A Conservation Genetic Study of *Cercopithecus mitis* in the Lomami Basin, Democratic Republic of the Congo

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Kate Detwiler, Department of Anthropology, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Dorothy F. Schmidt College of Arts and Letters and was accepted in partial fulfillment of the requirements for the degree of Master of Arts.

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Abstract

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The *Cercopithecus mitis* radiation has significant phenotypic and ecological diversity, making it a great candidate for evolutionary genetic studies. This study represents the first genetic survey of *C. mitis* from well provenanced wild populations, including *C. mitis heymansi* from the TL2 landscape, an area of remote tropical forest between the Tshuapa, Lomami, and Lualaba rivers in the Democratic Republic of the Congo. Tissue samples were collected from 7 male blue monkeys, DNA was extracted and surveyed at 919 bp of the Testis-Specific Protein Y-chromosome (TSPY), and added to a larger dataset including other *C. mitis* and *C. albogularis* specimens. Evolutionary analyses suggests TL2 *C. mitis* shared recent genetic contact with *C. albogularis* at the headwaters of the Congo River than with conspecifics to the northeast, indicating the Congo River is a significant biogeographic barrier to *C. mitis* and sheds new light on their genetic heritage, taxonomic assignment, and conservation status.

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Introduction

Guenons are a diverse and widely distributed primate group that present taxonomic challenges due to unresolved phylogenies and methodological disagreements about how best to classify closely related forms (Butynski, 2002). Of the guenons, the blue monkey clade, or *Cercopithecus mitis*, is well known for its taxonomic complexity because of its phenotypic diversity found across the African continent (Lawes et al., 2013). Research on blue monkeys reveals great variation within the group often sparking debates over the species and subspecies classifications.

Blue monkeys are found throughout sub-Saharan Africa ranging from Angola and Democratic Republic of the Congo (DRC) in the west, to Tanzania, Kenya and Ethiopia in the east, and Mozambique and South Africa in the south (see Fig. 1; Kingdon, 1997; Groves, 2001; Butynski, 2002; Grubb et al., 2003; Detwiler 2010a; Butynski et al., 2013; Mittermeier et al., 2013). Their pelage colors can vary from hues of blue and gray on the face and body to golden and russet-brown on the neck, crown, and back (e.g. Kingdon, 1997; Groves, 2001; Butynski et al., 2013; Mittermeier et al., 2013).

Recent surveys report new populations of blue monkeys living in central Africa and occupying a region of tropical forest in the Democratic Republic of the Congo surrounding the Tshuapa, Lualaba and Lomami Rivers known as the TL2 Conservation Landscape (see Figs. 2, 3 and 4). This area is incredibly remote and a significant distance from population centers. Surveys reveal that TL2 is an ideal habitat for a variety of faunal species (Hart *et al*., 2012), and has been only minimally explored by biologists.

Curiously, blue monkeys spotted in this region are phenotypically diverse and their taxonomy has yet to be confirmed. The Congolese nationals from this region have two names for these blue monkeys: ngoyi noir and ngoyi blanc. The ngoyi noir form has an overall darker pelage color on the under body, throat, limbs and tail with salt and pepper facial pelage, and a defined white diadem on the forehead (see Fig. 5; John Hart unpublished report). The distribution limits of ngoyi noir are the forests on both sides of the Lomami River from north of Opala to south of Katopa. Preliminary research based on published reports, known distributions, and phenotype suggests that ngoyi noir monkeys fit characteristics of the subspecies *Cercopithecus mitis heymansi*, also known as the Lomami River blue monkey (J. Hart, personal communication; Groves, 2001; Butynski, 2002; Colyn 1987). The ngoyi blanc form is lighter, and its taxonomic identity and distribution remain a mystery. It is not included in this thesis project as there are too few field observations and biological specimens available for study.

Guschanski et al. (2013) published a mitochondrial phylogeny of guenons (Tribe Cercopithecini) using DNA extracted from museum skins. The authors included one skin of *C. mitis heymansi* in their study that had a general source location from a town north of the TL2 study area. Currently, the *C. mitis heymansi*, or ngoyi noir form, from the TL2 forest have yet to be included in a genetic study to better understand their classification and evolution of their lineage.

A common goal when studying blue monkeys is to evaluate their taxonomic status. This is necessary for establishing conservation priorities and for making informed decisions regarding conservation actions (Butynski, 2002). Groves' (2001, 2004) taxonomy is particularly meaningful to this study because he focuses his analysis on

representing the total diversity within a lineage of animals. He recognizes variation as a source of diversity and aims to conserve and preserve that diversity by acknowledging distinct lineages as a species or subspecies. Groves follows the Phylogenetic Species Concept (PSC; Cracraft, 1983, 1997), an observable pattern based concept that identifies species as populations that are "diagnosably distinct" from each other (Groves, 2004). He defines subspecies as populations or geographic subdivisions that share genes of the same species (Groves, 2004). Groves tends to elevate more groups to the species and subspecies levels compared to other guenon classifications (Kingdon, 1997; Butynski, 2002; Grubb et al., 2003), especially in the blue monkey clade (Groves, 2001). In this study, I follow the taxonomy proposed by Groves (2001) and classify blue monkeys as its own species under the overarching species group *Cercopithecus mitis*.

The description of ngoyi noir from TL2 compares with published reports describing the distribution and phenotype of *Cercopithecus mitis heymansi* (Groves, 2001; Butynski, 2002; Colyn, 1987). This study investigates if genetic data from TL2 show a distinct lineage for *Cercopithecus mitis heymansi* that is different from all other *C. mitis* populations surveyed. Thus, my objective is to look for genetic variation between the TL2 individuals and other members from the blue monkey group. This is a between population study, not a within population study. Subsequently, the null hypothesis is no significant genetic differences between the blue monkeys from TL2 and other known *Cercopithecus mitis* groups in Africa.

The samples used in this study represent well-preserved high quality tissue specimens of *Cercopithecus* taxa, including *Cercopithecus mitis heymansi*, from wild populations in the Central Congo Basin. This research is the first conservation genetic

survey of *Cercopithecus mitis heymansi* from the TL2 landscape. This study contributes to our understanding of the blue monkey taxonomic conundrum by using phylogenetic methods to answer fundamental questions about the distribution and variation of blue monkeys in the TL2 area.

To test for variation, I extracted DNA from 10 tissue samples and examined sequence data from the Testis-Specific Protein on the Y-chromosome (TSPY). The TSPY is a conserved locus known for low recombination that has been used in previous studies to recognize guenon lineages and to identify genetic distinctiveness (Tosi et al., 2005; Detwiler, 2010a; Hart et al. 2012). I compared seven new *C. mitis* sample sequences to 34 genetic sequences from 25 different taxa including *Cercopithecus mitis stuhlmanni* (a blue monkey population to the north and east of TL2 in the DRC) and three new *Cercopithecus wolfi* samples from TL2. I conducted phylogenetic analyses on all sequences to evaluate taxonomy and ancestry in relation to geographic location. As mentioned above, I followed the taxonomy of Groves (2001), who acknowledges evidence of genetic distinctiveness as a unit of conservation, and support for speciation. Finally, I compared my findings to existing *Cercopithecus* genetic studies.

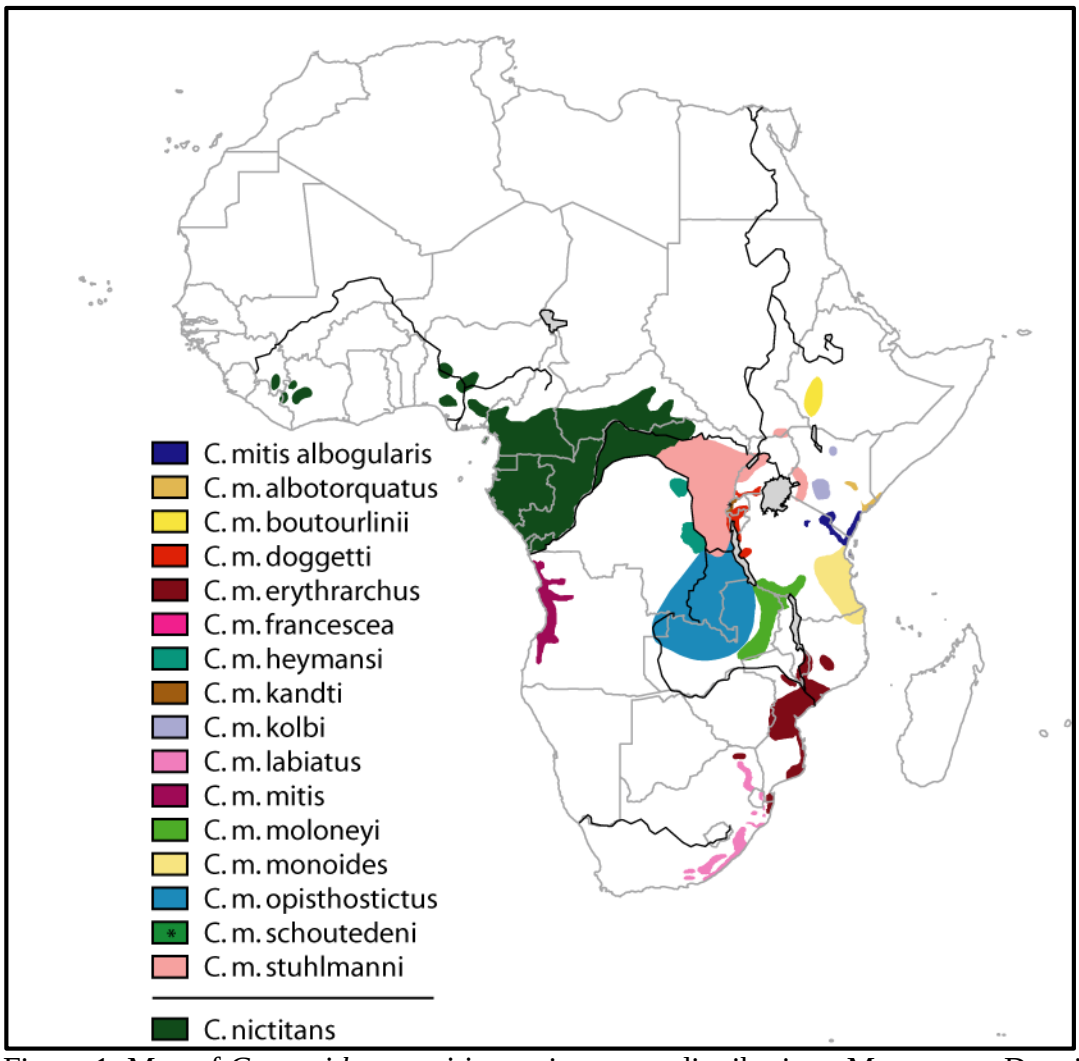

Figure 1: Map of *Cercopithecus mitis* species group distribution. Map source Detwiler, 2010a; modified from Lawes et al., 2013.

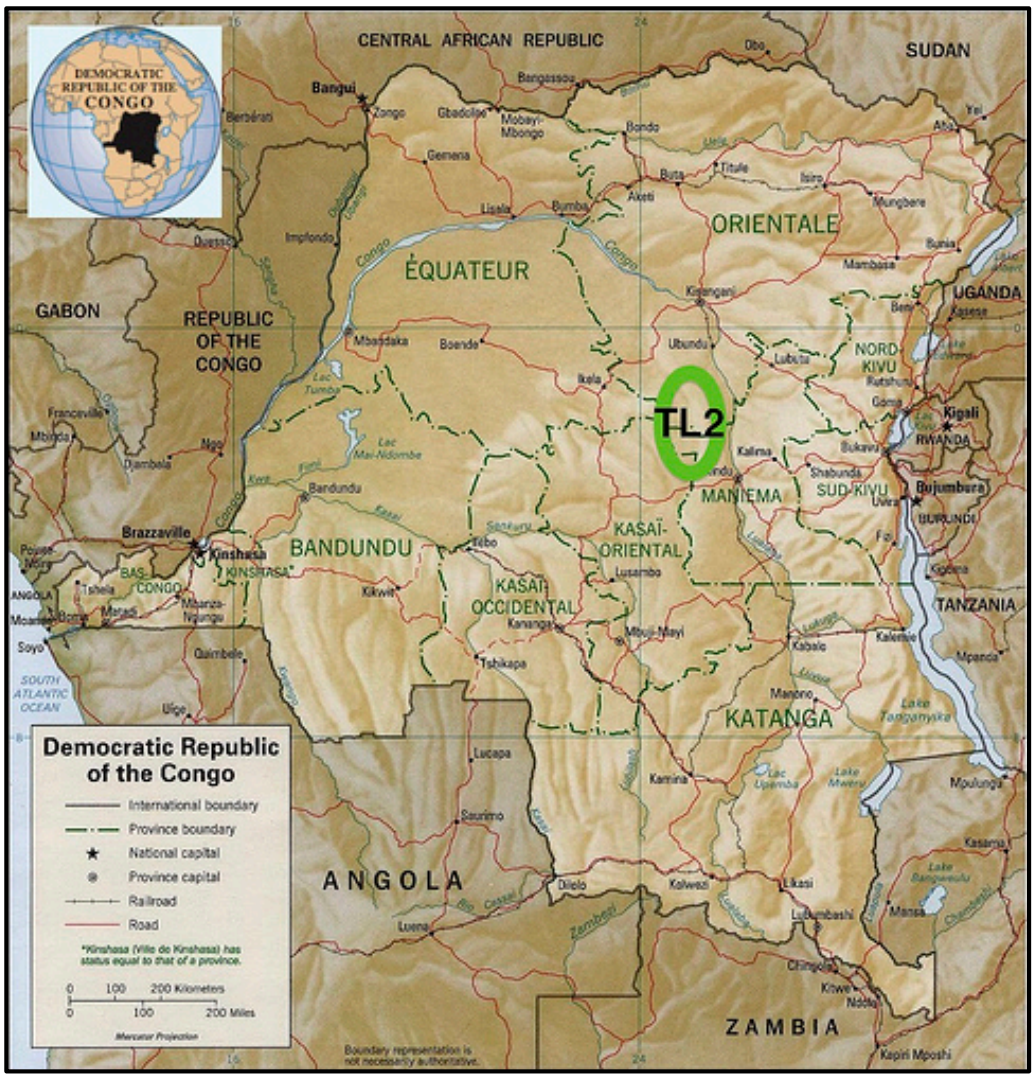

Figure 2: Map of TL2 region in central Democratic Republic of the Congo, Africa. Map by Lukuru Foundation, TL2 Project.

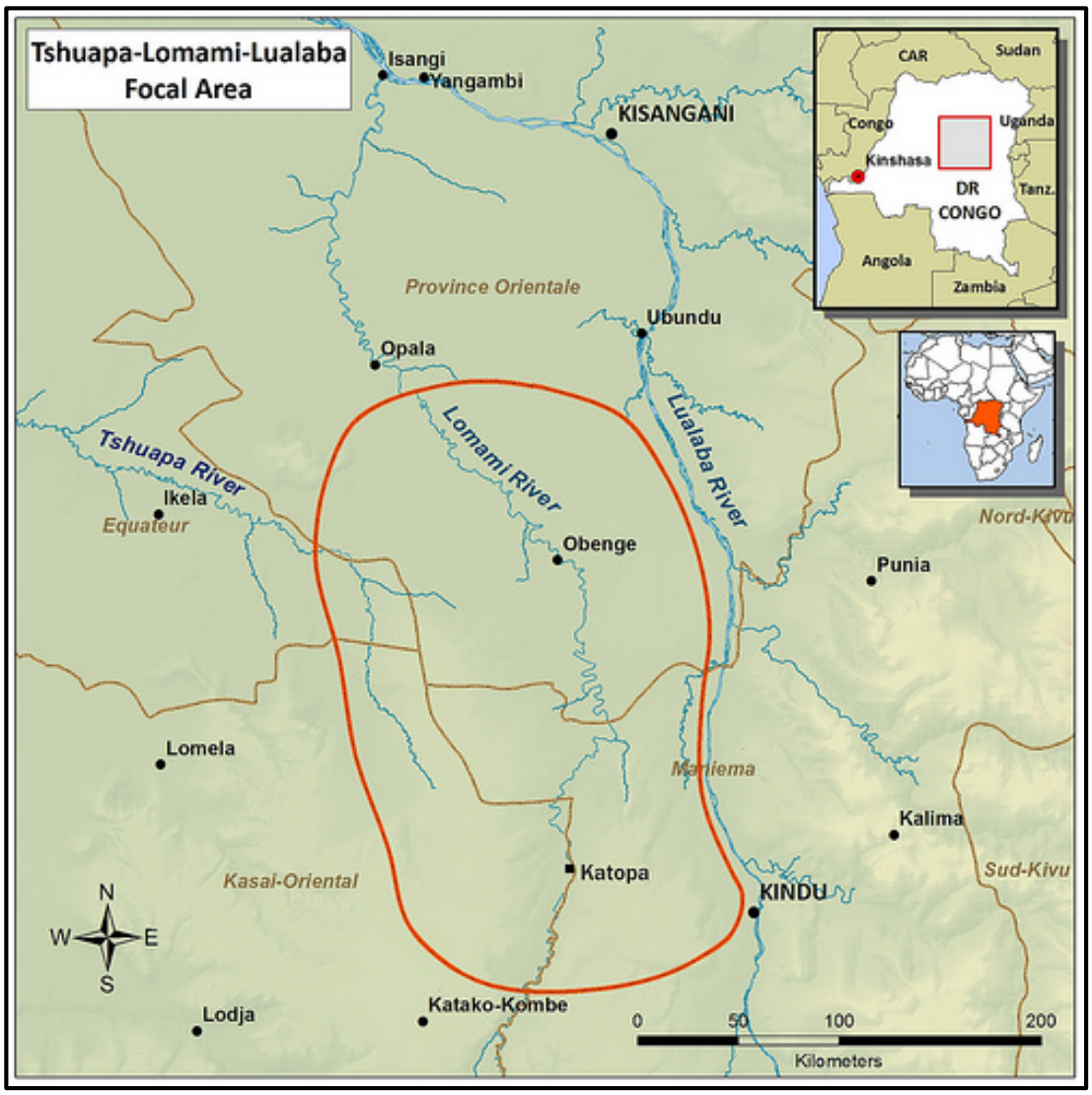

Figure 3: Enlarged map of the TL2 area between the Tshuapa, Lualaba, and Lomami Rivers outlined in red and the focal area for this study. Map by Lukuru Foundation, TL2 Project.

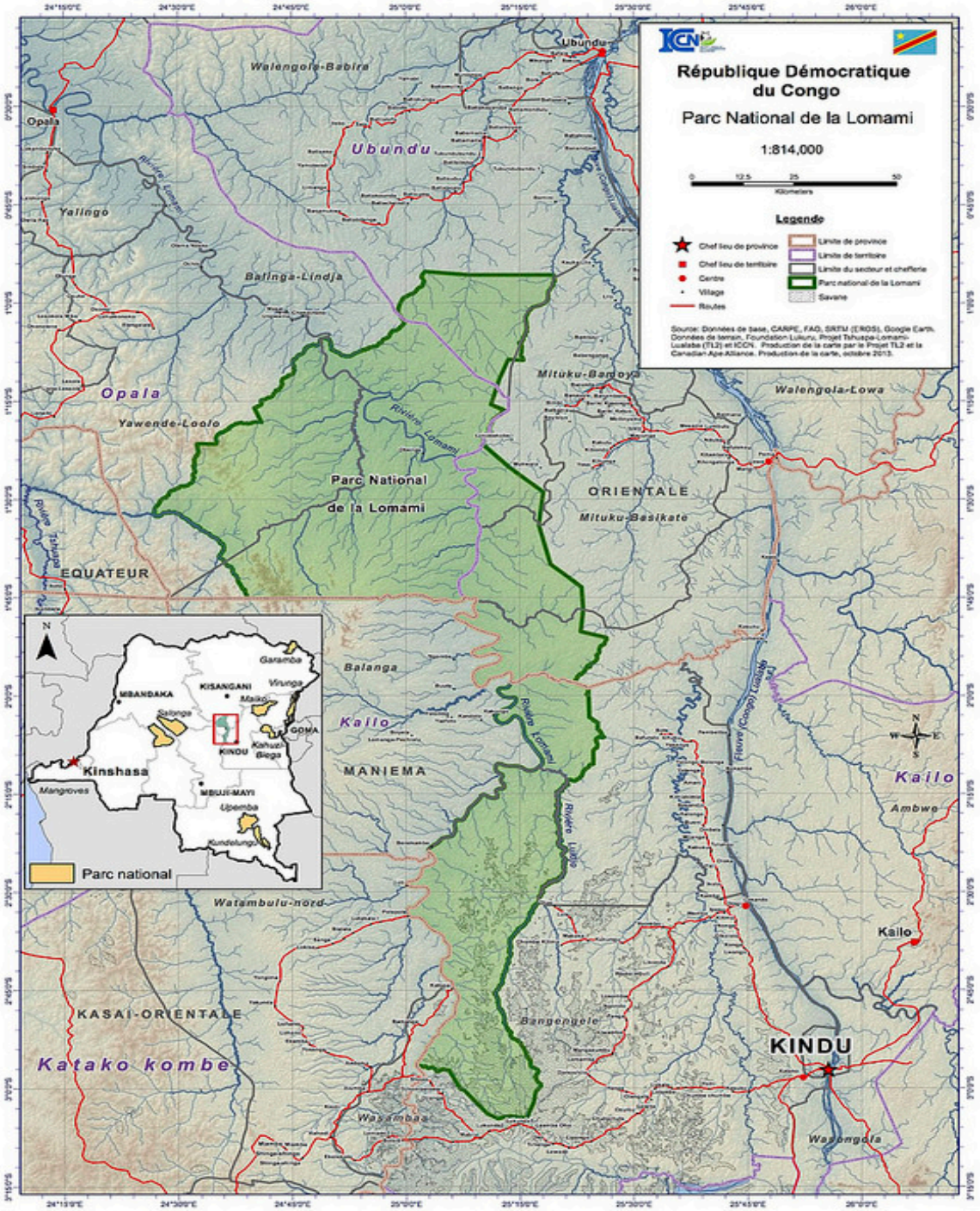

Figure 4: Map of Lomami National Park within the TL2 Conservation Landscape. Map by Lukuru Foundation, TL2 Project.

Figure 5: Photos of TL2 blue monkey known locally as ngoyi noir showing its uniquely dark pelage on the underbody, limbs and tail, with salt and pepper facial pelage. Preliminary research suggests it is the blue monkey subspecies *Cercopithecus mitis heymansi*. This monkey's death was a result of bushmeat hunting. Photo source: John Hart.

Background

Biogeographic significance: geologic and climatic history leads to distribution and phenotype variation in guenons

An important factor to consider when examining the lineage and distribution of any species is the biogeography of a region and its change over time. Geological and climatic processes can work together to expand and contract forest environments, which in turn shape the series of events leading to the origin, dispersal, and distribution of a species (Middleton, 1997; Lehman et al., 2006b). In this section, I address the environmental and ecological changes that occurred in Africa, which gave rise to the current distribution of *Cercopithecus mitis* and other closely related guenon lineages we know today.

Beginning in the Tertiary period, geological processes and tectonic activity dramatically changed continental formations on earth. These transformations altered ocean currents and corresponded with major climatic shifts around the world producing glacial temperatures at the poles and warm tropical conditions around the equator (Benefit et al., 2002; Walker-Pacheco, 2010; Butynski et al., 2013). An expansion of wet forest environments throughout central Africa emerged spreading with it many ancestral primate and faunal populations (Benefit et al., 2002; Walker-Pacheco, 2010). Following the Miocene epoch were prolonged periods of cooler and dryer temperatures worldwide. This climate change produced retreating and fragmented forests in equatorial regions

with more arid terrain in northern and southern regions marked by seasonality (Walker-Pacheco, 2010; Butynski et al., 2013).

Taking into consideration their generalized diet and arboreal morphological adaptations, guenons evolved into a diverse and widely distributed primate group making them one of the best documented faunal groups for biogeographic research in central Africa (Colyn and Deleporte, 2002). However, new discoveries, like ngoyi noir in TL2, warrant the reassessment of guenon and blue monkey distributions into regions that have not been thoroughly explored by biologists. Early data surveys of African forests led to distribution maps of biodiversity in specific locations (Colyn et al., 2002). Unfortunately, TL2 was not included in these early surveys because the TL2 area was not easily accessible for research. Now, after several years of biological surveys conducted in TL2, we have observational data of unique populations of blue monkeys living outside their known range.

It should be considered that the TL2 region could be an area with a long established tropical forest that did not undergo severe climatic changes throughout the past (Hamilton, 1988; Butynski et al., 2013); therefore, having the ability to harbor a variety of species for many years. If this is the case, then there is reason to speculate that blue monkeys in the TL2 area could be a source population for which all other populations may have branched off and dispersed out.

On the other hand, blue monkeys may have traveled into the TL2 region seeking to escape from areas of unfavorable habitats. Forest fragmentation created by climate fluctuations, geographic barriers like rivers and mountains, and resource deficient areas can affect the growth and dispersal of a population. Blue monkeys are highly arboreal

and will not thrive in fragmented forests (Kingdon, 1997; Butynski, 2002; Detwiler, 2010a). Resource scarcity could also force individuals of a group to weigh the costs of staying within their normal range or leaving to find a more suitable range. Blue monkeys prefer a frugivorous rich diet, but can defer to a non-specialized diet and will range outside their known areas in search of suitable food sources (Twinomugisha et al., 2007). Those individuals of a population who migrated into TL2 could have become separated and isolated, ceasing gene flow between them and the rest of the population, and over time producing allopatric populations. Blue monkeys in the TL2 region could be a result of evolutionary forces that include migration and founder effect due to genetic drift.

Colyn and Deleporte (2002) studied the distribution of central African primates, including blue monkeys, in relation to biogeographic barriers like rivers. Their research focuses on primate species that are effectively restrained by major river barriers, and excludes primate groups that are situated on the banks of rivers and are known to be good swimmers, such as Allen's swamp monkey, Talapoin monkeys, and drill-mangabeys. They concluded that the Congo River is a significant geographic barrier that influences distribution patterns of primates and assists in the dispersal and constitution of primate communities in the central African basin (Colyn et al., 2002). More specifically, the Congo River manifests a clear demarcation of species on one side of the river and not on the other. This study is meaningful to my research because it emphasizes the prominence of the Congo River as a real geographic barrier that can separate populations resulting in genetic isolation, and over time, allopatric speciation and phenotypic variation.

Consequently, the Congo River is a considerable biogeographic feature and barrier for the *Cercopithecus mitis* group. In general, the Congo River creates four

distinct biogeographic regions: the eastern tropical mountain region, the central basin containing a closed canopy rainforest where TL2 is located, and transitional woodlands in the north and south with gallery forests that follow rivers and tributaries (Middleton, 1997). The Congo River begins as the Lualaba River in the southeastern region of the Democratic Republic of the Congo. It flows north through Kisangani, then rounds a northwestern route through Bumba and Lisala where it changes its name to the distinguished Congo River. It continues rounding west then south past Mbandaka, bordering the Republic of the Congo, passing Kinshasa, and ultimately coming to an end near Soyo, Angola emptying into the South Atlantic Ocean. At its broadest point, the Congo River can reach widths of 15 kilometers being quite shallow and having visible patches of islands in the middle. Contrastingly, there are points where the river narrows to choke points, becoming very deep, rapid-like, and difficult to cross (Middleton, 1997).

The distribution map of *Cercopithecus mitis* species (see Fig. 1) illustrates the differentiation of populations on both sides of the Congo River. There is a general consensus among scientists regarding the region and phenotype for each species and subspecies in central Africa. However, the distribution limits of ngoyi noir in TL2 represents a new range on the left bank of the Lualaba River to the forests on both sides of the Lomami River, north of Opala to south of Katopa. The subspecies closest in distance to ngoyi noir are *Cercopithecus mitis heymansi* and *Cercopithecus mitis opisthostictus*. There is little documented information about these two subspecies, yet their known distributions west of the Congo River make them the best candidates for comparing the newly discovered ngoyi noir population.

Cercopithecus mitis heymansi (henceforth *C. m. heymansi*), also known as the Lomami River blue monkey, is the nearest known population of blue monkeys just to the north and south of the ngoyi noir distribution in TL2. *C. m. heymansi* is found mostly between the Lualaba and Lomami Rivers in the canopy rainforests of the central Congo basin, reaching as far north as 2°S of the equator, and in some cases extending west of the Lomami River (see Fig. 1; Colyn, 1988; Groves, 2001; Mittermeier et al., 2013; Butynski et al., 2013). The Lomami River blue monkey is best identified by its white frontal diadem that narrows at the edges with contrasting blue-gray crown, nape, and shoulders, black arms, and lighter colored underside than upper (Groves, 2001; Mittermeier et al., 2013; Butynski et al., 2013). Butynski et al. (2013) hypothesize that the area between the Lualaba and Lomami Rivers, where *C. m heymansi* resides, could be a remnant range with implications that its members belong to a group once widespread throughout Africa. Thus, it is expected that the TL2 samples show a distinct genetic lineage for *C. m. heymansi*.

The other population to strongly consider due to its proximity to ngoyi noir is *Cercopithecus mitis opisthostictus* (henceforth *C. m. opisthostictus*), or the rump spotted blue monkey. This blue monkey has a large distribution range south of TL2 in the southeastern portion of the DRC beginning from approximately 6°S of the equator spreading down into northwestern Zambia, between the west bank of the Lualaba River to the Itombwe Mountains in the east (see Fig. 1; Groves, 2001; Mittermeier et al., 2013; Butynski et al., 2013). *C. m. opisthostictus* is classified as a blue monkey, however, its phenotype is distinguishable from ngoyi noir. This rump spotted blue monkey can be diagnosed by its light gray and broad frontal diadem, lighter gray back and hind limbs,

dark crown and nape with black arms, underside, and end of tail (Groves, 2001; Mittermeier et al., 2013; Butynski et al., 2013). Solely based on geographic distribution, *C. m. opisthostictus* could be considered a viable candidate for ngoyi noir.

A third population to keep in mind is *Cercopithecus mitis stuhlmanni*, or Stuhlmann's blue monkey (henceforth *C. m. stuhlmanni*). Although the distribution of this blue monkey is on the east bank of the Congo/Lualaba River, its range abuts the eastern range of *C. m. heymansi* and the northern range of *C. m. opisthostictus*. The distribution limits for *C. m. stuhlmanni* stretch from west Kenya and Uganda into northeastern DRC between the Uele River in the north and the Lualaba and Lukuga Rivers in the south (see Fig. 1; Groves, 2001; Mittermeier et al., 2013; Butynski et al., 2013). This is an interesting group of blue monkeys because the range in altitude throughout its distribution has produced noticeable variations in phenotype within this subspecies (Groves, 2001; Mittermeier et al., 2013; Butynski et al., 2013). Typically, *C. m. stuhlmanni* can be characterized by its black crown with contrasting broad and even width diadem across the forehead, black arms, and partially black legs with a lighter colored underside than back (Groves, 2001; Mittermeier et al., 2013). However, Colyn (1987) describes *C. m. stuhlmanni* that live in the mountains west of Lake Edward as having a distinct black interorbital region, which is rarely found in those in lower elevations. Also, specimens in high altitudes have fully developed cheek whiskers with a white zone from the nose to chin, not typically seen in the lowland populations. Lastly, blue monkeys found in the Semliki Valley have white ear tufts that are usually earth toned in more western populations (Colyn, 1987; Groves, 2001; Mittermeier et al., 2013).

Overall, the geologic and climatic history of central Africa played a direct role in creating natural geographic barriers, such as the Congo River and the eastern mountainous region, affecting the evolution of species by separating individuals through the act of migration and dispersal that resulted in isolated regional subpopulations (Walker-Pacheco, 2010; Kingdon et al., 2013). The evolutionary adaptive advantages of *Cercopithecus mitis* as a species over time allowed them to thrive and survive many climatic changes and migration waves that led to a mosaic distribution of blue monkey habitats we know today, and ultimately their genetic and phenotypic diversity in the central African region. Thus, it is easy to see why the taxonomic classification of blue monkeys can be confusing.

Taxonomy, phylogeny and genetic distinction

It is apparent that the blue monkey radiation is complex and polytypic making it an ideal candidate for evolutionary genetic studies. Mentioned in the previous section, blue monkeys, or *Cercopithecus mitis*, are a topic of taxonomic debate because their range of variation creates a challenge to resolve their ancestry. To add, primatologists who study blue monkeys vary in their agreement on the species and subspecies classification because different schools of thought - lumpers versus splitters - influence the classification of blue monkeys (Dutrillaux et al., 1988; Lernould, 1988; Butynski, 2002; Detwiler, 2002; Grubb et al., 2003; Detwiler 2010). In order to avoid miscommunication and misunderstanding, I believe it is important to explain precisely my taxonomic statements and my concept of what is a species.

First, Carl Linnaeus created taxonomy in the eighteenth century to help classify the natural world into categories of related organisms. Long since modified to include higher categories and new organisms, classification still plays a pertinent role in conservation management and identifying species. In general, taxonomy uses relatedness to define membership within and among species. Individuals that are closely related have a more recent common ancestor, but no species can belong to more than one taxon and cannot overlap or cross into another taxa (Groves, 2004). More specifically, organisms are classified in a nested order of hierarchy (Groves, 2004). Starting at the lowest level, every organism must belong to a single species, every species to a particular genus, every genus to one family, order, class, phylum, and finally into one kingdom.

Defining the concept of a species becomes more intricate. For most of the twentieth century, Mayr's Biological Species Concept (BSC; Mayr, 1942) was the dominant scientific paradigm for how best to define a species:

"A group of actually or potentially interbreeding natural populations which is reproductively isolated from other such groups."

Misinterpretation of Mayr's definition has implied that if these populations are reproductively isolated, then they cannot overlap or come into contact with one another. If they do meet in the future, they would need to be divergent enough to not have the propensity to interbreed so as to keep their species status designation (Groves, 2004; Freeman et al., 2007). It also does not account for the natural processes of evolution that can muddle the line between species and subspecies (Groves, 2004). Moreover, the BSC considers only a unitary mechanism for how a species is maintained (Groves, 2004).

These gaps and assumptions surrounding the BSC and Mayr's definition of a species create a need to further define what is a species.

For this research, I will follow Groves' (2004) explanation of a species. Groves (2004) describes the Phylogenetic Species Concept (PSC) as one of the most successful theoretical concepts to grasp the holistic idea of a species. Created by Cracraft (1983, 1997), Groves (2004) summarizes the PSC as requiring a species to have diagnosable heritable characteristics defined by patterns and observable evidence. This means that every member can be properly identified. Groves (2004) states that any characteristic will suffice, such as a DNA sequence, color, vocalization, size, so long as the characteristic is heritable and is a fixed difference. The PSC does not go beyond available evidence. In fact, it is completely objective and can be falsifiable (Groves, 2004). Most other species concepts infer processes that involve how the pattern arrived and is maintained, leading some scientists to speculate or extrapolate beyond what is actually observable (Groves, 2004).

Groves' taxonomy of blue monkeys is unique and names *Cercopithecus mitis* as the species group (equivalent to a superspecies classification) including five species and 21 subspecies: *C. nictitans* with two subspecies, *C. mitis* with seven subspecies, *C. doggetti*, *C. kandti*, and *C. albogularis* with twelve subspecies (Groves, 2001). Groves' *Cercopithecus mitis* species group classification deviates from the common *Cercopithecus nictitans* superspecies classification offered by other well-known blue monkey classifications (Kingdon, 1997; Butynski, 2002; Grubb et al., 2003; Butynski et al., 2013). *Cercopithecus nictitans* is known to have a distribution north of the Congo River separate from all *C. mitis* distributions, a distinct white nose spot not found in *C.*

mitis, and 2n=70 chromosomes that is two less than *C. mitis* making it diagnosably distinct and prompting other authors to name this as the superspecies to *C. mitis* (see Fig. 1; Groves, 2001; Butynski et al., 2013). Groves reasons that *C. m. opisthosticus*, a blue monkey subspecies, also has 2n=70 chromosomes, yet still shares characteristically distinct phenotypes and a distribution more closely linked to *C. m. stuhlmanni* and *C. doggetti* that could represent an early form of *C. nictitans* (Groves, 2001; Butynski et al., 2013) making *C. mitis* the applicable superspecies designation.

Groves tends to elevate more groups to the species and subspecies levels compared to other guenon classifications (Kingdon, 1997; Butynski, 2002; Grubb et al., 2003), especially in the blue monkey clade (see Table 1; Groves, 2001). This is noticeable in his recognition of *doggetti, kandti,* and *albogularis* as separate species, which are classified as subspecies in most other taxonomies (Kingdon, 1997; Butynski, 2002; Grubb et al., 2003; Butynski et al., 2013). Groves suggests enough fixed heritable characteristics to differentiate each group and warrant each their own species with subspecies classification. Groves (2004) goes on to define subspecies as populations or geographic subdivisions that share genes of the same species (Groves, 2004). Species can be divided into subspecies, but not all species have subspecies associated within them. Subspecies should have enough differences as a population to be scientifically considered worthwhile to be granted a trinomial name (Groves, 2004). In a sense, Groves can be considered a scientific splitter because he attempts to represent the total diversity within a lineage of animals. He recognizes variation as a source of diversity and aims to conserve and preserve that diversity by acknowledging distinct lineages as a species or subspecies.

In 2012, Hart et al. (2012) founded a new guenon species known locally as lesula (or *Cercopithecus lomamiensis*), which was also discovered in the TL2 area. Consistent with the PSC, Hart et al. (2012) describe the lesula monkey as having enough statistically significant genetic, morphological, and behavioral distinctions to categorize them as their own species. In this study, molecular data reveal *Cercopithecus lomamiensis* accumulated enough genetic differentiation on the Y-chromosome from long term biogeographic separation between the Congo (Lualaba) and Lomami Rivers to be distinguishable from its sister lineage, *Cercopithecus hamlyni*. Overall, allopatric speciation lead to distinct genetic variation that allowed lesula to be elevated to the species level, again representing TL2 as a location rich in biodiversity with a need for biological study and research.

In opposition, not all taxonomies recognize the same number of taxa as Groves (see Table 1). In fact, some scientists do not recognize the blue monkey group as a species at all, rather they lump them into groups or clusters equivalent to subspecies rankings. Kingdon (1997) published maps denoting phenotype and geographic location of blue monkeys throughout Africa, but does not formally recognize them as a species or subspecies. Instead, Kingdon refers to blue monkeys as a cluster under the overarching gentle monkey group. Butynski (2002) compiled taxonomic information from the International Union for Conservation of Nature-Species Survival Commission (IUCN/SSC) Primate Specialist Group workshop and described blue monkeys as a subspecies under the *C. nictitans* species group. Grubb et al (2003) similarly concluded that blue monkeys are not a species, but rather sections of monkeys under the larger

Cercopithecus nictitans species group encompassing several closely related and allopatric populations. Clearly, the taxonomy of blue monkeys is highly variable.

Table 1: Summary of classifications of guenon species.

Species	Kingdon (1997)	Groves (2001)	Butynski (2002)	Grubb et al (2003)	Hart et al (2012)	Mittermeier et al (2013)
***Cercopithecus mitis* superspecies/group**		X			X	X
Cercopithecus nictitans	X	X	X	X	X	X
Cercopithecus mitis	X	X	X	X	X	X
Cercopithecus dogetti		X				X
Cercopithecus kandti		X				X
Cercopithecus albogularis		X	X		X	X
TOTAL SPECIES RECOGNIZED	2	5	3	2	3	5

X indicates a species is recognized by author and publication year. Groves recognizes several blue monkey populations as distinct species, such as *C. doggetti*, *C. kandti*, and *C. albogularis*. The other authors recognize these populations as groups or clusters equivalent to subspecies (modified from Butynski 2002 in Glenn & Cords 2002: Kingdon, 1997; Groves 2001; Butynski, 2002; Grubb et al., 2003; Hart et al. 2012; Butynski et al., 2013; Mittermeier et al. 2013).

In order to articulate membership in a species and to help solve the classification discrepancies, genetics provides a useful tool to look at diversity within *Cercopithecus mitis*. Specifically, the rise of molecular phylogenetics provides an objective analysis of genetic patterns for taxonomic identification. The most comprehensive phylogenetic study was published in 2013. Guschanski et al. (2013) applied the newest next-generation DNA sequencing to find mitochondrial genomes from preserved museum specimens in order to infer estimated divergence times and evolutionary relationships among almost all guenon taxa. The authors assess phylogeny, not taxonomy, and are clearly lumpers recognizing all populations as subspecies within the *Cercopithecus mitis* species complex.

Guschanski et al. (2013) gives us good insight into guenon phylogeny, although, it is based only on mitochondrial DNA (mtDNA). Here, the mtDNA phylogeny groups

C. m. heymansi in the silver (*C. doggetti*) and blue monkey (*C. m. stuhlmanni*) clades, which according to their distribution map is the central African region, and is a distinct lineage from the Syke's monkey clade (*C. albogularis*) found in eastern and south Africa. Also, the mtDNA for *C. m. opisthostictus* falls outside the *C. mitis*, *C. albogularis*, and *C. nictitans* clades, and thus is basal to the entire *C. mitis* species group revealing a deep ancestry. This provides an interesting story to compare findings from my genetic analysis. However, a note about the museum skin samples used in their study is that the geographic information is limited as the museum skins have been preserved for years and some may only have minimal info from where exactly the specimens were collected. By contrast, the samples for my research each have a source population locality attached to them and contribute new info from the TL2 area that allow us to know where in the phylogeny ngoyi noir fit, and who they are closely related.

For my thesis research, I analyzed genetic sequence data from tissue samples of 10 male monkeys from the TL2 area to determine their taxonomic identity and evolutionary history. The 10 male samples are from monkeys identified by their phenotypes, and include five from the ngoyi noir population that most closely fit characteristics of the subspecies *C. m. heymansi*, also known as the Lomami River blue monkey. The other samples include two from the *C. m. stuhlmanni* clade, two from *C. wolfi wolfi*, and one from *C. wolfi elegans* (total 3 from the *C. mona* species group). I included the three *C. wolfi* samples in my study because they were new samples from the TL2 area, and could be used as a comparison to analyze the sequence differences for another pair of TL2 *Cercopithecus* species that have different phenotypes.

I isolated portions of the Testis-Specific Protein on the Y-chromosome (TSPY), amplified and sequenced each sample, then looked for variation via single nucleotide polymorphisms. The TSPY locus is a window into the population history of male inheritance. Previous studies have published phylogenetic information on the guenon groups using the TSPY gene and provide a baseline to compare my findings (Hart et al. 2012; Tosi et al 2005; Detwiler, 2010a).

More specifically, the TSPY marker is a gene family located on the non-recombining portion of the primate Y-chromosome (Hart et al. 2012, Tosi et al 2005), which reveals noticeable clade specific nucleotide mutations. The TSPY has very low variation, which means even one shared mutation is significant and thus single nucleotide polymorphisms can be used in phylogenetic studies.

In addition to my 10 samples, I included 34 genetic sequences of *Cercopithecus* species from GenBank, as well as unpublished sequences available in the FAU Molecular Anthropology Lab's database to help build my phylogenetic trees and provide context for my samples. I compared my trees to what is known about the blue monkey radiation. Ultimately, my molecular and phylogenetic analyses allowed me to test my hypothesis to determine if blue monkeys from TL2 are genetically distinct from other known *C. mitis* groups in Africa, or accept the null hypothesis that there is no significant genetic difference. In the next sections, I discuss the molecular methods used to obtain my sequences, and the phylogenetic analyses employed to make my evolutionary trees.

Table 2: List of source samples of Y-DNA sequences used to construct phylogenetic trees. Samples highlighted in yellow represent the 10 new sequences generated in this study. SD202 was used as a positive control to verify sequence results.

	Sample ID	Taxonomy	Sex	Origin	Source	GenBank Accession Number
1	R14697	*Allenopithecus nigroviridis*	M	Congo	National Museum, Scotland	AF284280
2	41137B	*Cercopithecus ascanius*	M	Central Africa	Louisiana Purchase Zoo	EF517804
3	DM3376	*Cercopithecus ascanius*	M	Kibale, Uganda	Columbia University	AY048054
4	FK105	*Cercopithecus ascanius schmidti*	M	Democratic Republic of Congo	TL2 Project, FAU Primate Lab	n/a
5	AK05IM04	*Cercopithecus ascanius schmidti*	M	Kenya, Kakamega Forest Reserve	FAU Primate Lab	n/a
6	AT05MM42	*Cercopithecus ascanius schmidti*	M	Tanzania, Mahale Mountains National Park	FAU Primate Lab	n/a
7	CcephusZoo	*Cercopithecus cephus*	M	Central Africa	Gulf Breeze Zoo	AY450874
8	CerythroChar	*Cercopithecus erythrogaster*	M	Mulhouse Zoo	Mulhouse Zoo	AY665648
9	1534	*Cercopithecus petaurista*	M	West Africa	Central Florida Zoo	AY897616
10	FK112	*Cercopithecus mitis heymansi*	M	Lutanga, east bank Lomami River	TL2 Project, FAU Primate Lab	n/a
11	GP603	*Cercopithecus mitis heymansi*	M	Lomami Forest	TL2 Project, FAU Primate Lab	n/a
12	JH015	*Cercopithecus mitis heymansi*	M	Camp Bonobo	TL2 Project, FAU Primate Lab	n/a
13	ME406	*Cercopithecus mitis heymansi*	M	Lomami Forest, TL2	TL2 Project, FAU Primate Lab	n/a
14	**SD202 AL**	*Cercopithecus mitis heymansi*	M	**Captured swimming across the Lomami River right to left bank**	TL2 Project, FAU Primate Lab	n/a
15	**SD202 KD**	*Cercopithecus mitis heymansi*	M	**Captured swimming across the Lomami River right to left bank**	TL2 Project, FAU Primate Lab	n/a
16	JH014	*Cercopithecus mitis stuhlmanni*	M	Ituri, DRC	TL2 Project, FAU Primate Lab	n/a
17	ME403	*Cercopithecus mitis stuhlmanni*	M	Tshopo Rainforest, DRC	TL2 Project, FAU Primate Lab	n/a
18	MK05IM19	*Cercopithecus mitis stuhlmanni*	M	Kenya, Kakamega Forest Reserve	FAU Primate Lab	n/a
19	5311	*Cercopithecus mitis stuhlmanni*	M	Dr. C. Lehn	Dr. C. Lehn	AY048057
20	MR05UM32	*Cercopithecus doggetti*	M	Nyungwe National Park	FAU Primate Lab	n/a
21	MT05AM112	*Cercopithecus albogularis*	M	Tanzania, Arusha, Mt. Meru	FAU Primate Lab	n/a
22	MK06GM92	*Cercopithecus albogularis*	M	Kenya, Gede Ruins	FAU Primate Lab	n/a
23	89M009	*Cercopithecus albogularis*	M	Africa	Cheyenne Mountain Zoo	EF517803
24	Newton	*Cercopithecus nictitans*	M	Central Africa	Baton Rouge Zoo	AY450878
25	SABF3	*Cercopithecus mona*	M	Grenada	Dr. M. Glenn and Dr. K. Bensen	AF284281
26	JH010	*Cercopithecus wolfi elegans*	M	Kindu, west bank Kasuku River	TL2 Project, FAU Primate Lab	n/a
27	GP601	*Cercopithecus wolfi wolfi*	M	Lomami Forest, TL2	TL2 Project, FAU Primate Lab	n/a
28	ME407	*Cercopithecus wolfi wolfi*	M	West side of Lomami River	TL2 Project, FAU Primate Lab	n/a
29	Nguma	*Cercopithecus wolfi wolfi*	M	Central Africa	San Antonio Zoo	AY450880
30	GAB108G	*Cercopithecus pogonias*	M	CIRMF, Gabon	CIRMF, Gabon	EF517805
31	GAB17	*Cercopithecus pogonias*	M	CIRMF, Gabon	CIRMF, Gabon	AY048059
32	CdianaZoo	*Cercopithecus diana*	M	West Africa	Gulf Breeze Zoo	AY195580
33	OR1646	*Cercopithecus hamlyni*	M	Central Africa	San Diego Zoo	AY450875
34	ME404	*Cercopithecus hamlyni*	M	Tshopo Rainforest	TL2 Project, FAU Primate Lab	JN106053
35	GP600	*Cercopithecus lomamiensis*	M	Lomami Forest, TL2	TL2 Project, FAU Primate Lab	JN106054
36	JH005	*Cercopithecus lomamiensis*	M	Obenge area, TL2	TL2 Project, FAU Primate Lab	JN106056
37	ME408	*Cercopithecus lomamiensis*	M	Lomami River, TL2	TL2 Project, FAU Primate Lab	JN106057
38	Sukari	*Cercopithecus neglectus*	M	Central Africa	Baton Rouge Zoo	AY450877
39	OMBOUE	*Allochrocebus solatus*	M	CIRMF, Gabon	CIRMF, Gabon	AY450879
40	Antwerp	*Allochrocebus lhoesti*	M	Central Africa	Antwerp Zoo	AY048055
41	VE98007	*Chlorocebus aethiops*	M	East Africa	Deparment of Anthropology, NYU	AY450872
42	R230	*Erythrocebus patas*	M	Africa	Department of Anthropology, CUNY	AY048064
43	PR00980 CypA3	*Colobus guerez*	M	Central Africa	unknown	JX896147
44	108806	*Macaca silenus*	M	Southwest India	National Zoo, Washington, D.C.	AF284237

Materials and Methods

Source material of biological non-human primate samples: field collection protocol

Between 2007 and 2010, fieldwork conducted in the TL2 area by the Lukuru Foundation's TL2 Project field teams yielded tissue samples from phenotypically different arboreal monkeys believed to be part of the *Cercopithecus mitis* or blue monkey group. Types of tissue samples included kidney, liver, ear, and muscle. These tissue samples were collected opportunistically from dead monkeys that resulted from bushmeat hunting. A clean razor blade was used to dissect pieces of tissue into 0.5 cubic cm or less. Samples were stored in 8 ml plastic tubes containing RNAlater buffer. Samples preserved in this way are stable for at least a week at room temperature, minimizing the need to immediately process or freeze the tissue. Also, RNAlater does not jeopardize the quantity and quality of the DNA obtained during the extraction process. To help the RNAlater buffer permeate the tissue, each sample was sliced before being submerged in five times or more volumes of buffer then shaken. The tissue samples were shipped to New York University, and transferred to Florida Atlantic University in Boca Raton, Florida, and stored at -20°C for future genetic processing and analysis. This study used 10 male monkey samples from the TL2 collection, which are highlighted in yellow on Table 2.

Molecular methods: DNA extraction to sequencing

During the months of June and July 2014, I conducted molecular analysis on 10 male monkey tissue samples in the new Molecular Anthropology Lab in FAU's Sanson Life Sciences Building. I used the Qiagen DNeasy Blood and Tissue Kit for DNA purification from animal tissue (Qiagen 69504; Valencia, CA). I extracted whole genomic DNA from all 10 samples making only minor modifications to the manufacturer protocol in the final elution step to maximize DNA yield and concentration.

I began the extraction process by macerating 25 mg of each tissue sample with a sterile scalpel on a sterile surface. Each tissue sample was then loaded into a 1.7 ml microcentrifuge tube with 180 ul of manufacturer provided buffer ATL and 20 ul of proteinase K. The addition of proteinase K to the sample is an important step in the DNA extraction process because it is a lysis buffer that allows for the release of nucleic acids from within the cell's nucleus and aids in the digestion of proteins and nucleases that could degrade nucleic acids. All 10 sample tubes were incubated overnight in a dry bath at 56°C, or until there were no visible pieces of tissue left in the lysate.

After incubation and digestion, the next step was to precipitate the DNA with ethanol. I added 200 ul of manufacturer provided buffer AL to each sample tube and mixed by vortex. Then, with a pipette I carefully transferred the sample mixture to a manufacturer provided spin column with 2 ml collection tube. The mixture was centrifuged at 8,000 rpm at room temperature for one minute and the flow-through in the collection tube was discarded. This step was repeated two more times, adding buffer and discarding the flow-through each time to ensure that no residual ethanol was carried over

during the final elution step. Residual ethanol can interfere with subsequent reactions downstream.

In the final step, I modified the manufacturer protocol to obtain the highest concentration and yield of DNA from our samples. The protocol suggests eluting 200 ul of DNA into one tube. Instead, I performed two successive elution steps opting for two tubes at 100 ul each. My purpose for performing two elutions was to obtain the maximum DNA yield in the first elution to use for research. Normally, the first 100 ul will have a higher concentration of DNA than the second elution because most of the DNA was washed through in the first 100 ul leaving the second elution less concentrated overall.

To confirm DNA was eluted, and to estimate nucleic acid concentration per sample, I used a spectrophotometer to measure the amount of ultraviolet light absorbed by the bases at a wavelength of 260 nm (Bio-Rad 170-2501; Hercules, CA). Optimal measurements at this wavelength should fall within the range of 0.1 to 1.0. After recording all spectrophotometer measurements, I used these values in the equation below to estimate the concentration of DNA for each sample.

Estimate DNA Concentration = (260 nm measurement)(Dilution Factor)(50)

Equation 1: Formula to estimate nucleic acid concentration determined by multiplying the 260 nm measurement by a 1:25 dilution factor by a mass/absorption conversion factor of 50 ul/ml.

The practical objective for this step was to determine the concentration of our samples and to dilute them for use in downstream applications. The minimum amount of DNA needed in a polymerase chain reaction (PCR) is 5 ng. Concentrations over 5 ng/ul contain excess genetic material. All of my sample DNA extractions resulted in

concentrations well over 5 ng/ul (see Table 3). I chose to make a working stock of DNA at a 1:10 dilution factor for each sample to increase the working amount of DNA and to not waste DNA by overloading each reaction with too much genetic material.

Table 3: List of spectrophotometer measurements and estimated DNA concentrations for all 10 sample elutions.

Sample ID	Spectro-photometer measurement at wavelength 260 nm	Estimated concentration of DNA	Working stock DNA at 10x dilution (brought up with H2O)
FK112	0.325	406.25 ng/ul	40.625 ng/ul
	0.133	166.25 ng/ul	
GP603	*0.073	91.25 ng/ul	9.125 ng/ul
	*0.045	56.25 ng/ul	
JH015	0.356	445 ng/ul	44.5 ng/ul
	0.124	155 ng/ul	
ME406	0.267	333.75 ng/ul	33.375 ng/ul
	0.159	198.75 ng/ul	
**SD202	0.112	280 ng/ul	28.0 ng/ul
JH014	0.442	552.5 ng/ul	55.25 ng/ul
	0.297	371.25 ng/ul	
ME403	0.473	591.25 ng/ul	59.125 ng/ul
	0.132	165 ng/ul	
**JH010	0.132	165 ng/ul	16.5 ng/ul
GP601	0.841	1051.25 ng/ul	10.51 ng/ul
	0.478	597.5 ng/ul	
ME407	0.301	376.25 ng/ul	37.625 ng/ul

Notice the first elution resulted in higher spectrophotometer measurement, and thus concentration. For each sample, only the first elution of DNA was used at 1:10 dilution factor. Single asterisk indicates a spectrophotometer measurement was outside the optimal range. Double asterisk indicates no second elution for that sample.

The next step involved isolating regions of the Testis-Specific Protein on the Y-chromosome (TSPY) and preparing for amplification via polymerase chain reaction. Below, I describe the primers and protocols used to amplify portions of the TSPY from my samples.

I used TSPY amplification primer pairs 170F and 691R, and 1710F and 2160R (Table 4 provides each primer's oligonucleotides). I labeled these amplification primers pairs as Y1 (170F and 691R) and Y2 (1710F and 2160R) for PCR purposes. These primers were used to prime the forward and reverse ends of specific gene fragments on the TSPY. The length of each gene fragment is 533 bp for Y1 and 445 bp for Y2.

Table 4: TSPY primers used in PCR reactions (Tosi et al., 2005; Detwiler, 2010a).

Amplification primers	**5' to 3'**
170F	GGC GTC GTT GTG ACC ATT TG
691R	GTG GTT TGG AAT CTG ACT GAG GTC
*primers 170F/691R = Y1	
1710F	AAC TGT GGA GTC TTA TGC CCA
2160R	GCA TCT CCT CTG AAC CAC CAT
*primers 1710F/2160R = Y2	

Next, amplification was achieved via PCR using 5.5 ul nuclease free water, 3 ul of 1:10 diluted DNA, 2 ul each forward and reverse primers, and 12.5 ul GoTaq Green Master Mix 2x (Promega M7121; Madison, WI) for a total volume of 25 ul per reaction (see Table 5).

Table 5: PCR protocol reagents and concentrations used in each PCR reaction.

PCR protocol		
Ingredient	**Working Concentration**	**Volume (ul)**
H20		5.5 ul
DNA	see Table 3	3 ul
F primer	10 uM	2 ul
R primer	10 uM	2 ul
GoTaq MM2X	2x	12.5 ul
TOTAL		25 ul

Polymerase chain reaction was performed twice on all samples, for Y1 and Y2, using 60 cycles of the following parameters: denature at 94°C for 25 seconds, anneal at 56°C for 36 seconds, and extend at 72°C for 40 seconds (see Table 6).

Table 6: PCR cycling parameters (Detwiler, 2010a)

PCR cycling parameters:				
	Denature	**Denature**	**Anneal**	**Extend**
Temperature	94	94	56	72
Time	0:50	0:25	0:36	0:40
Repeat	1x		60x	
		Extend	**End**	
Temperature		72	22	
Time		7:00	infinity	
Repeat		1x	1x	

After PCR, I performed DNA electrophoresis on a 2% agarose gel for each PCR product. This is an important step to confirm that the targeted fragments of the gene amplified and that the size of each fragment matched the expected band length on the gel. To determine the proper gel percent for electrophoresis, I needed to know the total number of base pairs in each of my gene fragments. Short fragments move faster through the gel than longer fragments. Increasing the percentage of agarose creates a more viscous matrix slowing down the travel of short fragments so they do not run off the gel. According to the manufacturer of my gel rigs (ThermoScientific B1A; www.thermoscientific.com), DNA fragments that range from 100 to 3,000 base pairs should be run on a 2% agarose gel. For my gene fragments, Y1 is approximately 533 bp and Y2 is approximately 445 bp. Both fall within the base pair range for a 2% agarose gel. Therefore, I ran 2% gels for all PCR products, one for Y1 and one for Y2.

For each gel, I used a 1 kb ladder in the first lane on the left. Then, I carefully load 5 ul of PCR product for each sample into each well via pipette, labeled my lanes, and then set the power supply to 120 V for 40 minutes per gel. When the run was complete, I visualized my gels on an ultraviolet transilluminator to confirm that my DNA fragments migrated to the appropriate position based on the 1kb ladder reference.

Unfortunately, I was not able to sequence my samples in the Molecular Anthropology Lab because we did not have access to sequencing equipment. Instead, I prepped my PCR products to be shipped out to Eton BioSciences in Research Triangle Park, North Carolina for Sanger sequencing. In order for Eton BioSciences to sequence my PCR products, I needed to provide them with sequence primers.

I used sequence primers 202F and 667R to sequence Y1 PCR products. I used sequence primers 1745F and 2119R to sequence Y2 PCR products (Table 7 provides each sequence primer's oligonucleotides). Sequences obtained from my PCR products were used to build my phylogenetic trees.

Table 7: Sequence primers per Tosi et al. 2005 and Detwiler 2010a sent to Eton Biosciences to sequence my PCR products.

Sequence primers	**5' to 3'**
202F	GAA CGA GGG TGA GTT TCC ACA G
667R	AGA GCC TTG AGA TGC AAT GGG A
*primers 202F/667R used to sequence Y1	
1745F	TGT CCA CAC TAA CTG AGA AGT A
2119R	ACT GCC TGC TGA GAA AAG ACT ACC
*primers 1745F/2119R used to sequence Y2	

Phylogenetic methods: sequence and contig assembly to estimating trees

Eton BioSciences sequenced my PCR products and produced raw sequence results. These raw sequences required editing and trimming prior to contig assembly and tree construction. First, I visualized my sequence chromatograms using the software 4Peaks (Griekspoor et al., 2006). This software allowed me to compare the forward and reverse for each sample's Y1 and Y2 gene fragments. I analyzed peak height and clarity for each base call and trimmed flanking regions of each sequence that were not readable or reliable.

Next, I transferred my edited sequences from 4 Peaks into the software program Sequencher (2014). In Sequencher, I made final trims by eye for Y1 and Y2 and assembled contiguous alignments of overlapping sequences for each sample. Final edits brought each gene fragment total to 513 bp for Y1 and 406 bp for Y2, 919 bp in total.

After Sequencher, I finalized contigs in TextWrangler (Siegel et al., 2014) and then imported all sequences into the last software program, MEGA 6.06 (Tamura et al., 2013). In addition to my 10 samples, I included 34 genetic sequences of *Cercopithecus* species from GenBank, as well as unpublished sequences from Detwiler's dissertation (2010a) to build my phylogenetic trees and provide context for my samples. I also ran a positive control on sample SD202, a male blue monkey from TL2, to confirm my results with Dr. Detwiler's previous research on the same individual. My results verified repeatable sequences for SD202. This verification provided me with positive reinforcement that my methodology for processing my samples worked and validates variation in my samples. In MEGA, I aligned all 44 sample sequences using MUSCLE,

and then conducted phylogenetic analyses using neighbor joining (NJ) and maximum likelihood (ML) with 1000 bootstrap replications to estimate the reliability of my trees.

Results

Informative Sites

Results of the sequence alignments for this portion of the TSPY gene revealed clade specific mutations (see Table 8). There are 12 informative variable sites, six within the *Cercopithecus mitis* (*C. mitis*) species group, and six within the *Cercopithecus mona* (*C. mona*) species group.

All *C. mitis* sequences in this study share a derived clade specific mutation at site 163 to the exclusion of other *Cercopithecus* species, demonstrating strong support for monophyly of the *C. mitis* species group. Interestingly, the five *C. mitis heymansi* (samples FK112, GP603, JH015, ME406 and SD202) do not cluster with other conspecific *C. mitis* from DRC (*C. mitis stuhlmanni*) or those close to the TL2 region (*C. doggetti*). Instead, the five *C. mitis heymansi* samples share a synapomorphic single nucleotide polymorphism at site 802 with *C. albogularis,* a population in East Africa near the Indian Ocean far from TL2 (see Fig. 6). Although the TL2 *mitis* Y-DNA is most closely related to the Y-DNA haplotype of *C. albogularis,* the five *C. mitis heymansi* samples are distinct from *C. albogularis* in having one mutation shared among them, a synapomorphy at site 828.

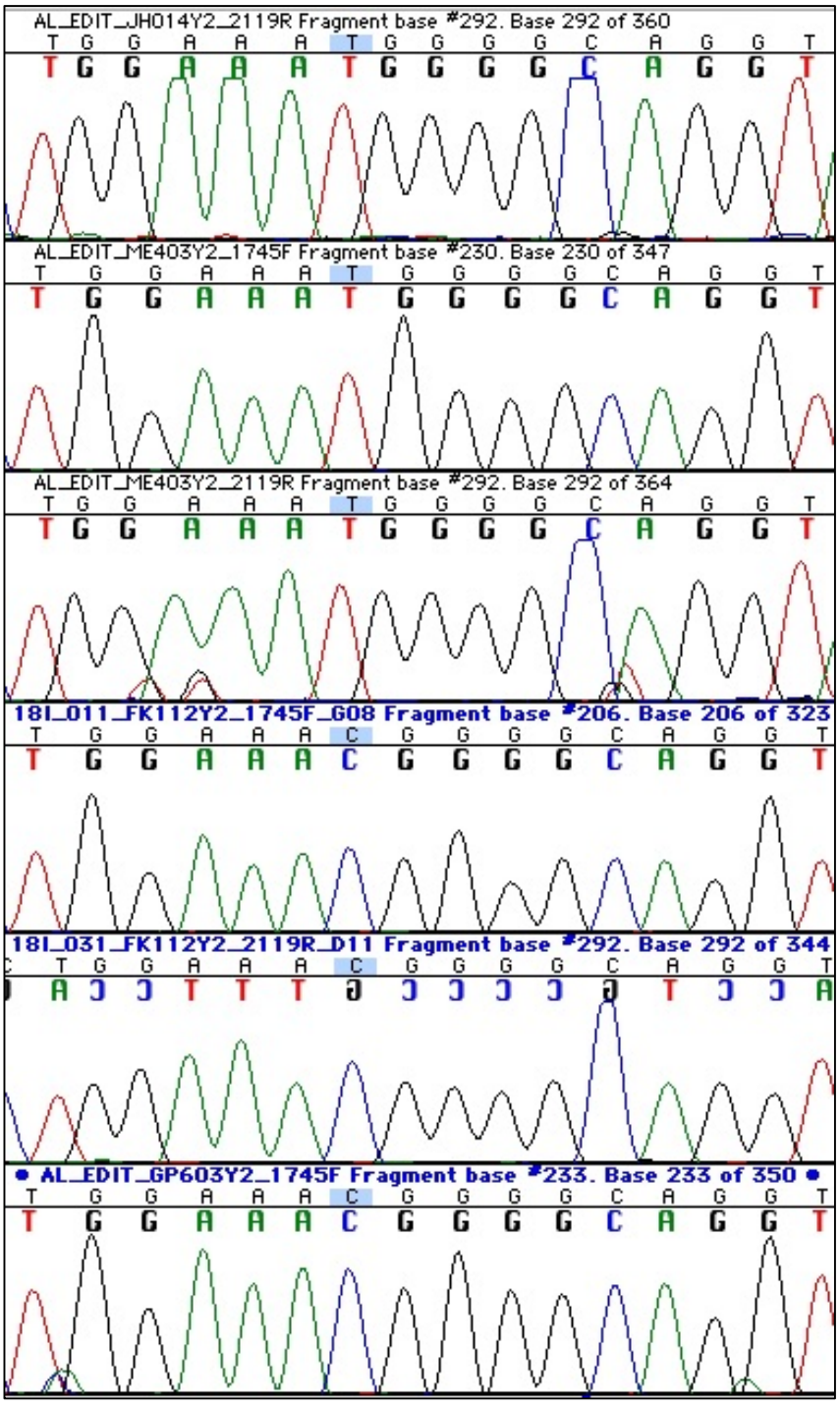

Figure 6: Chromatogram with shared derived mutation (T changed to C) at site 802 for *C. m. heymansi* and *C. albogularis*.

The *C. mitis stuhlmanni* sequences from the DRC (JH014 and ME403) generated in this study have two nucleotide substitutions (sites 46 and 840) in common with the Y-DNA haplotype shared by *C. mitis stuhlmanni* from Kenya and *C. doggetti* from Rwanda. These new sequences also share a distinct mutation at site 188 to the exclusion of the *C. mitis stuhlmanni* and *C. doggetti* samples. Further examination of sites 46 and 840 reveals no differentiation between *C. doggetti* from Nyungwe and *C. mitis stuhlmanni* of Kenya. These lineages are identical for all 919 base pairs. This indicates there are multiple Y-DNA lineages existing within *C. m. stuhlmanni*, and this subspecies appears to be paraphyletic.

For the same fragment of the TSPY gene, the sequence data for *C. mona, C. wolfi elegans, C. wolfi wolfi*, and *C. pogonias* reveal three clade specific mutations at sites 327, 338 and 411, demonstrating strong support for monophyly of the *mona* species group. It appears that *C. mona* retains an ancestral trait at site 207 while all others in the clade have the derived mutation. *C. wolfi wolfi* from central Africa and *C. wolfi elegans*/*C. wolfi wolfi* from TL2 (JH010, GP601 and ME407) also appear to retain ancestral traits at sites 187 and 339, and show no variation within them. However, *C. mona* and *C. pogonias* from Gabon in West Africa are genetically distinct sharing two mutations at sites 187 and 339. Overall, both *C. mitis* and *C. mona* species groups reveal genetic variation with regard to their geographic locations.

Table 8: TSPY synapomorphies and derived clade specific mutations for *mitis* and *mona* species groups. Samples highlighted in yellow represent the 10 new sequences generated in this study. Grey highlight identifies derived clade specific mutations for the *C. mitis* species group. Blue highlight indicates the TL2 *C. m. heymansi* autapomorphy. Purple highlight indicates the synapomorphy clustering *C. m. heymansi* with *C. albogularis*, commonly known as the Sykes' monkeys. Green highlight indicates the synapomorphies for the blue and silver monkey cluster represented by *C. m. stuhlmanni* and *C. doggetti* respectively. Red highlight indicates the DRC *C. m. stuhlmanni* autapomorphy. Orange highlight identifies mutations for the *C. mona* species group.

Sample ID	Taxonomy	Site 188	Site 46	Site 840	Site 163	Site 802	Site 828	Site 327	Site 338	Site 411	Site 207	Site 187	Site 339
R14697	*Allenopithecus nigroviridis*	T	A	A	A	T	T	C	A	A	G	A	C
41137B	*Cercopithecus ascanius*	T	A	A	A	T	T	C	A	A	G	A	C
DM3376	*Cercopithecus ascanius*	T	A	A	A	T	T	C	A	A	G	A	C
FK105	*Cercopithecus ascanius schmidti*	T	A	A	A	T	T	C	A	A	G	A	C
AK05IM04	*Cercopithecus ascanius schmidti*	T	A	A	A	T	T	C	A	A	G	A	C
AT05MM42	*Cercopithecus ascanius schmidti*	T	A	A	A	T	T	C	A	A	G	A	C
CcephusZoo	*Cercopithecus cephus*	T	A	A	A	T	T	C	A	A	G	A	C
CerythroChar	*Cercopithecus erythrogaster*	T	A	A	A	T	T	C	A	A	G	A	C
1534	*Cercopithecus petaurista*	T	A	A	A	T	T	C	A	A	G	A	C
FK112	*Cercopithecus mitis heymansi*	T	A	A	G	C	C	C	A	A	G	A	C
GP603	*Cercopithecus mitis heymansi*	T	A	A	G	C	C	C	A	A	G	A	C
JH015	*Cercopithecus mitis heymansi*	T	A	A	G	C	C	C	A	A	G	A	C
ME406	*Cercopithecus mitis heymansi*	T	A	A	G	C	C	C	A	A	G	A	C
SD202 AL	*Cercopithecus mitis heymansi*	T	A	A	G	C	C	C	A	A	G	A	C
SD202 KD	*Cercopithecus mitis heymansi*	T	A	A	G	C	C	C	A	A	G	A	C
JH014	*Cercopithecus mitis stuhlmanni*	C	G	G	G	T	T	C	A	A	G	A	C
ME403	*Cercopithecus mitis stuhlmanni*	C	G	G	G	T	T	C	A	A	G	A	C
MK05IM19	*Cercopithecus mitis stuhlmanni*	T	G	G	G	T	T	C	A	A	G	A	C
5311	*Cercopithecus mitis stuhlmanni*	T	G	G	G	T	T	C	A	A	G	A	C
MR05UM32	*Cercopithecus doggetti*	T	G	G	G	T	T	C	A	A	G	A	C
MT05AM112	*Cercopithecus albogularis*	T	A	A	G	C	T	C	A	A	G	A	C
MK06GM92	*Cercopithecus albogularis*	T	A	A	G	C	T	C	A	A	G	A	C
89M009	*Cercopithecus albogularis*	T	A	A	G	C	T	C	A	A	G	A	C
Newton	*Cercopithecus nictitans*	T	A	A	G	T	T	C	A	A	G	A	C
SABF3	*Cercopithecus mona*	T	A	A	A	T	T	G	C	G	G	G	G
JH010	*Cercopithecus wolfi elegans*	T	A	A	A	T	T	G	C	G	C	A	C
GP601	*Cercopithecus wolfi wolfi*	T	A	A	A	T	T	G	C	G	C	A	C
ME407	*Cercopithecus wolfi wolfi*	T	A	A	A	T	T	G	C	G	C	A	C
Nguma	*Cercopithecus wolfi wolfi*	T	A	A	A	T	T	G	C	G	C	A	C
GAB108G	*Cercopithecus pogonias*	T	A	A	A	T	T	G	C	G	C	G	G
GAB17	*Cercopithecus pogonias*	T	A	A	A	T	T	G	C	G	C	G	G
CdianaZoo	*Cercopithecus diana*	T	A	A	A	T	T	C	A	A	G	A	C
OR1646	*Cercopithecus hamlyni*	T	A	A	A	T	T	C	A	A	G	A	C
ME404	*Cercopithecus hamlyni*	T	A	A	A	T	T	C	A	A	G	A	C
GP600	*Cercopithecus lomamiensis*	T	A	A	A	T	T	C	A	A	G	A	C
JH005	*Cercopithecus lomamiensis*	T	A	A	A	T	T	C	A	A	G	A	C
ME408	*Cercopithecus lomamiensis*	T	A	A	A	T	T	C	A	A	G	A	C
Sukari	*Cercopithecus neglectus*	T	A	A	A	T	T	C	A	A	G	A	C
OMBOUE	*Allochrocebus solatus*	T	A	A	A	T	T	C	A	A	G	A	C
Antwerp	*Allochrocebus lhoesti*	T	A	A	A	T	T	C	A	A	G	A	C
VE98007	*Chlorocebus aethiops*	T	A	A	A	T	T	C	A	A	G	A	C
R230	*Erythrocebus patas*	T	A	A	A	T	T	C	A	A	G	A	C
PR00980 CypA3	*Colobus guerez*	T	A	A	A	T	T	C	A	A	G	A	C
108806	*Macaca silenus*	T	A	A	A	T	T	C	A	A	G	A	C

Phylogenetic relationships and Y-chromosomal patterns

Neighbor joining and maximum likelihood analyses confirmed the above mentioned clade specific mutations and resulted in congruent Y-chromosomal topologies depicting a five way polytomy within the genus *Cercopithecus* (see Figs. 7 and 8). For my outgroup taxa, I chose terrestrial guenons (*Allochlorcebus*, *Chlorocebus*, and *Erythrocebus*), *Macaca silenus*, and *Colobus guereza*, of which all fell basal to the arboreal guenons in both analyses.

The *C. mitis* species group clade clearly shows the *C. mitis heymansi* samples clustering with *C. albogularis* forming a monophyletic sister lineage to *C. mitis stuhlmanni* and *C. doggetti*. In addition, both trees depict the genetic distinctiveness of the DRC *C. mitis stuhlmanni* samples. Both topologies support three lineages in the *C. mitis* species group: a Sykes' monkey lineage recognized as *C. albogularis*, a blue and silver monkey cluster recognized as *C. mitis stuhlmanni* and *C. doggetti* respectively, and *C. nictitans*.

The *mona* species group topology reveals lineage branching patterns based on geographic locations. *C. mona* from West Africa is separated by one lingering ancestral trait and forms a lineage with its closest neighbor *C. pogonias* from Gabon in West Africa. Furthermore, *C. wolfi wolfi* and *C. wolfi elegans* from TL2 appropriately share a common ancestor with *C. wolfi wolfi* from Central Africa, which separates them from the western most lineages and places them into their own monophyletic clade with respect to their geographic origins. A robust node value at the base of the entire *mona* species group provides strong support for this clade.

Some nodes indicate high bootstrap values, which are a result of how many times the statistical algorithm was able to replicate the selected tree. Higher numbers provide stronger statistical support that the branching pattern likely occurred based on the sequence data. For instance, high bootstrap values at the base of the *C. mitis heymansi/C. albogularis* clade, and at the base of the *C. mitis stuhlmanni/C. doggetti* clade show strong support for distinct lineages (see Figs. 7 and 8). The TSPY marker is highly conserved with little variation, any samples that have at least one change should be considered unique for that clade and provides a robust phylogenetic signal for support of that clade.

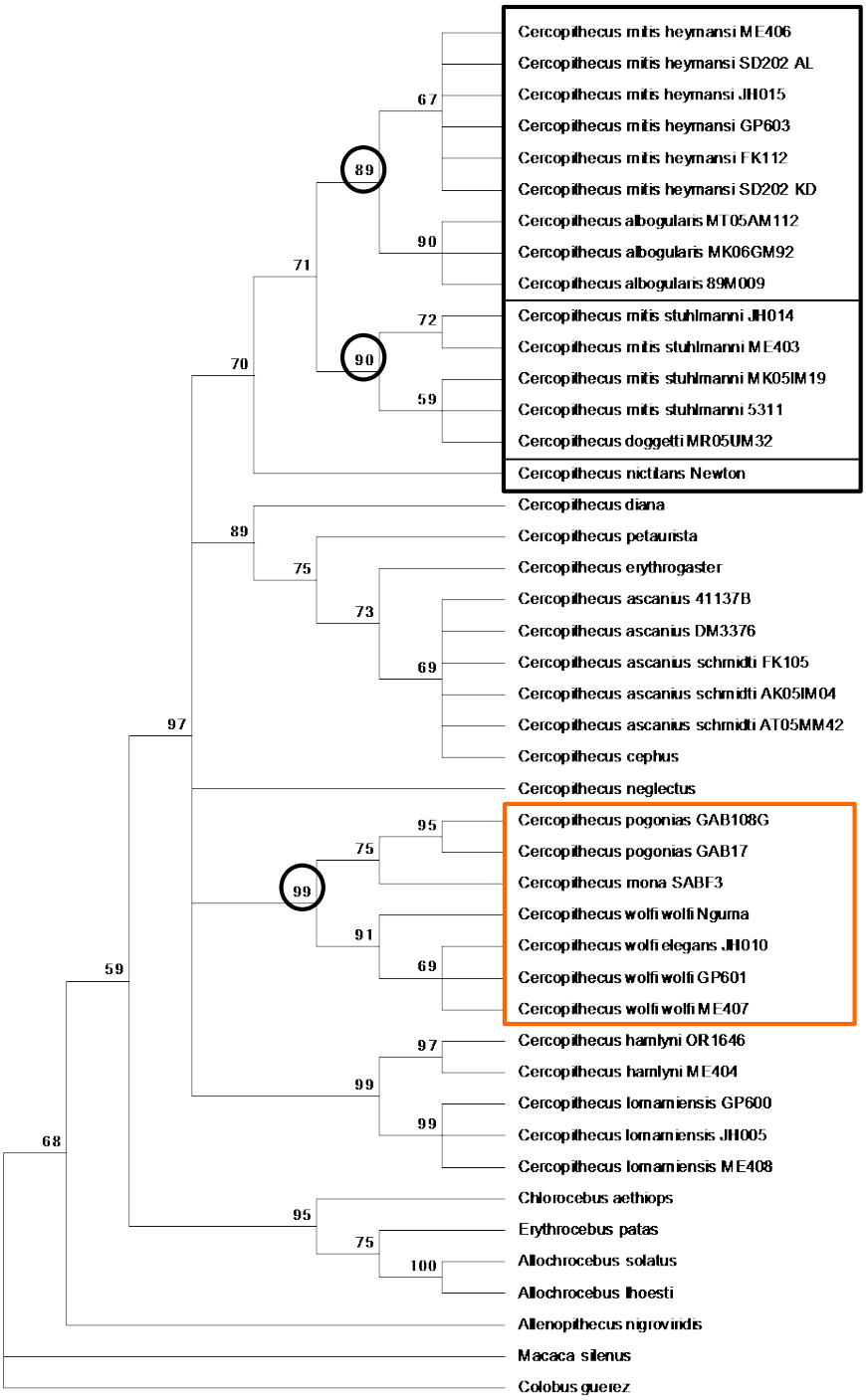

Figure 7: Evolutionary relationships of guenon taxa and relative branch lengths of the different lineages inferred using the Neighbor-Joining method with 1000 bootstrap replicates; bootstrap values are shown above the branches. The evolutionary distances were computed using the p-distance method. The analysis involved 44 nucleotide sequences. Two fragments of the TSPY were trimmed to a total of 919 bp in the final dataset. This topology is congruent with the Maximum Likelihood tree in Fig. 8.

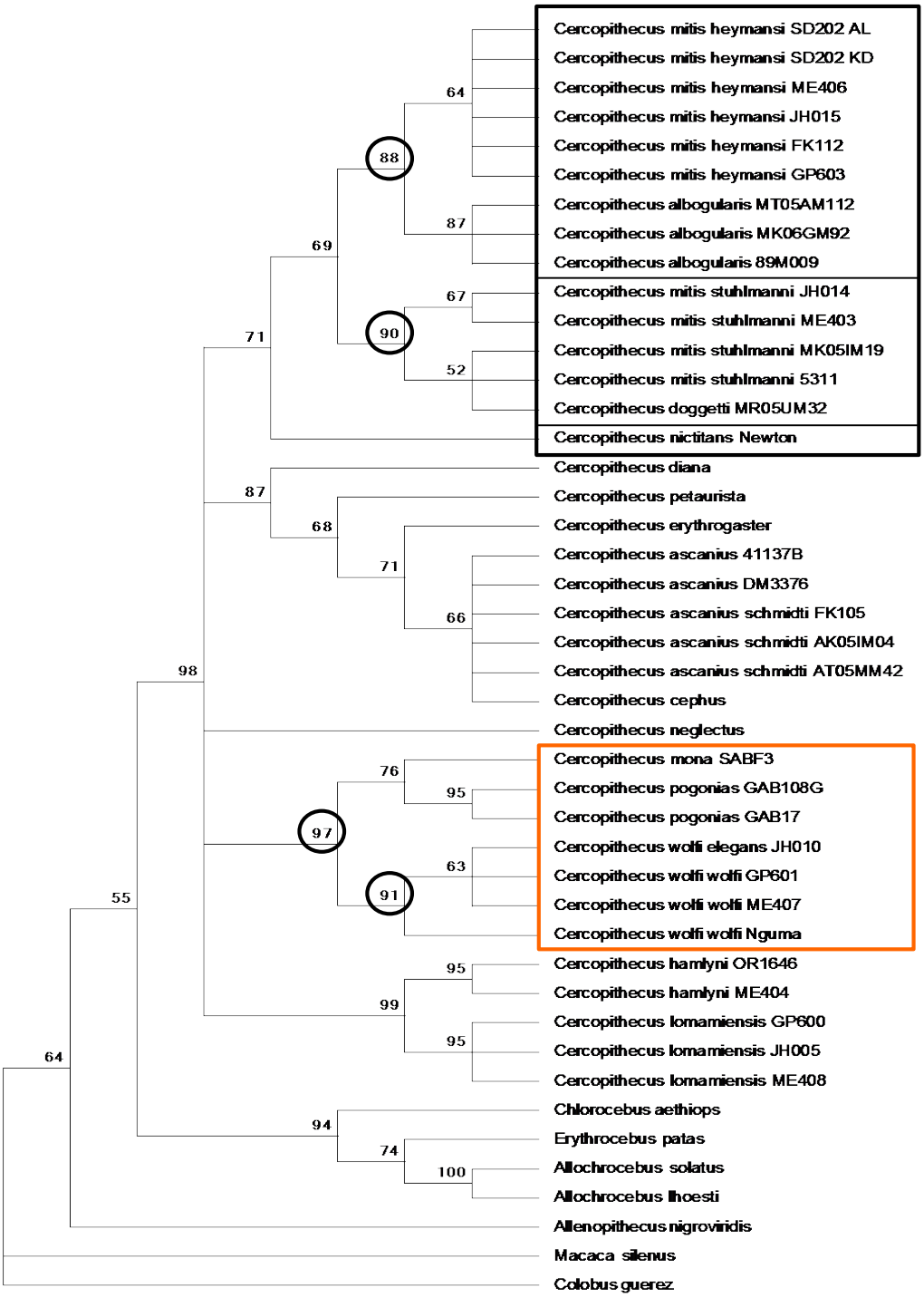

Figure 8: Maximum Likelihood tree based on the Jukes-Cantor model with 1000 bootstrap replicates; bootstrap values are shown above the branches. The tree with the highest log likelihood (-2097.3124) is shown. The analysis involved 44 nucleotide sequences. Two fragments of the TSPY were trimmed to a total of 919 bp in the final dataset.

Discussion

The focus of this discussion is to provide potential explanations for distribution and biogeography of the blue and mona groups, and their taxonomic evaluation and identification.

Distribution and biogeography

A strong pattern emerged with the five ngoyi noir blue monkey samples from TL2 that interestingly placed them in a clade with *C. albogularis* from East Africa near the Indian Ocean. This Y-chromosomal pattern suggests that the ngoyi noir, or Lomami River *C. m. heymansi*, may have shared more recent genetic contact with their *C. albogularis* neighbors at the headwaters of the Congo River than with conspecifics further to the northeast (*C. m. stuhlmanni*) signaling the Congo River as a biogeographic barrier for the blue monkey group, and a southern dispersal route around the central basin and Congo River as a possible answer for a shared Y-DNA haplotype.

My assessment agrees with Guschanski et al. (2013) on the biogeography of guenons. Their time range overlap test is consistent with allopatric speciation and dispersal eastward due to climatic fluctuations throughout the last 10my. Guschanski et al. (2013) reconstructed ancestral geographic ranges, which indicate guenons originated in West Africa where several lineages still remain. Furthermore, Butynski et al. (2013) states that the area between the Lualaba and Lomami Rivers, where *C. m heymansi*

resides, is an ideal forest habitat that could harbor many remnant ranges with members now widespread throughout Africa.

The TL2 ngoyi noir samples, or *C. m. heymansi*, used in this study have one synapomorphic mutation shared among them indicating *C. m. heymansi* is an isolated lineage in the DRC central basin to the exclusion of eastern populations. This could also explain TL2 as a relic range of tropical forest with long established climatic stability where ancestral lineages ultimately dispersed outward and eastward.

Current distribution maps (see Fig. 1) depict the Congo River as an important biogeographic barrier for populations of blue monkeys and other guenons. Hypotheses of dispersal events track guenon lineages eastward in a northern route around the Congo River toward the Rift Valley, or following a southern route down around the Congo basin crossing at the headwaters of the Congo River (Tosi, 2008; Guschanski et al., 2013). In the past, the Congo basin's relatively low altitude and many river tributaries may have coalesced into a larger body of water becoming impenetrable to guenons and other species (Tosi, 2008; Guschanski et al., 2013), or trapping and isolating populations within the central basin. This would have made dispersal through the basin impassable, instead forcing dispersal patterns around either to the north or south. Populations coming back into contact with one another either from a direct crossing or circumnavigation of the river may explain the current pattern of overlap of Y-DNA haplotypes.

As for the TL2 *C. wolfi elegans* and two *C. wolfi wolfi* samples, there is strong support for geographic origin and a common ancestor in Central Africa, which separates them from the western lineages of *C. mona* and *C. pogonias* from West Africa. In opposition to the movement east of *C. mitis*, the *C. mona* species group shows evidence

of a western dispersal pattern (Guschanski et al., 2013) beginning northwest of the Congo River and moving into Gabon then towards Cameroon, Nigeria, and Ghana.

The geographic locations of these groups may be linked to a male dispersal pattern. It is common among *Cercopithecines* for males to leave their natal group to seek out new social groups of conspecific females to mate with (Stanford et al., 2013). This could be the reason for paraphyletic lineages on the Y chromosome. Over time males may venture far beyond their natal range possibly following female mating opportunities, as well as ideal forest habitats and feeding resources.

Taxonomic implications

This study provides new research on the Y-DNA of TL2 *C. mitis heymansi*. Guschanski et al. (2013) published a mtDNA phylogeny on guenons from areas north and south of the TL2 study area. I assumed our phylogenetic stories would be congruent, but interestingly they are not: my Y-DNA phylogenetic analysis is different from the mtDNA results.

In Guschanski et al. (2013), *C. m. heymansi* mtDNA groups with all other *C. mitis* subspecies. However, according to my data, Y-DNA for TL2 ngoyi noir, or *C. m. heymansi*, groups with *C. albogularis* and forms a reciprocally monophyletic clade to the exclusion of the other blue monkeys. Even though my data show a close Y-DNA haplotype link for *C. m. heymansi* and *C. albogularis* indicating these lineages share a recent common ancestor, there is one synapomorphic mutation within the *C. m. heymansi* samples that infers their distinct lineage within the TL2 landscape. Thus, these results address the objective of the study, identifying a genetic difference of the TL2 ngoyi noir population from all other *C. mitis* populations.

On the other hand, there is no distinction between the Kenyan *C. m. stuhlmanni* and *C. doggetti* sequences. This indicates a need for further genetic loci and samples to be screened to assess the species level classification for *C. doggetti* due to lack of variation between itself and the *C. m. stuhlmanni* subspecies.

In the same light, the TL2 *C. wolfi wolfi* and *C. wolfi elegans* samples do not reveal any variation between all three sequences. Although they have different phenotypes, my Y-DNA data show there is no genetic variation between these populations warranting reevaluation of these as two separate subspecies and possibly combining them into one classification.

In order to construct the most accurate tree, I analyzed homologous traits and identified characteristics that qualify as synapomorophies. I used species-specific synapomorphies in the TSPY of my samples based on previously published studies of guenon phylogeny using the full TSPY locus (>2kb pairs), which is a gene located on the non-recombining portion of the primate Y-chromosome (Tosi et al., 2005). It is noteworthy to mention that the marker used in this study is relatively conserved, but does exhibit enough variation to detect differences between closely related taxa. Thus, samples with at least one substitution shared in common can be identified as a genetically distinct lineage.

Another important point to keep in mind, and a possible explanation for the discrepancies in my nuclear compared to mtDNA phlylogenetic trees, is mtDNA is linked through maternal lineages only and does not cross over with paternal DNA. However, nuclear DNA from the Y chromosome is linked through paternal lineages only and is the male version of mtDNA undergoing minimal recombination as well (Stanford et al.,

2013). The results of the nuclear versus mtDNA phylogenetic trees are from opposing sexes that could have opposing dispersal and migration stories.

Ultimately, taxonomy is not definitive and species are always being redefined. Even estimating the right phylogenetic tree doesn't truly exist. Instead, the reason for estimating phylogenetic trees is to try to create the closest approximate order of taxa from a common ancestor using genetic sequences, and to calculate change within each branch and between diverging events (Hall, 2011). However, this may not always be a complete and accurate story of historical events or the absolute branching pattern (Hall, 2011). Rather, estimating a correct phylogenetic tree will mostly approximate what may have happened in the past.

Conclusion and Future Research

Guenons are a model system for studying diversity within a group and the evolutionary processes that act on a population. The objective of this research was to shed light on the diversity of blue monkeys in the TL2 area, a relatively new study location, and to add to the existing knowledge about their lineage and classification. This research falls under the larger discipline of conservation genetics because it used genetics to identify the uniqueness of the TL2 *C. mitis heymansi* population. More specifically, the importance of conservation genetics in anthropology is to recognize the variety of primate species and to manage and sustain their diversity. Climate fluctuations and the biogeography of landscapes shape habitats over time, and with it the distribution of forest fauna. Studying the Y-chromosomal biogeography of these new lineages of blue monkeys found in the TL2 area provides new information on their evolutionary history.

My research used Y-DNA to confirm the new population of *Cercopithecus mitis heymansi* in the TL2 region is an evolutionarily distinct lineage, and is indeed different from other surveyed *C. mitis* lineages in Africa. We can use this new information to extend the known range and distribution of *Cercopithecus mitis* into this gap area where TL2 falls between known populations of blue monkeys. More research is needed to complete the story of *C. m. heymansi,* and *C. wolfi*. Future research should include sequencing mtDNA of the 10 TL2 samples in order to build trees and compare phylogenies. There is also a need to sequence the entire TSPY locus. I examined only a small portion of TSPY, which still showed variation, but when expanded could reveal

more genetic distinction due to geographic location. Lastly, *C. m. opisthostictus* is clearly very distinct and a basal lineage that is a deep ancestral outgroup to *C. mitis*. It would be very interesting to see what the Y-DNA story is for this subspecies considering its geographic location and proximity to *C. m. heymansi*.

There is an urgency to conduct research on blue monkeys in TL2 because several real pressures threaten their survival. Mittermeier et al. (2013) mention that *C. m. heymansi*, or the Lomami River blue monkey, population status is unknown and is expected to have experienced significant decline over the past two decades due to relatively small and isolated distributions threatened by human activity. Human activity, such as bushmeat hunting and deforestation by logging for timber and agriculture, creates a severe problem in the DRC and has resulted in ecological destruction, habitat loss, local extirpation, and forest fragmentation separating faunal populations (Keonig, 2008; Mittermeier et al., 2013). Blue monkeys are arboreal creatures living a life in trees that prefer a diet rich in fruit. They are also important seed dispersers of forest tree species, making blue monkeys a critical element in the long-term survival and rejuvenation of the TL2 forest and ecosystem.

Ultimately, wildlife species are a source of scientific and educational value. It is important to consider the proper approach for conservation management to preserve biodiversity and to prevent extinction of species. The goal of this study is to provide new and valuable contributions of molecular data to the biogeographic and phylogenetic story of *Cercopithecus mitis*. By studying these newly documented forms of blue monkey in TL2, we can better articulate their genetic diversity, taxonomic identification, evolutionary history, and geographic distributions. In turn, this information can provide

valuable insight into the genetic diversity of one of the most diverse radiations of primates.

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