sorting and/or hybridization. The unique and divergent mitochondrial lineage in Miss Waldron's red colobus (*waldroni*) also requires further investigation to establish whether or not this taxon is truly an evolutionary divergent red colobus lineage.

Chapter 4 discussed how the inferred red colobus monkey mitochondrial relationships can be used to test previous hypotheses concerning the biogeographic history of the African rainforest. The relationships between the East African and Central African forms suggest the East African coastal forests were most recently connected to the forests of the Albertine Rift and Congo Basin. Support is found for Kingdon's (1971) hypothesis of two former connections between these forests – one through the northern highlands and another through the southern highlands, with the southern connection becoming isolated first. Relationships among the Central African forms suggest that large rivers within the Congo Basin (Congo River, Lomami River, Lualaba River) have not always impeded gene flow in rainforest taxa. It appears that red colobus monkeys were either able to cross these rivers or circumvent their headwaters at certain times during the Pleistocene. The relationship between the Niger Delta red colobus (*epieni*) and the Central and East African taxa suggest a prior forest connection between these regions. The geographic presence of an unrelated red colobus clade in between the Niger Delta and Central Africa requires further explanation. Finally, the distant relationship of the

Western red colobus group to the other red colobus forms supports a biogeographic barrier in the vicinity of the Dahomey Gap or western Nigeria, and the distinct mitochondrial lineage found in Miss Waldron's red colobus (*waldroni*) may indicate that the Bandama River, Baoulé V, or both have been larger biogeographic barriers than previously thought.

3. What red colobus classification is most consistent with their phylogeny and molecular diversity, and how does it affect the designation of conservation priorities?

This question was addressed in Chapter 5 (classification) and Chapter 6 (conservation implications). Chapter 5 compared likelihood branch lengths between closely related groups in order to infer a genus and species level classification for the red colobus monkeys. At the genus level, it was unclear whether branch length differences between the red colobus (*Piliocolobus*) and olive colobus (*Procolobus*) were greater than those between the well-defined Asian colobine genera. Because of this ambiguity and the numerous similarities between the olive colobus and red colobus in morphology and natural history, it is best to retain *Piliocolobus* as a subgenus of *Procolobus*. Within the red colobus group, there were seven mitochondrial lineages as divergent from one another as those found between well-defined black-and-white colobus biological species (sensu Mayr 1942). Two of these were inferred to be mitochondrial patterns that are not tracing the organismal phylogeny, which led to the recognition of five red colobus monkey species (*P. badius, P. epieni, P. pennantii, P. kirkii, P. rufomitratus*) whose limits loosely correspond to biological species boundaries in the black-and-white colobus group. Aspects of this classification are similar to some previously proposed arrangements, but primary differences are in the recognition of the Niger Delta red colobus as a distinct species (*P. epieni*) and in grouping the Udzungwa (*gordonorum*) and Zanzibar (*kirkii*) forms into one species (*P. kirkii*). This classification also differs substantially from the most recent arrangements proposed by Groves (2001, 2007), which recognize 9 and 16 species, respectively. Further work must be done to determine if the divergent mitochondrial lineage found in Miss Waldron's red colobus merits elevation of this taxon to species status, and the phylogenetic position and taxonomic status of Bouvier's red colobus (*bouvieri*) still needs to be assessed.

The Chapter 5 classification is appropriate for use in the designation of red colobus monkey conservation priorities because the recognized species reflect phylogenetic relationships and distinctiveness and were diagnosed using objective and quantitative criteria applied consistently across the whole group. Recognition of the inferred species in the IUCN Red List would ensure that species-based and area-based conservation efforts prioritize the protection of divergent lineages. This would minimize the loss of evolutionary history and guide conservation planning so that limited conservation resources are not wasted and effective policies are implemented. Species such as *P. epieni, P. pennantii,* and *P. kirkii* that possess fragmented populations and restricted geographic ranges would merit high priority for action as they are particularly threatened with extinction. *P. badius* is also threatened and lacks many large and secure populations, and the *waldroni* form may be a distinct species and thus merits the highest priority for conservation action (if it is not already extinct). Although *P. rufomitratus* has a large range and likely a large population size, some subspecies (*P. r. rufomitratus* and

P. r. tephrosceles) require further protection of they are to survive. The Chapter 5 classification also shows that western Africa contains just as many, if not more, distinct red colobus lineages as East and Central Africa, which is in contrast with several other previous taxonomic arrangements. Use of this classification to identify areas of species endemism will aid in identifying areas for protection that would preserve taxonomic distinctiveness.

Overview and Conclusion

This dissertation provides new insight into the evolutionary history of the African colobines, especially of the red colobus group. Mitochondrial data were collected from an extensive sample of colobus monkey populations to address questions concerning evolution, systematics, biogeography, and conservation planning. The inferred divergence dates provide expectations for what can be found in the fossil record. The mitochondrial relationships represent testable hypotheses concerning the evolution of the colobus monkeys and the biogeographic history of the African rainforest. The inferred species level classification reflects the phylogenetic relationships and diversity of the red colobus group, and the recognized species can be used in the designation of conservation priorities for these endangered monkeys.

This dissertation also produced new questions that need to be addressed. The evolutionary origins of *C. vellerosus* may have been complex and require further investigation. A phylogeographic study of the Central African red colobus forms would further elucidate the evolution of the red colobus monkeys and provide much needed insight into the forest history of the Congo Basin. Further research needs to be conducted on certain red colobus forms that are poorly known and highly threatened with extinction. In particular, Miss Waldron's red colobus and Bouvier's red colobus may represent distinct lineages that require further study, if they are not already extinct. This dissertation also places very high priority for conservation action on the Niger Delta red colobus and *P. pennantii*. While the *kirkii* and *gordonorum* forms are not as distinct as formerly thought, they require conservation attention to prevent further population declines, as do the *P. badius* and some *P. rufomitratus* subspecies. Scientific data on these animals need to be collected in order to make informed decisions concerning conservation planning and to aid in the prevention of further losses in primate biodiversity.

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