Genetic consequences of occupying a highly fragmented landscape among ring-tailed lemurs (*Lemur catta*) in south-central Madagascar

by

Tara Anne Clarke
MA, New Mexico State University, 2009
BA, Stony Brook University, 2005

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in the Department of Anthropology

Supervisory Committee

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Abstract

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Global climate change and habitat fragmentation represent two of the greatest threats to biodiversity and ecological processes worldwide. It is predicted that anthropogenic induced climate change could represent a key factor for extinctions in the near future, considering that the Earth is set to become warmer than at any period in the past 40 million years. Habitat fragmentation and isolation pose a number of challenges for the fauna inhabiting degraded areas, including lack of dispersal opportunities leading to inbreeding resulting in a loss of genetic diversity, reduced reproductive fitness; increases in vulnerability to predation, hunting, and disease, and an inability to deal with or respond to environmental changes and/or disease.

Madagascar, the fourth largest island in the world, is home to unprecedented levels of endemism, including over 100 species of lemur. The island has undergone a range of historical and contemporary landscape transformations, both natural and anthropogenic. These landscape transformations combined with additional human-induced disturbances, such as the illegal pet and bushmeat trades, have had devastating effects on the island's extant primate populations. Thus, Madagascar's lemurs have been deemed the most endangered group of mammals and now represent the highest primate conservation priority in the world.

The ring-tailed lemur (*Lemur catta*) is endemic to the southern regions of the island and occupies an array of habitats. *L. catta* is known for its remarkable behavioral and ecological flexibility, which contributes to its ability to exist in a mostly fragmented landscape. While this species represents one of the most well studied Malagasy strepsirhines, there has been a paucity of research regarding the population and conservation genetics of this endangered species. The goal of my dissertation was to

examine the influence of habitat fragmentation and isolation on the genetic diversity and population structuring of this flagship species in three populations living in the central highlands of Madagascar: Anja Reserve, Sakaviro, and Tsaranoro Valley. Non-invasive fecal samples from 30 individual lemurs were collected from three fragmented forests and genotyped at six polymorphic microsatellite loci.

Population genetic analyses were examined via *GenAlEx* software and revealed a moderate level genetic diversity. Genetic differentiation (F_{ST}) among the three fragmented populations ranged from 0.05-0.11. These data suggest that the *L. catta* populations within south-central Madagascar have not yet lost significant genetic variation.

To examine past and recent demographic declines or genetic bottlenecks, I employed three approaches, including mode-shift and M-Ratio tests, as well as a test to detect heterozygosity excess using three mutation models: the two-phase model (TPM), step-wise mutation model (SMM), and the infinite allele model (IAM). Results were equivocal depending on the test that was applied; however, a mode-shift was detected for Anja, signifying this population underwent a historical bottleneck. M-ratio tests revealed that all three populations suffered historical bottlenecks. A population bottleneck was indicated via heterozygosity excess under the IAM for both the Anja and Sakaviro populations.

To understand the impact of natural (e.g., mountains) and anthropogenic disturbances (e.g., roads, habitat fragmentation) on male reproductive strategies (dispersal) and population structuring, I utilized both *GenAlEx* and STRUCTURE software. Population assignment analyses suffered from a likely 'lack of signal'. Therefore, individuals were unable to be reliably assigned to their *population of origin*. Genetic population structure was ambiguous. These data suggest that that these three fragmented populations are not genetically differentiated enough for proper population assignment, or perhaps the sample is not robust enough for population assignment analyses to produce unequivocal results.

My research represents the first population genetic data for ring-tailed lemurs within the central highlands, and thus, serves as a baseline for future investigations into the genetic health of these populations. These data support the suggestion that these three

fragments represent areas in which concerted conservation efforts are necessary if genetic diversity is to be maintained and future demographic declines are to be prevented. My results are informative for the local community conservation associations working within south-central Madagascar and can now be applied to determine areas of conservation priority and where forest corridors will be the most beneficial for maintaining gene flow. The loss and fragmentation of habitat continues across Madagascar, including the central highlands; thus, all remaining *L. catta* populations should be considered a high conservation priority. If we are to safeguard the long-term viability of this species, continued conservation and research initiatives will be crucial.

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Acknowledgments

This dissertation has been a journey, made up of many twists and turns, peaks and valleys, and even the occasional dead end. It is the product of dedication, passion, sacrifice, laughter, tears, occasional panic attacks, way too many taxi brousse rides, and countless insect bites. It could not have been achieved without the endless support of my amazing family, friends, and colleagues.

To my supervisor, Lisa Gould, where to begin… You have been more than just a 'supervisor'; you have been my mentor and my friend. I thank you for your never-ending support, thoughtful constructive criticisms and your wonderful sense of humor, friendship, and patience. Thank you for sharing your lemur passion with me, as well as your vivid and remarkable stories from your prolific career. Thank you for believing in this project and my abilities as an anthropologist, primatologist, conservationist, and aspiring geneticist. Finally, thank you for making me a better scientist and writer. I am forever grateful.

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Dedication

For Lawrence-

Thank you for always believing in me and for your endless support through this long journey.

To My Lemurs-

I will always be your voice, thank you for inspiring me to do more, to be better, and to not give up hope.

Chapter 1. Introduction

1.1. Project Introduction

While *Lemur catta* (ring-tailed lemur) represents one of the most well studied primates within the suborder Strepsirhinni, the majority of information regarding this species' life history comes from two main gallery forest study sites in Madagascar: Berenty Private Reserve and Beza Mahafaly Special Reserve (Jolly, 1966; Sussman, 1991, 1992; Sauther et. al., 1999; Jolly et al., 2002; Koyama et al., 2001, 2002; Gould et. al., 2003; Gould, 2006a). Consequently, our current understanding of this species' behavior and documented adaptability corresponds to these sites (Gould et. al., 1999; Koyama et. al., 2001; Gould et. al., 2003; Gould, 2006a; LaFleur and Gould, 2009); however, studies examining *L. catta* in alternative habitats (e.g., spiny bush and forest) are slowly beginning to accumulate, which has begun to allow for a greater understanding of this species' behavioral and ecological plasticity (Gould et al., 2011; Kelley, 2011, 2013; LaFleur, 2012; Cameron and Gould, 2013; Gabriel, 2013a,b).

Gabriel (2013a,b) recently examined the ecological flexibility of *L. catta* in two anthropogenically disturbed habitats (i.e., Anja and Tsaranoro) located in south-central Madagascar, specifically behavior and health ecology (i.e., stress and parasitism) with respect to forest fragmentation. Results revealed substantially lower plant species diversity at both sites; Anja had a total of 24 and Tsaranoro a total of 32 plant species, in comparison to other *L. catta* habitats, such as Berenty, which has a total of 109 (Gabriel, 2013a,b). The author suggests that low levels of species diversity are most likely a result of the historical anthropogenic disturbance. Furthermore, *L. catta* at both sites were found to have a considerable reliance on introduced non-native species (*Melia* and *Ficus*), and between-site differences in feeding effort were found to be significant, where *L. catta* at Tsaranoro dedicated more time to feeding (Gabriel, 2013a,b; Gould and Gabriel, under review). Additional between-site differences in behavior included significantly higher rates of territorial marking/defense behaviors, as well as intergroup encounters for *L. catta* inhabiting the Anja fragment (Gabriel, 2013a,b). Moreover, ring-tailed lemurs at Anja were found to have significantly higher fecal glucocorticoid (fGC) levels, which is

suggested to be associated with the higher population density (6.6. lemurs/ha), leading to an increase in social interactions, as well as higher rates of intergroup encounters (i.e., competition for space and resources) (Gabriel, 2013a). Gabriel's (2013a,b) work highlights the extreme behavioral and ecological flexibility of the species in relation to inhabiting a disturbed landscape, yet, cautions us to *not* mistake this behavioral and ecological plasticity for stability, and emphasizes the need for continued research in alternative habitats.

To date, just two investigations of wild ring-tailed lemur population genetics have been conducted (Parga et al., 2012; Pastorini et al., under review). Parga and colleagues (2012) examined two populations of *L. catta* that occupy continuous forests in southwestern Madagascar (i.e., Beza Mahafaly and Tsimanampetsotsa reserves), where regular male dispersal has been documented (Sussman, 1992, Gould et al., 2003; LaFleur, 2012). Their study revealed evidence of a genetic bottleneck signal in the southwestern *L. catta* populations within the recent past (Parga et al., 2012). Comparative data on ring-tailed lemur genetics is imperative to fully grasp the species' long-term viability in the wild. Due to a rapidly increasing fragmented landscape and the cascading effects that follow (e.g., reduced heterozygosity, inbreeding depression) (Fahrig, 2003), it is crucial to gain an understanding of how different species respond to habitat disturbance so that more informed conservation decisions can be made regarding their future viability and aid in establishing more and better protected areas for biodiversity (Marsh, 2003; Fahrig, 2003; Marsh and Chapman, 2013).

Considering that my three study fragments (see Chapter 2) in south-central Madagascar are far smaller in size compared to the populations examined by Parga et al. (2012), and are isolated from each other with no available corridors, resident males within the fragments experience far less opportunity for dispersal. Thus, the health of *L. catta* populations in such fragments may be compromised by a loss of heterozygosity due to dispersal limitations.

1.2. Microsatellite Markers: Challenges and Solutions

The advent of non-invasive DNA sampling has presented researchers with a valuable tool that can be used in parallel with field observations of wild populations

(Vigilant and Guschanski, 2009); however, there are several limitations to be considered and addressed when utilizing such samples (Taberlet et al., 1999; Di Fiore, 2003; Vigilant and Guschanski, 2009). The main limitations involving non-invasive samples, such as feces, include low quality DNA and/or a low quantity of DNA (i.e., degraded DNA), poor extraction quality (Taberlet et al., 1999; Di Fiore, 2003; Vigilant and Guschanski, 2009), and the fact that most DNA from fecal extractions comes from exogenous organisms like bacteria (Perry et al. 2010). Additional concerns are the risk of contamination during extraction and amplification, and the difficulty of amplifying long sequences when the majority of DNA is reduced into short fragments (Taberlet et al., 1999).

Microsatellites, also referred to as simple sequence repeats (SSRs) and short tandem repeats (STRs), consist of a variable number of tandem repeats of a 1-6 base pair nucleotide motif distributed randomly within a given species genome (Di Fiore, 2003; Selkoe and Toonen, 2006). These markers are particularly useful for a number of reasons, including: 1) their fast evolution (high mutation rates) in comparison to the rest of the genome; 2) they are hyper-variable within populations; 3) they are selectively neutral; 4) they have co-dominant inheritance; and 5) only small amounts of template DNA is required for genotyping of these markers (Di Fiore, 2003; Selkoe and Toonen, 2006). More specifically, microsatellite loci represent one of the most variable types of DNA sequence within a given genome (each marker can be considered a sample of the genome) (Ellegren, 2004; Selkoe and Tonnen, 2006). These genetic markers have high mutation rates and co-dominant inheritance, which contribute to their high levels of allelic diversity resulting in an abundance of genetic information (Selkoe and Toonen, 2006). Additionally, microsatellites are neutral in that they do not code for proteins and are therefore not under selection (Selkoe and Toonen, 2006). All of these traits contribute to the popularity and ubiquity of their use in answering a variety of genetic based questions (Selkoe and Toonen, 2006). However, some drawbacks do exist. Primers must be available for a given species in order to carry out molecular analyses (e.g., PCR), and there is the problem of "null alleles", which can result from a mutation at one or both primer binding sites, resulting in no PCR amplification (Di Fiore, 2003). Taberlet et al. (1999) emphasize that researchers must be conscious of the potential errors that can occur

with “allelic dropout” in addition to the misinterpretation of amplification artefacts as “true alleles”. Allelic dropout occurs when an allele fails to amplify; if this happens to one allele of a heterozygous individual, that individual will falsely appear to be homozygote (Taberlet et al., 1999). Therefore, allelic dropout can result in incorrect genotypes being obtained, resulting in an overall reduction of observed heterozygosity− leading to inaccurate data regarding a population’s genetic diversity (Taberlet et al., 1999).

To combat any drawbacks or limitations, and to ensure accurate results, several steps can be taken during sample collection and genotyping in the lab, including: 1) collecting samples (i.e., hair, feather, feces) directly after an animal deposits them and using proper recommended storage methods; 2) multiplex or co-amplify (via QIAGEN Multiplex PCR Kit) several loci during a PCR, or 3) a more conservative, but time consuming and expensive approach, replicate each DNA amplification independently for each locus several times (i.e., multiple tubes approach) (Taberlet et al. 1999; Di Fiore, 2003). By collecting fresh samples and following recommended storage protocols, one can potentially avoid or lessen degradation of DNA (Di Fiore, 2003, Di Fiore et al., 2011; Vigilant and Guschanski, 2009). The co-amplification via QIAGEN Multiplex PCR Kit will allow for the most efficient use of limited amounts of DNA (Taberlet et al., 1999). Employing a ‘multiple tubes approach’ increases the overall accuracy of results by providing a way to detect and monitor for genotyping errors (e.g., allelic dropout, false alleles) (Taberlet et al., 1999). It is important to note that the flexibility of microsatellites far out weigh their limitations and that these genotyping errors occur in less than 5% of PCRs (Taberlet et al., 1999; Selkoe and Toonen, 2006).

My study is the first population and conservation genetics investigation of *Lemur catta* in the central-highland region of Madagascar. I examined how *L. catta*, a species known for its remarkable behavioral and ecological flexibility, has responded and adapted to existing in a severely fragmented and isolated landscape, within three study sites located in the central-highlands of Madagascar (see Chapter 2), in regards to: (1) genetic health (i.e., genetic diversity); (2) genetic structuring; (3) past and recent genetic bottlenecks; and (4) reproductive strategies (i.e., male dispersal). My study assists in identifying how genetic health, in the face of limited dispersal opportunities, can affect

animal populations experiencing marked habitat change and varying degrees of isolation. This work is also informative for conservation programs in Madagascar in relation to assessing the viability of *L. catta* (and potentially other lemur species) populations in situations of ever-increasing fragmentation. Furthermore, there is the potential for the identification of conservation priority areas (i.e., areas with known gene flow) within the Tsaranoro Valley and the Anja-Andranombe region in south-central Madagascar.

My dissertation is composed of six chapters. Chapter two provides a background and literature review. Chapters three through five represent independent manuscripts that address specific themes. In Chapter 3, I examine the genetic variability within and between the three study populations, as well as the population structure. In Chapter 4, I investigate evidence of historical and recent genetic bottlenecks, and compare these results with population bottleneck data from two *L. catta* sites in southwestern Madagascar (see Parga et al., 2012). In Chapter 5, I investigate whether male dispersal in the face of occupying a severely fractured landscape has been compromised. By utilizing assignment tests or indices (AIs) I determine the mean probability that a particular individual (i.e., individual's genotype) is from either the sampled population (i.e., natal) or whether that individual is an immigrant (Di Fiore, 2003; Lawson Handley and Perrin, 2007). Finally, In Chapter 6, I first provide a summary of the dissertation results. In addition, I briefly discuss various community conservation projects within primate habitat countries. Lastly, I discuss a community-based conservation plan for the endangered ring-tailed lemur throughout its geographic range.

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Chapter 2. Background

May I announce to you that Madagascar is the naturalist's promise land? Nature seems to have retreated there into a private sanctuary, where she could work on different models from any she has used elsewhere. There you meet bizarre and marvelous forms at every step….

Philibert Commerson

(Teissier, 1859)

2.1. Background

Madagascar, once a part of the supercontinent Gondwanaland, began its separation from the African continent during the middle Jurassic (~165 million years ago (mya); approximately 125 mya ago the process was complete (Tattersall, 2006). Thirty million years later, India separated from Madagascar, leaving the island in complete geographic isolation for approximately 88 million years (Storey, 1995 as cited in Martin, 2000). This extended period of isolation resulted in an immense and unparalleled level of unique biological diversity and endemism (Martin, 2000; Myers et al., 2000). In comparison to other biodiverse-rich countries, Madagascar remains unmatched in regards to endemism levels, with 26 endemic families and 480 endemic genera (Schwitzer et al., 2013). Human colonization of the island occurred rather recently, approximately 4000 B.P. (Dewar et al., 2013). Late human arrival allowed for the persistence of numerous late prehistoric mammalian megafauna, including eight families of lemurs, three of which have gone extinct (Archaeolemuridae, Palaeopropithecidae and Megaladapidae), a rare aardvark-like mammal (Plesiorycteropodidae), the flightless elephant bird (Aepyornithidae), three species of pygmy hippopotamuses (Hippopotamidae); and the giant fossa (Eupleridae) (Burney et al., 2004; Perez et al., 2005; Godfrey and Irwin, 2007). Numerous studies have shown a clear and undisputed temporal overlap of humans and the now extinct megafauna (Burney, 1999; Burney et al., 2004; Perez et al., 2005; Godfrey et al., 2006; Godfrey and Irwin, 2007; Dewar et al., 2013). Due to the island's unique history, two main questions have generated an ongoing debate: 1) *How did*

Madagascar come to evolve such a unique and diverse group of mammalian fauna; more specifically, how and when did the ancestral populations arrive?; and 2) *What role did humans play in the extinction of the Malagasy megafauna?*

2.1.1. Rafting Versus Land-Bridges: How Did Ancestral Lemurs Colonize Madagascar?

Simpson's (1940) 'sweepstakes process' and McCall's (1997) 'land-bridge hypothesis' represent the two foremost early explanations for how the oceanic island of Madagascar came to host such a unique and diverse biota, characterized by high levels of endemism. Simpson (1940) suggests, over-water dispersal whereby small mammals, most likely possessing low-metabolic rates and/or the capacity to enter seasonal torpor, rafted to Madagascar via eastern Africa on large pieces of floating vegetation. Conversely, McCall (1997) proposed that animals dispersed from mainland Africa to Madagascar via prominent land-bridges formed by the Davie Ridge in the Mozambique Channel.

While the land-bridge hypothesis has received some support (Stankiewicz et al., 2006; Masters et al., 2006; Tattersall, 2006), evidence to the contrary is substantial and comes from an array of disciplines, including biogeography/geology, molecular phylogenetics, palaeogeographic reconstructions, and palaeo-ocenanographic modeling (Wiley, 1988; Martin, 2000; Kappeler, 2001; Yoder, 1997; Yoder and Nowak, 2006; Ali and Huber, 2010). Specifically, our understanding of plate tectonics (Wiley, 1988) combined with the apparent dearth of mammalian taxa on Madagascar, suggests a single colonization event for Madagascar's endemic primates (Simpson, 1952; Yoder et al., 1996; Krause et al., 1999; Martin, 2000; Yoder and Yang, 2004; Yoder and Nowak, 2006; Yoder, 2013). Additionally, incontrovertible molecular phylogenetic data illustrating lemuriform monophyly, with African taxa representing the closest sister group (Yoder, 1997; Yoder et al., 1996; Martin, 2000; Yoder and Nowak, 2006; Yoder, 2013) and divergence age estimates which revealed that the lemur clade descended from an early Cenozoic ancestor (Yang and Yoder, 2003; Yoder and Yang, 2004; Yoder, 2013) call the validity of the land-bridge hypothesis into question. Furthermore, evidence of successful contemporary over-water dispersals by various species of plants and animals, such as green iguanas (*Iguana iguana*) on the island of Anguilla in the Caribbean lends additional support to the over-water hypothesis (Censky et al., 1998, also see de Queiroz,

2005). One of the more recent and compelling pieces of evidence comes from Ali and Huber (2010), in which palaeo-oceanographic models were employed to simulate Cenozoic (i.e., Eocene) ocean currents and test whether past currents would have supported an over-water dispersal route from mainland Africa via the Mozambique Channel to Madagascar. The simulation models demonstrated that ocean currents during the Eocene would have been markedly different than modern day currents. These differences are due to the past geographic position of the continents; for example, Madagascar and Africa were both >10° further south in relation to Australia (also further south than today), which placed Madagascar in a "convergence zone" consisting of a system of ocean currents that circulate counterclockwise, thus resulting in an eastward trajectory from the African coastline. Furthermore, Ali and Huber (2010) note that during the Eocene the strongest current system in the southern ocean occurred east of Madagascar. Therefore, the strength of the southern ocean current combined with the eastward trajectory towards Madagascar strongly support Simpson's model (1940) of an over-water dispersal (Ali and Huber, 2010). Thus, in light of overwhelming evidence, it can be concluded that Madagascar's "living biota is predominantly comprised of neoendemics that have evolved from transoceanic dispersers" (Yoder and Nowak, 2006, 422).

2.1.2. Holocene Exinctions: What Happened To The Malagasy Megafauna?

Until recently, it has generally been accepted that human arrival to the island of Madagascar occurred ca. 2300 B.P. (Burney, 1997a, 1999; Burney et al., 2004; MacPhee and Burney, 1991). However, new archeological evidence presented by Dewar and colleagues (2013) suggest an earlier period of stone tool-using foragers, dating to as early as 4000 B.P., thus doubling the length of the island's occupancy. Investigations regarding the genetic origins of the Malagasy people are scarce, however recent studies reveal an admixed ancestry of African and Indonesian descent (Hurles et al., 2005; Tofanelli et al., 2009; Ricaut et al., 2009; Pierron et al., 2014), which is consistent with linguistic studies confirming components of both East African (i.e., Bantu) and Indonesian (i.e., Maanyan, spoken in southern Borneo) languages (Dahl 1951, Dahl 1988 as cited in Hurles et al., 2005; Adelaar 1995 as cited in Hurles et al., 2005).

A temporal overlap of Malagasy megafauna (except *Babokotia radofilai*) and the first human inhabitants to the island has been well documented (Burney, 1999; Burney et al., 2004;Godfrey et al., 2006; Godfrey and Irwin, 2007; Crowley, 2010) and both direct and indirect evidence confirm a dramatic decline in the now-extinct megafauna following human arrival (Burney et al., 2003; Burney et al., 2004; Perez et al., 2005; Godfrey et al., 2006; Crowley, 2010; Dewar et al., 2013). However, it should be noted that while the decline of megafaunal species was typically thought to be precipitous, radiocarbon dating suggests the process of extinction was more gradual depending on the species and its geographic locality (Burney et al., 2003; Crowley, 2010; Dewar et al., 2013). The Holocene extinctions included 40-50 species of vertebrates and all terrestrial species with body masses >10kg, including the three families of sub-fossil lemurs (i.e., Archaeolemuridae, Palaeopropithecidae and Megaladapidae) (Burney, 1999; Burney et al, 2004; Crowley, 2010; Dewar et al., 2013). The impact of human arrival, occupation and settlement on the endemic megafaunal communities was devastating. In order to elucidate our understanding of the Holocene extinctions, we must examine how humans interacted with the landscape and the megafauna.

Several hypotheses have been proposed to explain the mass extinction of the megafaunal communities, including: 1) human-introduced hypervirulent diseases (MacPhee and Marx, 1997), 2) over-hunting (Walker, 1967), 3) natural aridification of the landscape (Mahe and Sourdat, 1972 as cited in Crowley, 2010), 4) habitat degradation and loss via fire (Humbert, 1927 as cited in Crowley, 2010), and 5) an amalgamation of both anthropogenic and environmental factors (e.g., hunting, climate change, habitat loss) (Burney, 1999; Godfrey et al., 2006; Dewar et al., 2013). The 'synergistic hypothesis' (hypothesis 5) is now the most widely accepted and there are numerous data to support it (Burney, 1993; 1999; Simons et al., 1995; Burney et al., 1997a,b, 2003, 2004; Perez et al., 2005; Godfrey and Irwin, 2007; Crowley, 2010; Virah-Sawmy et al., 2010).

For example, indirect evidence in the form of fossil pollen and charcoal samples obtained from sediments in south-eastern Madagascar, combined with climatic (e.g., records of droughts that occurred throughout the Holocene), archaeological, and faunal records, revealed marked environmental change in the form of climatic desiccation,

resulting in extensive habitat degradation and vegetation transformations (Virah-Sawmy et al., 2010). It has been suggested that these pronounced environmental changes prompted an increase in the hunting of megafauna populations, as well as heightened competition for resources and land space from introduced cattle, ultimately leading to the mass extinctions of the megafaunal communities of Madagascar (Virah-Sawmy et al., 2010).

Burney et al. (2003) utilized fossil spores of the coprophilous fungus *Sporomiella spp*. via sediment cores from different locales across the island (southeastern northern, and central Madagascar) to investigate changes in Malagasy megafaunal biomass. *Sporomiella* represents a successful proxy for megafaunal density due to its inability to complete its lifecycle without the presence of feces from large animals (Godfrey et al., 2006). Fungus spores analyzed from sediments dated to pre-human arrival, revealed high percentages of *Sporomiella,* whereas a decrease or absence of spore presence was found at sites coinciding with human arrival (Burney et al., 2003). Additionally, the decrease in *Sporomiella* was followed by dramatic increases in charcoal presence, which suggests human modification to the landscape (Burney et al., 2003). These data, when considered with fossil evidence and paleoclimate data, strongly support the idea that the megafauna extinctions were driven by multiple interacting factors (e.g., aridification, fires, hunting) (Burney et al., 2003).

Lastly, Perez and colleagues (2005) provide us with the first conclusive and direct evidence of butchery, signifying hunting and consumption of the now extinct sub-fossil lemurs. Specimens collected throughout southwestern Madagascar during the early 1900s (both extinct and extant species) and held in three museum collections, were examined for cut marks, tooth marks, and percussion marks (Perez et al., 2005). In regards to the sub-fossil lemur specimens, all three collections revealed evidence of butchery (10/28 specimens); specifically, cut and/or chop marks were present indicating that they were produced by sharp metal instruments on fresh bone, and the patterns of these marks are suggestive of human processing for cooking (Perez et al., 2005). An examination of extant lemur specimens (77/269 specimens) was also consistent with butchery (Perez et al., 2005). While the evidence of butchery does not solve the questions surrounding the

megafauna extinctions, it serves as an important piece of a complex puzzle.

When evidence from archaeology, paleontology, palynology, and paleoclimatology are considered collectively, it is possible to gain a clearer understanding of *how* and *why* the Malagasy megafaunal communities went extinct. It therefore seems reasonable to hypothesize that, not one, but a multitude of interacting and cascading factors led to the Holocene extinctions. A clearer understanding of past climatic events and anthropogenic pressures on Madagascar's biodiversity contributes to a more comprehensive interpretation and understanding of modern impacts on the island's extant flora and fauna. This knowledge will aid in the development and implementation of more effective conservation initiatives across the island.

2.1.3. Contemporary Madagascar: Forest Fragments, Lemurs, And Conservation

Habitat fragmentation and global climate change represent the two greatest threats to biodiversity and ecological processes worldwide (Travis, 2003). Predictions for global climate change suggest a maximum global temperature increase of 5.2°C by 2100; this increase is likely to result in changes in the frequency and distribution of rainfall and droughts (IPCC, 2001 cited in Opdam and Wascher, 2004). Additionally, many species are expected to experience a northward shift in their range boundaries (Honnay et al., 2002), which will be confounded by landscape configuration (e.g. habitat fragmentation, roads) (Opdam and Wascher, 2004; Thomas et al., 2004). It is predicted that anthropogenic induced climate change could represent a key factor for extinctions in the near future, considering that the rise in global temperatures are expected to be greater than any period throughout the last 40 million years (Thomas et al., 2004).

Habitat fragmentation can lead to an array of dramatic landscape-scale changes including decreases in remnant patch size, higher edge to forest interior ratios, increased patch isolation and variation in the degree of connectivity of patches (Fahrig, 2003; Gehring and Swihart, 2003), and changes in microclimate and plant diversity (Irwin, 2008). Such severe alterations to the structure of the landscape poses a number of challenges for the fauna inhabiting these degraded areas, including lack of dispersal opportunities leading to inbreeding and, thus, loss of genetic diversity (Dietz et al., 2000),

reduced reproductive fitness (Charpentier et al., 2008), increased vulnerability to predation, hunting, and disease (Fahrig, 2003), and an inability to deal with or respond to environmental changes and/or disease (Oklander et al., 2010). Moreover, species' biological responses to landscape modifications (e.g., fragmentation) are variable and can occur immediately or exhibit a time-lagged response or relaxation time, eventually leading to an *extinction debt*, defined as the number or proportion of species expected to become extinct after a new equilibrium has been achieved, post environmental perturbation (Brooks et al., 1999; Metzger et al., 2009; Kussaari et al., 2009). This dichotomous response to landscape modifications pertains to both plants and animals; for example, tropical African bird communities inhabiting small fragments were reported to have a relaxation period of 25-100 years, during which time these communities *paid off* 50% of their total extinction debt (Brooks et al., 1999). In contrast, calcareous grasslands of southern Belgium had a more rapid response to new deforestation and fragmentation versus historical, resulting in an absence of an extinction debt (i.e., responded immediately to disturbance and had no relationship between species richness and historical landscape modifications) (Adriaens et al., 2006). It is important to recognize that species richness, distribution patterns, abundance, and ultimately, survival, are influenced by a variety of factors, such as a species life history traits, historical and recent landscape modifications, fragment size, level of connectivity, surrounding matrix configuration, forest re-growth, and the rate of change of a forest fragment; thus, all of these variables need to be examined when developing conservation management plans (Metzger et al., 2009). Lastly, when species or populations are found to exhibit either immediate or short time-lag responses to environmental perturbations, conservation action plans should be implemented immediately in order to prevent future extinctions and ensure the population's future viability (Metzger et al., 2009; Kussaari et al., 2009).

During the period from 1950-1979, more than 300 million hectares (ha) of the world's tropical forests were destroyed, and while deforestation rates have slowed since then, more than 100 million ha were lost from 1996-2010 (FAO, 2012). The last remaining habitats supporting 44% and 35% of all plant and vertebrate species, respectively, exist in the world's 25 biodiversity hotspots or areas that contain

incomparable levels of endemism and are experiencing extensive habitat loss (Myers et al., 2000).

Madagascar is one of 91 countries in the world that contains wild primate populations and while significantly smaller than Central and South America, Africa, and Asia, it is considered one of the high priority biogeographic regions for primate conservation (Schwitzer et al., 2013). This oceanic island is considered one of the top priority hotspots in the world and is characterized by its unmatched levels of species diversity and endemism (Myers et al., 2000). In fact, 20% of all primate taxa can be found in Madagascar: 5 endemic families, 15 genera, and 103 species (including four subspecies) have been confirmed, and more are still being discovered (Schwitzer et al., 2013). Human-induced disturbance, especially slash and burn agriculture or *tavy* (Malagasy term for slash and burn) and illegal logging, have had detrimental effects on the local flora and fauna. For example, during the 1950s, approximately 160,000 km^2 of the island was forested and by the year c. 2000, that number decreased to 89,000 km^2 (i.e., ~45% loss of forest) (Harper et al., 2007). The dramatic decline in original forest cover has continued, and today less than 10% remains, resulting in a patchwork of forest fragments across the island (Harper et al., 2007; Schwitzer et al., 2013). If current rates of deforestation are maintained, Madagascar is predicted to lose countless species to extinction in the near future (Brooks et al., 2002). In 2009, a coup d'état cast Madagascar into political turmoil, giving rise to a marked increase in illegal logging of precious hardwoods, such as rosewood (*Dalbergia* spp.), and an emergence of a bushmeat market (Barrett et al., 2010; Schwitzer et al., 2013, 2014; LaFleur, 2013). Following the breakdown in government, international aid and monies for environmental programs were halted, which only intensified the problems at hand (Schwitzer et al., 2013, 2014). According to Barrett et al. (2010) up to 20,450 ha have been negatively affected by the illegal rosewood trade, and the demand for furniture and musical instruments produced from this hardwood has not diminished. Studies investigating local bushmeat consumption and trade in Madagascar are scarce; however, Golden (2009) examined bushmeat hunting across 14 villages surrounding the Makira Forest in northeastern Madagascar, one of the last remaining contiguous forests on the island. Twelve species of lemur were hunted, including the critically endangered black and white ruffed lemur

(*Varecia variegata*) and the endangered indri (*Indri indri*), as well as other threatened mammal species (e.g. tenrecs, bats) (Golden, 2009). A more recent report noted that a burgeoning pet trade and growing bushmeat trade is rapidly becoming an issue for the endemic species in the southern regions of Madagascar, particularly for ring-tailed lemurs (LaFleur, 2013). Due to an influx of migrant workers, traditional *fadys* or taboos, which at one time functioned to protect the lemurs, have undergone a breakdown, resulting in fewer people holding onto traditional values (LaFleur, 2013). Regardless of their protected status, ring-tailed lemur infants and juveniles are now being targeted and sold as pets or as tourist attractions at local hotels (LaFleur, 2013). Lastly, smoked lemur carcasses are sold for approximately 5,000 Ariary ($2 USD), due to an increasing demand for luxury bushmeat across the island's cities (LaFleur, 2013).

In July of 2012, lemur researchers and conservationists from around the world gathered in Madagascar on behalf of the International Union for the Conservation of Nature (IUCN) Red List to conduct a re-assessment of the conservation status of all known lemur species, as well as to develop a conservation strategy to help to ensure their future viability (Schwitzer et al., 2013, 2014). The outcome was alarming, yet not surprising, considering the current political and environmental state of the island. The enormity of the conservation threat and urgent need for action is painfully obvious when one compares the last (2005) and most current (2013) IUCN Red List assessments. According to the 2005 assessment, 66% of the then-known 68 lemur species, for which there were sufficient data, were classified as *under threat of extinction*; in contrast, the 2013 assessment revealed that almost 94% of the 103 lemur taxa are now threatened with extinction (Schwitzer et al., 2013). Thus, the Malagasy strepsirrhines are now considered the most endangered group of mammals (Schwitzer et al., 2013, 2014). Considering Madagascar's past and recent landscape transformations, ongoing anthropogenic threats, unstable political and economic climate, and the fact that the majority of the island's endemic lemurs that are occupying forest fragments have an *extinction debt* that has yet to be paid (i.e., due to long generation times, they have not fully responded to the habitat disturbance and are experiencing a time lag until extinction) (Irwin et al., 2010), time is clearly of the essence. The survival of these unique primates is tenuous, as they are living on borrowed time; in order to ensure their future survival, immediate conservation action

and implementation is imperative.

2.2. Background of Study Species

2.2.1. Social Organization

Lemur catta is a monomorphic, medium-sized strepsirrhine with an average adult body mass of 2.2 kg that live in multi-male/multi-female social groups, typically containing 10-20 individuals (Jolly, 1966; Sussman, 1991; Sauther et al., 1999; Sauther et al., 2006). Adult sex ratios at Beza Mahafaly Special Reserve are 0.92 (male to female) (Gould et al., 2003; Gould, 2006) and range from 9:3-1:5 (male to female) at Berenty Reserve (Jolly et al., 2002). Sex ratio can be quite variable depending on various factors, such as environmental stresses (e.g., droughts, cyclones), predator abundance, and availability of resources (Wright, 1995; Gould et al., 1999). It should be noted that these two reserves are significant because they represent the two research sites where the majority of our knowledge of ring-tailed lemur behavior, biology, and ecology come from. *L. catta*, like the majority of Malagasy strepsirhines, exhibits strict seasonal breeding (Jolly, 1966; Sauther, 1998; Gould et al., 1999; Sauther et al., 1999; Wright, 1999; Koyama et al., 2001; Gould et al., 2003). Females are receptive for approximately 6-24 hours (Sauther, 1998; Sauther, 1991; Koyama et al., 2001), and synchronous receptivity occurs among female group members with estrus cycles occurring within 1-3 weeks of each other (Sauther, 1998; Sauther et al., 1999). During receptivity females typically mate with more than one male (group males, non-group males, and transferring males) and give birth annually (Koyama, 1988; Sauther, 1991; Sussman, 1992; Gould, 1994). Infant mortality rates are variable depending on the site and the year, with recorded rates ranging between 30% to 51% at Beza Mahafahly and 38% at Berenty, but reaching as high as 80% during a drought year at Beza Mahafaly Reserve (Gould et al., 1999, 2003; Sauther et al, 1999; Koyama et al., 2001). Female ring-tailed lemurs wean their infants at four to five months of age (Gould, 1990; Sauther, 1998; Sauther et al., 1999).

L. catta exhibits “true” female dominance in which females have feeding priority as well as social dominance in non-feeding contexts (Jolly, 1984). Within ring-tailed lemur groups there is a top-ranking female, who is dominant to both males and other

females in the group (Jolly, 1966). Hierarchies can be linear and remain stable for long periods of time, however, changes within the female hierarchy can occur suddenly (Sauther, 1992; Nakamichi and Koyama, 1997; Sauther et al., 1999; Gemmil and Gould, 2008). The female philopatric groups are comprised of a core of related adult females and their offspring, one or two central males, and peripheral males (Jolly, 1966; Gould, 1997; Sauther et al., 1999). Larger groups have been found to contain more than one matriline (Taylor and Sussman, 1985; Taylor, 1986; Sauther, 1992) and when this occurs, one matriline will be dominant to the other(s) (Taylor and Sussman, 1985; Taylor, 1986; Sauther, 1992; Sauther et al., 1999; Gould et al., 2003; Gould, 2006). According to Gould et al. (2003) when group size reaches a "critical number", which is approximately 14-21 individuals, fissioning will occur (also see Sussman, 1991, 1992). Typically this is achieved by one matriline exhibiting targeted aggression towards another (Hood and Jolly, 1995; Vick and Pereira, 1989; Sauther et al., 1999; Gould, 2006).

Male ring-tailed lemurs first disperse from their natal groups at sexual maturity (3-4 years); some males may remain in their natal groups until the age of five, although this is rare (Sussman, 1992). Dispersal events take place several times throughout a male's life and on average occur every 3.5 years (Sussman, 1992; Sauther et al., 1999). Partnerships are formed by dispersing male ring-tailed lemurs (Sussman, 1991; Sussman, 1992; Gould, 1994, 1997; Sauther et al., 1999) and upon emigrating into a new group they are always low-ranking, peripheral and consistently challenged by male and female group members for several months (Sussman, 1991; Gould, 1996; Sauther et al., 1999). Dominance hierarchies among males are not always linear and changes in rank are common (Budnitz and Dainis, 1975; Kappeler, 1993; Gould, 1996; Gould, 1997; Sauther et al., 1999; Parga, 2009). Male dominance rank changes can also be non-transitive (Gould, 2006b). In a study of the migration/integration period at Beza Mahafaly, Gould (2006b) noted that one emigrating male was successful in quickly achieving dominance over the highest-ranked resident male, while simultaneously remaining subordinate to the other resident male.

Intergroup encounters occur regularly at gallery forest sites and typically involve females (Sauther, 1992; Hood and Jolly, 1995; Nakamichi and Koyama, 1997; Sauther et al., 1999; Jolly et al., 2000). For example, in a 1997 study at Berenty, females were

reported to be responsible for more than three-quarters (78%) of the observed agonistic interactions during intergroup encounters (Nakamichi and Koyama, 1997). More specifically, female-female targeted aggression (e.g., chasing, charging, biting, cuffing) and infanticide, which is considered rare, both within and between groups have also been documented (Jolly et al., 2000). Jolly et al. (2000) suggest that due to the ecological and physiological pressures of inhabiting an unpredictable climate in southern Madagascar (e.g., droughts), females may benefit by eliminating the offspring of competing females. However, most encounters involve access to food resources or defending a core area of a particular group's home range (Sauther, 1992; Sauther et al., 1999). Conversely, intergroup encounters are rare among ring-tailed lemurs found in the Cap Ste. Marie region, a scrub and bush forest which is surrounded by *Opuntia monacantha* (i.e. introduced cacti species)(Kelley, 2011).

2.2.2. Habitat Types and Geographic Range

Ring-tailed lemurs are found in an array of habitat types throughout the south and southwestern parts of Madagascar including: spiny, brush/scrub, gallery and dry deciduous forests, anthropogenic savannah, and within the high-altitude regions of the Analavelona and Andringitra massifs (Figure 1) (Goodman and Langrand, 1996; Goodman and Rasolonandrasana, 2001; Sussman et al., 2003; Goodman et al., 2006; Gould, 2006). The Northwestern boundary for *L. catta* is suggested to be at Belo sur Mer, north of the Mangoky River (Goodman et al., 2006) and within the Parc Nationale de Kirindy-Mitea (Menabe Region) (Goodman et al., 2006; Gould, 2006). Sakaviro, located in the central highlands of Madagascar, represents the Northeastern limit (L.Gould pers. comm.). The Cap Ste. Marie Reserve, which is located at the southern tip of the island, represents the southern most portion of the species' range (Goodman et al., 2006; Gould, 2006). The southeastern limit of *L. catta* range lies 30 km west of Ft. Dauphin in Ambatotsirongorongo, which is represented by a mixture of humid/dry forest (Razafindramanana, 2011).

During the mid-1990s a high-altitude population of *L. catta* was discovered inhabiting the Andringitra Massif in south-central Madagascar (Goodman and Langrand, 1996; Goodman and Rasolonandrasana, 2001). This mountainous region represents one

of the most extreme habitats inhabited by this species (Goodman and Langrand, 1996; Goodman and Rasolonandrasana, 2001). Groups inhabiting the upper portions of the massif (above the forest line) experience daily temperatures of 30-35° C and nightly lows, which can fall to -16° C (Goodman et al., 2006), demonstrating the extreme ecological and behavioral flexibility of *L. catta*.

2.2.3. Population Density and Home Range Size

Depending on the habitat type, ring-tailed lemur biomass is variable (Jolly, 1966; Jolly et al., 2006; Gould et al., 1999, 2003). Generally, lower densities of *L. catta* are found within drier habitats while higher densities are found among gallery and mesic forests (Jolly et al., 2006). For example, at Berenty, within the gallery forest the density of ring-tailed lemurs is 2.5/ha whereas within the scrub and spiny forest it is 1.3/ha (Gould et al., 2011). The average density of lemurs within the Beza Mahafaly Reserve is 0.23/ha (Sauther and Cuozzo, 2008) and 1.3/ha for Parcel I (Gould et al., 2003). More recently, Axel and Maurer (2011) re-examined the density for the entire reserve, as well as the surrounding unprotected forest patches. Regions 1-6 (i.e., Parcel I) were classified as the 'protected' area and had a density of 2.16/ha, where as the 'unprotected' area or Regions 7-8 had a lower density of 0.36 (Axel and Maurer, 2011). In comparison to Berenty and Beza Mahafaly much lower densities have been reported for populations within Andohahela and Andringitra National Parks, 8-64 lemurs/ km^2 and 22.8 lemurs/ km^2, respectively (Rakotoarisoa, 2000; Gould, 2006). Likewise, lower population densities (~.017/ha) have recently been documented for ring-tailed lemurs inhabiting the Cap St. Marie region (Kelley, 2011), Tsimanampetsotsa National Park (0.10/ha) (LaFleur, pers.com), as well as the Tsaranoro Valley (1.1/ha) (Cameron and Gould, 2013). In contrast, the highest recorded population density of *L. catta* (6.6 lemurs/ha) occurs within the Anja Reserve located in south-central Madagascar (Cameron and Gould, 2013). This reserve consists of a markedly different forest type than that found in the south and southwestern parts of this species' range: 'rupicolous vegetation' which consists of mixed xerophytic and deciduous vegetation, and large granite (Cameron and Gould, 2013; Gabriel 2013a,b).

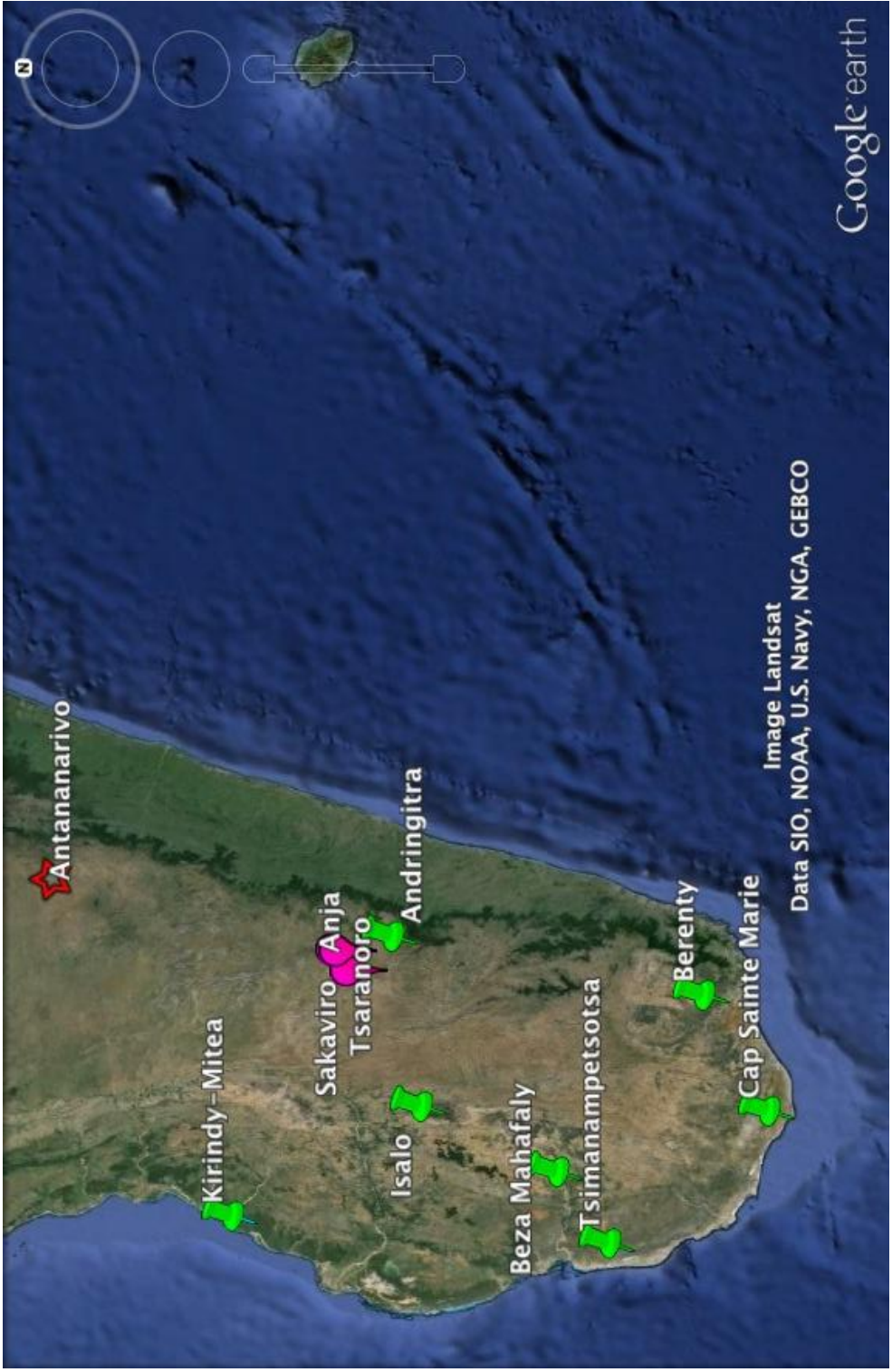

Figure 2.1. Map of *Lemur catta* geographic range.

L. catta home range size, like population density and group size, is dependent upon habitat type (Jolly and Pride, 1999; Gould et al., 2003; Gould, 2006). Ring-tailed lemurs inhabiting the Berenty Reserve gallery forests are found living in home ranges ranging from 3.9-16.7 ha while groups living in the xerophytic and scrub forests have home ranges varying from 12-25 ha (Jolly et al., 1993). Spiny forest groups within the reserve were found to have home ranges ranging from 7.9-11.2 ha (Gould et al., 2011). *L. catta*

living within the gallery forests at Beza Mahafaly have average home ranges of 17ha, whereas groups found within the drier parts of the reserve have a larger average home range of 32ha (Sussman, 1991; Gould, 2006). In contrast, *L. catta* inhabiting the Cap St. Marie region were found to have home ranges markedly larger (87-132 ha) that of ring-tailed lemurs living within gallery forests (Kelley, 2011, 2013). Similar to *L. catta* groups of the Cap St. Marie region, groups living in Tsimanampetsotsa National Park have also been documented to have very large home ranges (85-100 ha) (LaFleur, 2012).

2.2.4. Diet and Feeding Ecology

Lemur catta is characterized by its markedly flexible diet and is classified as an opportunistic omnivore (Sauther et al., 1999; Gould, 2006; LaFleur and Gould, 2009). A wide diversity of resources comprise the ring-tailed lemur diet, including fruit (ripe and unripe), leaves (young and mature), leaf stems, flowers, flower stems, spiders, spider webs, caterpillars, cicadas, insect cocoons, some small animal prey (birds and chameleons), soil, as well as cactus (*Opuntia*) (Sussman, 1972; Jolly, 1966; Sauther, 1992, 1993; Rasamimanana and Rafidinarivo, 1993; Oda, 1996, Sauther, 1998; Sauther et al., 1999; Yamashita, 2002; Simmen et al., 2003, 2006; Gould, 2006; Kelley, 2011).

Ring-tailed lemurs concentrate on particular resources depending on their availability across the wet and dry seasons (Sauther, 1998; Gould, 2006; Gould et al., 2011; LaFleur, 2012). In particular female *Lemur catta* rely heavily on specific phenological resources throughout the different phases of reproduction (Rasamimanana and Rafidinarivo, 1993; Sauther, 1992, 1998; Sauther et al., 1999; Gould, 2006; Gemmil and Gould, 2008; Gould et al., 2011). For example, female *L. catta* inhabiting the Beza Mahafaly Reserve gallery forest consume more fruit and flowers during gestation whereas lactating females rely more young leaves (Sauther, 1994, 1998; Gould, 2006). Conversely, females in the spiny forest consume vine leaves significantly more than any other food resource during early gestation. During the early to mid-lactation season spiny forest dwelling females consume significantly more flowers, leaves, and fruit (Gould et al., 2011).

Owing to the seasonal nature of the habitats in which ring-tailed lemurs are found and of Madagascar in general (Sauther, 1998; Wright, 1999; Gemmil and Gould, 2008) this species has come to depend upon essential keystone resources in periods of food

scarcity (Sauther, 1998; Gemmil and Gould, 2008). The tamarind tree (*Tamarindus. Indica*) (fruit and leaves) represents the most important keystone species for gallery forest dwelling *L. catta* due to its year-round availability (Sauther, 1998; Mertl-Millhollen et al., 2003; Sauther and Cuozzo, 2009). However, a study of *L. catta* inhabiting anthropogenic savannah showed a reliance on the fruit and leaves of *Melia azadirach* as their primary food resource (Cameron and Gould, 2013; Gould and Gabriel under review). Furthermore, adhering to their flexible nature, *L. catta* will often travel outside their home range during periods of low food availability to monitor the phenological cycle of desired keystone resources (Sauther, 1998; Gould, 2006), in addition to taking advantage of non-forest fallback foods (i.e. crop foods)(Gemmill and Gould, 2008; LaFleur and Gould, 2009; Cameron and Gould, 2013; Gabriel, 2013a,b). For example, *L. catta* inhabiting Anja, an isolated fragment in south-central Madagascar, regularly exploit food and water from agricultural crops and an anthropogenically-constructed lake (Cameron and Gould, 2013, Gabriel, 2013a,b).

2.3. Study Sites

The Anja Community Reserve (21°51'12 S 46°50'40 E) is a 34 ha fragment (Figure 2) with a population of ~220 *L. catta*, and a population density of 6.6. lemurs/ha- the highest population density of any known ring-tailed lemur site (Cameron and Gould, 2013; Gabriel, 2013a,b). The reserve is situated adjacent to Route Nationale 7, 13 km south of the town of Ambalavao in south-central Madagascar (Cameron and Gould, 2013; Gabriel, 2013a,b). Officially protected since 1999 and community-run since 2001 by the local association Anja Miray, it now represents one of the most popular community-run ecotourist sites in the country (Cameron and Gould, 2013). Approximately 12,000 tourists visit the reserve each year, generating $35,000-$45,000 USD annually (Gould, *in prep*) Revenues contribute to community development, as well as small-scale agriculture and pisciculture projects (Gould, *in prep*). Due to the success of the association's community-based conservation and development efforts, they have been recognized nationally and internationally; for example, in 2012 Anja-Miray received the prestigious UNDP Equator Prize (Gould, in prep). The reserve is completely isolated from other forest fragments due to the Route Nationale 7 highway to the west and a large mountain range to the east (Gabriel, 2013a,b). As a result of Anja's isolation, lack of corridors to

other forest patches (i.e., no meta-populations), and the challenging surrounding matrix (e.g., villages, highway) male dispersal is highly unlikely due to the risks (e.g. predators, cars, lack of food resources) involved in moving across such a fractured landscape. Elevations for the Anja Reserve are between 925 and 1,200m, however, the maximum ranging elevation observed for *L. catta* groups is 1,020m (Cameron and Gould, 2013). The eastern edge of the fragment is lined by large granite rock formations, which lie roughly 350m from the edge of the reserve (Cameron, 2010). Prior to 2012, village gardens ran parallel to an anthropogenically-constructed lake, making up the surrounding matrix on the south side of the reserve; however, due to consistent crop raiding by *L. catta* groups, the gardens have been relocated several times in an effort to deter the lemurs. The reserve is comprised of mixed xerophytic and deciduous vegetation, which includes a combination of endemic (e.g., *Erythroxylum platycladum, Aloe, Kalanchoe, Adina microcephala*) and introduced species (e.g., *Melia azedarach, Psidium cattleianum, Grewia, Passiflora incarnate*)(Cameron and Gould, 2013; Gabriel, 2013a,b; Gould and Gabriel, under review). As a result of selective logging, prior to gaining protected status, most of the native fruit trees, such as *Ficus* and *Adansonia spp.,* are absent (Gabriel, 2013a,b). The forest is now comprised mostly of introduced tree species, including *Melia azedarch* and *Psidium cattleianum* (guava) (Cameron and Gould, 2013; Gabriel, 2013a,b; Gould and Gabriel, under review). In fact, these introduced tree species represent more than 25% of the forest canopy and serve as a year-round food resource for *L. catta* (Gabriel, 2013b; Gould and Gabriel, under review). The matrix surrounding the fragment is a mixture of grasslands, rice paddies, rocky savannah, a small river to the north, as well as an anthropogenically-constructed lake to the south, which is utilized for cattle watering and pisciculture (Gabriel, 2013a,b).

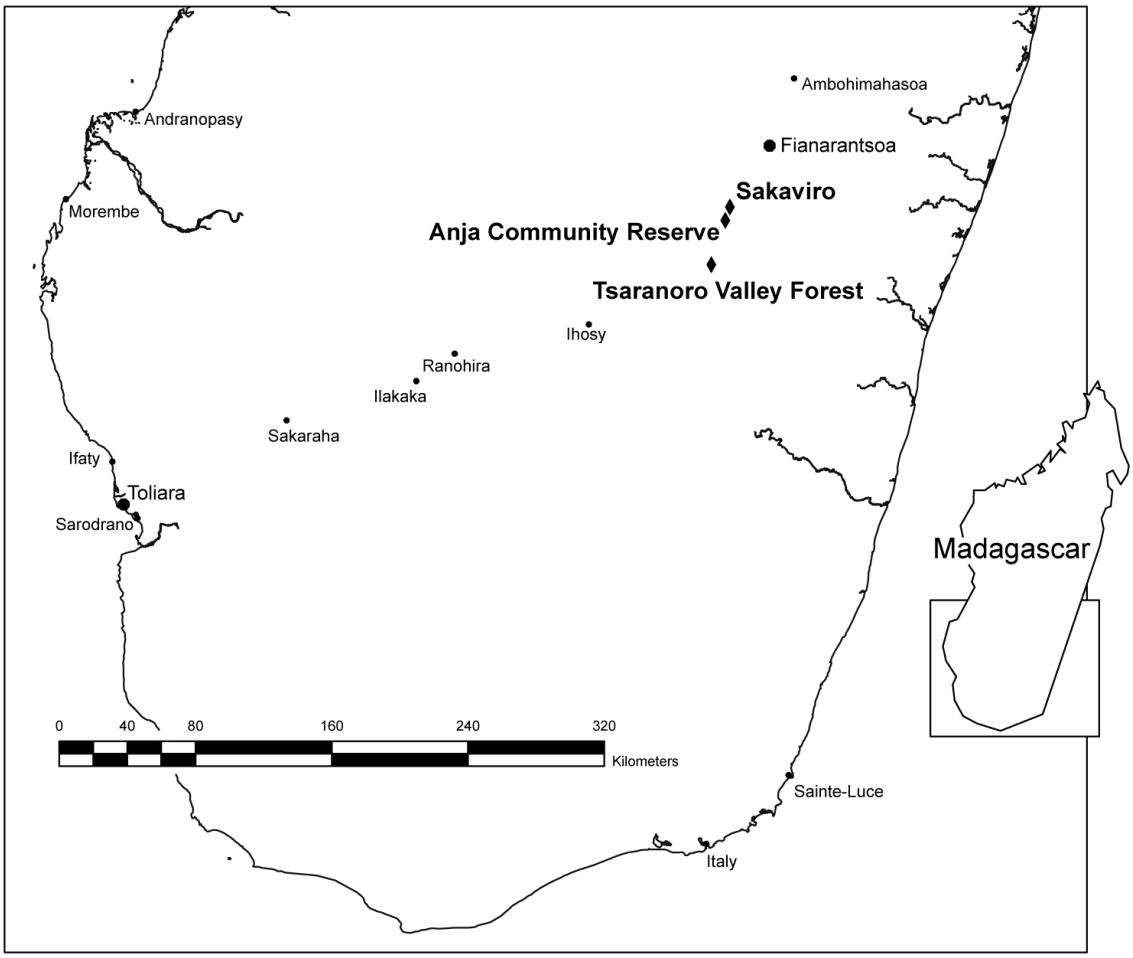

Figure 2.2. Geographic location of study sites (Map Credit: Marc Myers)

The Tsaranoro Valley Forest (22°05'11 S 46°46' 14 E), is larger than Anja at 46 ha, however, it hosts a much smaller population density of *L. catta*, 1.6 lemurs/ha (Cameron and Gould, 2013). The Tsaranoro fragment lies ~55 km SW of Ambalavao and 10 km east of Parc Nationale Andringitra, which also hosts populations of wild ring-tailed lemurs. The Ampiadianombilahy mountain chain, which Andringitra National Park is found within, may serve as a fragmented corridor to and from the Tsaranoro Valley. Furthermore, the valley contains two additional fragments that host *L. catta* groups, Chameleon (8ha) and Marody (3 ha) (Gould, in prep). In light of the landscape structure within the valley it is not improbable to assume that there is movement of individuals between the remaining fragments (i.e., gene flow). The Association Tantely, which is made up of residents from the 11 local villages, manages the fragments, trail and road maintenance, community conservation projects, as well as ecotourism within the valley (Gould, *in prep*). In addition, there are five ecotourism camps located within the valley that contribute a portion of their profits to Association Tantely, which has helped fund community projects, such as the construction of a church and medical clinic (Gould, *in prep*). The elevation range within the valley is variable, 762m and 1,104 m (Cameron and Gould, 2013). Tsaranoro is comprised of continuous and semi-continuous rocky-outcrop vegetation (e.g., *Aloe, Ficus, Dombeya, Kalanchoe*), as well as a mixture of xerophytic, temperate, and rainforest vegetation (Cameron and Gould, 2013; Gabriel, 2012a). The majority of native fruit trees (e.g., *Ficus* and *Adansonia spp.)* within the valley have been removed via selective logging, and in contrast to Anja, the fragments have not been renewed with introduced species (Gould et al., in prep). However, *M. azedarach, P. cattleianum*, and *Mangifera indica* can be found scattered throughout the matrix (Gould et al., in prep).

Sakaviro (21°47. 019' S 046°52.077' E), the smallest of the three study sites, is a 14.2 ha fragment with a population of ~30 *L. catta,* and a population density of 2.1 lemurs/ha (Gould et al., in prep). Sakaviro is located about 8 km north of the Anja Community Reserve, and like both Anja and Tsaranoro, is comprised of rocky-outcrop and xerophytic vegetation (Gould, *in prep*). The surrounding matrix is made-up of numerous villages, dirt roads, and anthropogenic savannah. Sakaviro gained protected

status in September 2012 and is managed by the community-run association, Communautés de Bases Sakaviro-Miray (Gould, *in prep*). The fragment is isolated from other nearby forest patches and at the time of data collection no forest corridors existed. However, the community association in charge of the reserve has plans to plant trees in the hopes of creating corridors for the ring-tailed lemurs. Funding from several NGOs has already allowed for the construction of a reception center for tourists, the establishment of tree nurseries, the creation of trail systems and maintenance, as well as placards for trail identification (Gould, *in prep*).

All of the fragments are similar in their ecological composition, however, Anja is more dominated by introduced fruit trees, including *M. azedarach* and *P. cattleianum* (Cameron and Gould, 2013; Gabriel, 2013b; Gould and Gabriel, under review). Due to year-round abundance of *Melia* within the Anja fragment, *L. catta* has been found to utilize this resource as a staple fallback food (Gould and Gabriel, under review). In addition, all of three sites are considered sacred forest fragments—as Betsileo ancestral tombs are located throughout the fragments. Local *fadys* or taboos restrict logging, burning and hunting within the fragments. Furthermore, the *maki* (Malagasy term for ring-tailed lemur) are highly regarded and protected by the local Betsileo (http://anjareserve.angelfire.com/aboutanja.html). These three study sites are unique among ring-tailed lemur habitat due to their small size and habitat type, rocky-outcrop forest surrounded by anthropogenically disturbed savannah (Gould, 2006; Cameron and Gould, 2013).

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Chapter 3. Genetic Variability of *Lemur catta* in South-Central Madagascar

3.1. Abstract

Madagascar's lemurs, now deemed the most endangered group of mammals in the world, represent the highest primate conservation priority in the world. Due to historical and recent anthropogenic disturbances across Madagascar, particularly slash and burn agriculture, only 10% of original forest cover remains. The endangered ring-tailed lemur *(Lemur catta)* is endemic to the southern regions of the island and occupies an array of habitats. *L. catta* is known for its remarkable behavioral and ecological flexibility, which contributes to its ability to exist in a mostly fragmented landscape. I examined the influence of habitat fragmentation and isolation on the genetic diversity and population structuring of this flagship species in three populations living in south-central Madagascar. Non-invasive fecal samples from 30 individual lemurs were collected from three fragmented forests and genotyped at six polymorphic microsatellite loci. Analyses revealed a moderate level genetic diversity. Genetic differentiation (F_{ST}) among the sites ranged from 0.05-0.11. These data suggest that the *L. catta* populations within south-central Madagascar have not yet lost significant genetic variation. However, due to ongoing anthropogenic threats faced by ring-tailed lemurs, continued conservation and research initiatives are imperative for long-term viability of the species.

3.2. Introduction

Habitat loss and fragmentation represent two of the greatest threats to global biodiversity (Fahrig, 2003). Madagascar, one of the world's preeminent biodiversity hotspots, hosts > 20% of all known primate species, while maintaining only 10% of primary forest cover (Myers et al., 2000; Schwitzer et al., 2013). Human-induced disturbances, particularly slash and burn agriculture has resulted in a severely fragmented landscape (Harper et al., 2007). If current rates of deforestation are maintained, Madagascar is predicted to lose countless species to extinction in the near future (Brooks et al., 2002). Thus, Madagascar's lemurs are now collectively deemed the most endangered group of mammals and represent the highest primate conservation priority in the world (Schwitzer et al., 2013).

Genetic diversity, an essential component of fitness and population viability is vital for animal populations to evolve, respond and adapt to environmental changes (Frankel, 1974; Wright, 1978; Fahrig, 2003). Anthropogenic habitat fragmentation has been shown to have negative impacts on the genetic diversity of wild primate populations (Grativol et al., 2001 *Leontopithecus rosalia*; Goncalves et al., 2003 *Mico argentatus*; Craul et al., 2009 *Lepilemur edwardsi*; Yang et al. 2012 *Rhinopithecus* sp; Brenneman et al., 2011 *Eulemur cinereiceps*). In particular, small, fragmented and/or isolated populations are known to have increased risks of experiencing genetic drift and inbreeding depression, ultimately compromising their evolutionary potential and future viability, and thus, possibly resulting in extinction (Keller and Waller, 2002; Balloux and Moulin, 2002; Frankham, 2003; for a review of inbreeding depression in primates see (Charpentier et al., 2007). For example, black and gold howler monkeys (*Alouatta caraya*) inhabiting forest fragments in Northern Argentina were reported to have compromised dispersal resulting in reduced gene flow and inbreeding (Oklander and Corach, 2013). An examination of captive ring-tailed lemurs house at the Duke Lemur Center revealed compromised health (e.g., increased prevalence of parasites), decreased survivorship, and a loss of genetic diversity due to inbreeding (Charpentier et al., 2008).

Loss of habitat has been described as an accurate predictor of species loss (Brooks et. al., 2002); however, these relationships are complex and poorly understood (Fahrig,

2003). Although some cross-specific similarities exist in terms of species' responses to fragmentation, it is important to realize that we cannot make broad generalizations (Fahrig, 2003). The broader literature regarding how species respond to habitat fragmentation and loss demonstrates that thus far, no clear patterns exist; responses are variable and generally negative (e.g., decreased species richness/abundance, species loss, sensitivity to microclimate changes) depending on taxonomic group (Fahrig, 2003; Ewers and Didham, 2006; Metzeger et al., 2009; Irwin et al., 2010). Certain traits, such as large body size, high trophic level, habitat specialization, and low mobility, have been hypothesized to amplify a species susceptibility to anthropogenic disturbance (Ewers and Didham, 2006). Behavioral and ecological plasticity, high mobility, and longer generation times are suggested to contribute to a species ability to cope with disturbance, resulting in a delayed response or extinction debt (Ewers and Didham, 2006; Kuussaari et al., 2009; Irwin et al., 2010). Additional influencing factors on species' responses to anthropogenic disturbance include historical and recent landscape modifications, level of connectivity, surrounding matrix configuration, population density, as well as life history traits (Kuussaari et al., 2009; Metzeger et al., 2009). In order to tease out confounding factors that may obscure fragmentation effects, Ewers and Didham (2009) highlight the importance of considering concomitantly individual species' traits, spatio-temporal scale, as well as synergistic interactions among other potential drivers of biodiversity loss (e.g., climate change, disease transmission).

While the majority of our knowledge regarding extinction debts comes from research focused on plants (Vellend et al., 2006; Adriaens et al., 2006; Helm et al., 2006) and birds (Brooks and Balmford, 1996; Brooks et al., 1999; Ford et al., 2009), Cowlishaw (1999) examined evidence for potential extinction debts in African primates in relation to historical deforestation. Approximately 50 years after fragmentation occurred, the estimated extinction debts are greater than 30% in the species that remain. In other words, the time-lagged response for African forest primates is extensive; however, it is probable that the majority of extinctions could occur shortly after isolation (Cowlishaw, 1999). Similarly, Metzger et al. (2009) examined the impact of historical and recent anthropogenic disturbance on various forest species (small mammals, birds, frogs, trees) in Brazil. They found that past landscape alteration and structure (i.e., severe

deforestation and fragmentation) had considerable influence on the distribution, richness and abundance of species, except for small mammals and avian edge species (Metzger et al., 2009). Furthermore, species loss was significant when compared to nearby primary and secondary forests (Metzger et al., 2009). These studies underscore the detrimental impact that historical anthropogenic disturbance can have on extant biodiversity.

The endangered ring-tailed lemur (*Lemur catta*) is endemic to the southern regions of Madagascar and occupies an array of diverse habitats (e.g., spiny bush, brush and scrub forest, gallery and dry deciduous forest, high altitude ericoid bush, rocky-outcrop rupicolous, and mangroves) (Jolly, 1966; Goodman et al., 2006; Gould, 2006; Cameron and Gould, 2013; Gould pers. comm.). While this species is characterized by its remarkable behavioral and ecological flexibility (Sauther et al., 1999; Gould et al., 1999; Gould et al., 2011, Kelley, 2011, 2013; Cameron and Gould, 2013; Gabriel, 2013a,b) it may be at particular risk as it is now primarily restricted to living in forest fragments throughout its geographic range (Sussman et al., 2003; Bodin et al., 2006; Cameron and Gould, 2013). *L. catta* has typically been considered ubiquitous throughout southern Madagascar (Sussman et al., 2006; IUCN Red List, 2014), however, habitat fragmentation is ongoing (Bodin et al., 2006), and population numbers in many areas are low and local extinctions have occurred (Sussman et al., 2003; IUCN Red List, 2014; Gould, in prep). Additionally, an emergence of hunting and consumption of *L. catta* in the southern part of the island (i.e., Tsimanampetsotsa National Park) (LaFleur, 2013) increases the need for immediate evaluations (e.g., censuses, forest assessments, and genetic analyses) and implementation of conservation measures. Furthermore, to date, only two investigations of wild ring-tailed lemur genetics have been conducted, focusing on two southwestern populations (Parga et al., 2012; Pastorini, under revision). Thus, comparative data on *L. catta* genetics is imperative to estimate the species' long-term viability in the wild throughout its geographic range.

In this dissertation, I report the first population genetic data utilizing non-invasive sampling and microsatellite genotyping for three populations of *Lemur catta* living in small forest fragments in south-central Madagascar. Researchers have been studying these populations (except Sakaviro) since 2009 (Cameron and Gould, 2013; Gabriel,

2013a,b, Gould and Gabriel, 2014). To understand the influence of habitat fragmentation and isolation on the genetic variability and population structure of *L. catta* populations, I measured the genetic variability within and between sites and examined the genetic structure of the populations within the fragmented landscape. I then discuss and compare my results with genetic data from two populations of *L. catta* in southwestern Madagascar (Parga et al., 2012).

3.3. Methods

3.3.1. Study Sites and Sample Collection

Non-invasive fecal samples were collected opportunistically from wild ring-tailed lemurs inhabiting three different forest fragments (Chapter 2, Figure 1, pg. 24) in south-central Madagascar. All three sites are comprised of mixed xerophytic, deciduous vegetation, and large granite outcrops (for more detail see Chapter 2) (Cameron and Gould, 2013; Gabriel, 2013a,b; Gould, et al., in prep). The Anja Private Reserve is an isolated 34 ha fragment with a population of approximately 220 *L. catta* (Cameron and Gould, 2013; Gabriel, 2013a,b). Sakaviro is 14 ha and maintains a population of approximately 30 ring-tailed lemurs (Gould, et al., in prep). The Anja and Sakaviro fragments are isolated due to surrounding roads and anthropogenic savannah. Lack of corridors to other forest patches means that dispersal is highly unlikely due to the risks (e.g., predators, cars) involved in moving across such a fractured landscape. In contrast, the Tsaranoro Valley is the largest at 49 ha with a population of approximately 80 *L. catta* (Cameron and Gould, 2013; Gabriel, 2013a,b). Unlike Anja and Sakaviro, the Tsaranoro valley contains two additional fragments with *L. catta* <5km away from the Tsaranoro fragment (Gould et al., in prep). Genetic samples were opportunistically collected during August-October 2012. The sample (n=30) utilized for this study is comprised of ten individuals per site (Table 1): 5 adult males, 4 adult females, and 1 unknown from Anja, 5 adult males and 5 adult females, from Tsaranoro, and 5 adult males, 1 adult female, 3 sub-adult females (~2 yrs. old), and 1 unknown from Sakaviro. Samples collected within the three sites came from various groups within each population: Anja samples were collected from three groups, Tsaranoro samples from four groups, and Sakaviro samples were collected during censusing. Upon collection, each individual fecal sample was immediately stored in RNA*later* (Ambion, Austin, TX)

solution to ensure proper preservation and minimize DNA degradation (Di Fiore, 2003). Each sample was logged and labeled, noting the date, site, group number, and sex of individual (if known). Samples were stored at ambient temperature until they could be brought back from the field. This time frame ranged from one to three months depending upon when the sample was collected. Samples were then banked at New York University's Molecular Anthropology Laboratory. The University of Victoria's Research Services Animal Care Committee has approved all research methods and protocols for this research (protocol # 2012-013).

Table 3.1. Sampled individuals (n=30), Animal ID, site where sample was collected, and sex of individuals

Animal ID	Site	Sex
1	Anja	Male
4	Anja	Male
6	Anja	Female
21	Anja	Male
22	Anja	Female
29	Anja	Female
30	Anja	Male
46	Anja	Male
68	Anja	Female
100	Anja	Unknown
3	Tsaranoro	Female
8	Tsaranoro	Male
9	Tsaranoro	Female
15	Tsaranoro	Female
17	Tsaranoro	Male
25	Tsaranoro	Female
27	Tsaranoro	Male
28	Tsaranoro	Female
33	Tsaranoro	Male
39	Tsaranoro	Male
11	Sakaviro	Female
12	Sakaviro	Male
13	Sakaviro	Male
14	Sakaviro	Sub- Female
23	Sakaviro	Male
24	Sakaviro	Sub- Female
35	Sakaviro	Male
36	Sakaviro	Unknown
51	Sakaviro	Sub- Female
52	Sakaviro	Male

3.4. Genetic analysis

3.4.1. DNA extraction

DNA was extracted from fecal samples using QIAampDNA Stool Mini Kits (Hilden, Germany). Extraction procedures followed the manufacturer's protocols, with the following modifications: starting material was increased to 300mg; Proteinase K was increased to 35 µl; incubation was increased to 30 minutes (70°C); and DNA was eluted twice in Buffer AE (75 µl and 50 µl, respectively).

3.4.2. Microsatellite genotyping

Samples were amplified at six highly polymorphic loci (Table 2). Polymerase chain reaction (PCR) was performed in a total reaction volume of 8 or 9 µl using 1µl of DNA extract. The 5' end of forward primers were fluorescently labeled and DNA was amplified using Qiagen Multiplex PCR kits following (Burrell, 2009), using annealing temperatures specified in Parga et al. (2012). PCR products were run on an ABI 3730 DNA Analyzer with Gene Scan ROX size standard (Applied Biosystems, Foster City, California). Allele calls were carefully performed by eye via Genemapper software *v. 4.0* (Applied Biosystems). In order to accurately confirm individual genotypes independent PCR amplifications were conducted a minimum of two to three times per marker/per individual (i.e., multiple tubes approach) (Taberlet et al., 1999). Specifically, heterozygous genotypes were confirmed with at least two independent reactions, whereas homozygous genotypes were confirmed with 3-7 independent reactions (Taberlet et al., 1999).

Table 3.2. Microsatellite sequence/markers used for molecular analyses.

Locus	Primer Sequence (5' to 3')	Annealing Temp. °C	Size Range (bp)	Reference
Lc5	F: tgggacacctttgttatcag	60	127-151	Pastorini et al., 2005
	R: gatttctcaggacatccatag			
Lc7	F: acctcccagcctattcacag	60	172-198	Pastorini et al., 2005
	R: ggagtggggacttgaatagc			
69HDZ267	F: acctccataacataagcacacg	55	156-178	Zaonarivelo et al., 2007
	R: agccagaataaagtcagggg			
Efr09	F: aatttcaggtatgcgtgt	54	92-126	Jekielek & Strobeck, 1999
	R: gtttggggcaagttgaatag			
L-2	F: atagacatccagagataagg	48	179-203	Merenlender, 1993
	R: ggcacctctagactctgtta			
Em12	F: gaacctgggtggctacattc	60	119-171	Pastorini, in prep.
	R: gtttgtattaggcttggctgc			

3.5. Statistical Analysis

Scoring errors (i.e., null alleles, allelic dropout) were assessed via Micro-Checker *v.2.2.3* (Van Oosterhout et al., 2004) and GENEPOP *v. 4.2* (Raymond and Rousset, 1995) was employed to confirm that loci were independent (i.e., test for linkage disequilibrium). Genetic diversity was measured using the mean number of alleles per population (MNA), observed (H_O) and expected (H_E) heterozygosity, as well as effective number of alleles (N_e) and inbreeding coefficient (F) via *GenAlEx v. 6.5* (Peakall and Smouse 2006, 2012). Departure from Hardy-Weinberg equilibrium (HWE) was performed using *GenAlEx v. 6.5* (Peakall and Smouse 2006, 2012), which follows Hedrick (2000). The level of genetic structure among the three fragments was estimated via Pairwise F_{ST} (Wright, 1978), following Hartl and Clark (1997). In addition, an analysis of molecular variance (AMOVA) was also performed to examine the level of population structure (Peakall and Smouse, 2006, 2012). Nei's *D* (Nei, 1972, 1973) was employed to assess the genetic distance among populations. The relationship between genetic distance and geographic distance was examined to assess isolation-by-distance (Slatkin, 1993) via a mantel test (Mantel, 1967) by employing the IBD web service *v. 3.23* (Jensen et al., 2005).

3.6. Results

Individual identity was confirmed for all samples across the six loci (i.e., no individuals were identical at the six markers). Null alleles, or non-amplified alleles, were not detected for Anja and Sakaviro; however, locus L-2 showed evidence of null alleles in the Tsaranoro sample. The presence of null alleles can result in genotyping errors; for example, a heterozygous individual could be incorrectly identified as homozygous due to non-amplification (van Oosterhout et al., 2004). Only one combination of loci showed evidence of linkage disequilibrium (L-2/Lc5), meaning that the alleles associated with these two loci have been inherited together due to their proximity on a single chromosome. All six loci used were highly polymorphic, with an average of 5.2 alleles (SD=0.40) per locus across all samples. Number of alleles per locus/per forest fragment ranged from 3-9 (Table 3). Allelic richness per population ranged from 4.8-5.8 (Table 4). The mean number of private alleles per population (alleles that are only found in a single population) was low and ranged from 0.5-1.83. Mean H_E ranged from 0.66 (SD=0.05) to 0.74 (SD=0.01). The mean H_O ranged from 0.72 (SD=0.09) to 0.88 (SD=0.04). Only one

locus deviated from Hardy-Weinberg Equilibrium (L-2, at Tsaranoro; $p<0.05$). Pairwise F_{ST} values between all populations ranged from 0.052 to 0.11 (Table 5). The highest Fst estimates were found between Sakaviro and Tsaranoro. Likewise, Nei's *D* values were the greatest for Sakaviro and Tsaranoro, whereas the lowest values were found between Anja and Sakaviro. The results of the AMOVA (Figure 2) revealed that 90% of the variation was found within populations, while 10% was attributed to among population variance ($P<0.001$). A tight, yet not significant correlation was found between genetic and geographic distance (r=0.99, p=1.0).

Table 3.3. Geographic locale, number of alleles, H_O and H_E per locus.

Forest Fragment	MNA	MNP	N_e	H_O	H_E	F	HWE
Anja	5.16	0.66	3.74	0.88	0.71	-0.25	NS/all loci
Sakaviro	4.83	0.50	3.40	0.79	0.66	-0.18	NS/all loci
Tsaranoro	5.83	1.83	3.97	0.72	0.74	0.03	NS/one locus*

NOTES: MNA, mean number of alleles; MNP, mean number of private alleles; N_e, number of effective alleles; F, inbreeding coefficient; HWE, Hardy-Weinberg Equilibrium, NS, not significant, *P<0.05

Table 3.4. Summary Statistics for the three study fragments.

Forest Fragment	Locus	# of Alleles	H_O	H_E
Anja	Efr09	3	1.00	0.62
	Lc5	6	1.00	0.80
	Lc7	8	0.77	0.80
	L-2	4	0.90	0.65
	Em 12	4	0.75	0.65
	69HDZ267	6	0.87	0.73
Tsaranoro	Efr09	4	0.71	0.78
	Lc5	4	0.83	0.76
	Lc7	9	0.50	0.68
	L-2	5	0.40	0.75
	Em 12	3	1.00	0.76
	69HDZ267	4	0.87	0.71
Sakaviro	Efr09	5	1.00	0.68
	Lc5	5	0.37	0.49
	Lc7	5	0.75	0.82
	L-2	6	0.88	0.74
	Em 12	6	0.75	0.55
	69HDZ267	8	1.00	0.69

NOTES: H_O is observed heterozygosity and H_E is expected heterozygosity.

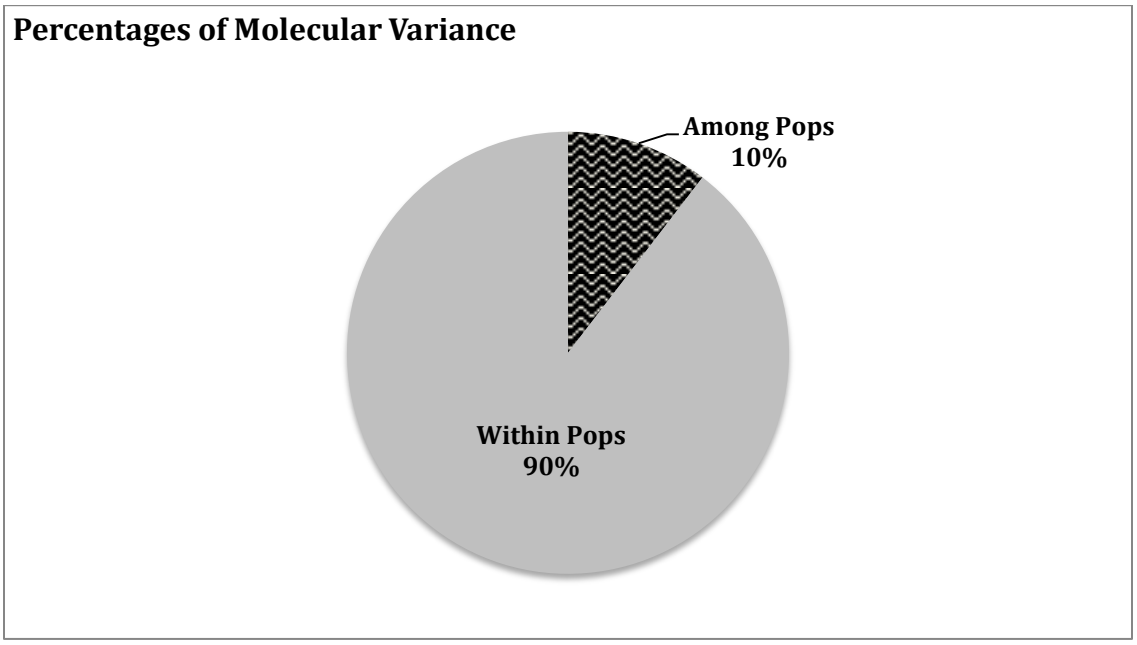

Figure 3.1. Analysis of Molecular Variance (AMOVA).

NOTES: Pie chart shows the proportion of variation present within (grey) and among (zig-zag pattern) populations. Analysis of Molecular Variance (AMOVA) was performed using GenAlEx 6.5.

Table 3.5. Pairwise F_{ST} and pairwise Nei's *D*.

Forest Fragments	Distance between fragments	F_{ST}	Nei *D*
Sakaviro & Tsaranoro	34 km	0.11	0.91
Sakaviro & Anja	12 km	0.05	0.26
Anja & Tsaranoro	27 km	0.09	0.77

NOTES: Pairwise F_{ST} and *Nei's D* were performed using GENALEX 6.5. F_{ST} measures the degree of genetic distance between populations; *Nei's* genetic distance, a measure of genetic divergence between two populations.

3.7. Discussion

3.7.1. Genetic Diversity

Genetic diversity is a key factor in a species ability to respond and adapt to environmental changes (Frankel, 1974). This diversity is lost more rapidly amongst smaller populations and loss is intensified when occupying a fragmented or isolated habitat (Lawes et al., 2000; Bergl et al., 2008). Moreover, the loss of genetic diversity can increase extinction risk (Frankel, 1974).

The three study populations of *L. catta* appear to maintain a moderate level of genetic diversity (MNA =4.8-5.8, H_O=0.72-0.88), despite their small population sizes and substantial level of fragmentation. As expected, the smallest isolated fragment, Sakaviro, showed the lowest level of allelic richness (MNA=4.8), whereas the largest non-isolated fragment, Tsaranoro, had the highest (MNA=5.8). The level of diversity found among *L. catta* in south-central Madagascar is similar to those found for other Malagasy strepsirrhines, including *Propithecus tattersalli* (MNA=6.3, H_O =0.69) and *Microcebus spp.* (MNA=4.38-6.50, H_O =0.47-0.69) (Olivieri et al., 2008; Quéméré et al., 2010). Higher H_O versus H_E values were found for the Anja and Sakaviro populations, as well as a trend of high H_O values for the majority of individual loci in both populations. Since Tsaranoro is the only study population with more opportunity for gene flow with other *L. catta* groups in nearby forest patches which contain *L. catta* groups (Chameleon (8ha) and Marody (3 ha)), the lower H_E versus H_O values is unexpected, and suggests compromised gene flow and possible inbreeding. The positive inbreeding coefficient (F=0.03) is further evidence for inbreeding in the Tsaranoro population. An alternative interpretation, the positive inbreeding coefficient is due to the sampling methodology. In other words, sampling individuals who live in groups where non-random mating is occuring resulting in a structured population (Sugg et al., 1996). In contrast, negative inbreeding coefficients, indicating no evidence of inbreeding, were found for Anja and Sakaviro (F= -0.25 and -0.18, respectively).

Similar average H_O levels (0.82 and 0.82) were found for *L. catta* populations within the markedly larger Beza Mahafaly Special Reserve (BMSR) (80 ha) and Tsimanampetsotsa National Park (TNP) (48,000 ha) in southwestern, Madagascar (Parga et al., 2012). However, in contrast, the ‘mean number of alleles per locus’ were greater

across all loci for *L. catta* populations within BMSR (MNA = 9.5) and TNP (MNA= 9.8) (Parga et al., 2012) than for Anja (MNA= 5.16), Tsaranoro (MNA= 5.83), and Sakaviro (MNA= 4.83). It should be noted that Parga and colleagues (2012) had a larger sample size (BMSR=20, TNP=25) and utilized eight microsatellite loci for their analyses. For my comparisons with the data from Parga et al. (2012), I only included the microsatellite markers that both studies shared in common.

3.7.2. Population Structure

A relatively low pairwise F_{ST} (0.05) value between Sakaviro and Anja indicate little population structuring, suggesting these populations likely share a history of gene flow between them and are more genetically similar. Conversely, higher pairwise F_{ST} values between Tsaranoro and Anja (0.09) and Tsaranoro and Sakaviro (0.11) suggest a greater degree of population structuring, indicating greater genetic differentiation. Patterns of genetic structuring and genetic differentiation will increase with geographic distance (Wright, 1943, 1946). Thus, these results are not unexpected considering the greater geographic distances that exist between Sakaviro and Tsaranoro (35 km) and Anja and Tsaranoro (27.3 km) versus the 7.8km distance between Anja and Sakaviro. Differences in population structuring are further supported by the strong pattern of *Isolation by Distance* (IBD) found to be present. My results suggest that genetic population structuring between Anja and Tsaranoro (and separately, Tsaranoro and Sakaviro) are a product of geographic distance and fragmentation. Likewise, a significant pattern of IBD and similar F_{ST} values (0.01-0.15) were found for *Microcebus ravelobensis* and are proposed to reflect a situation in which fragments had previously been connected prior to recent habitat disturbance (Olivieri et al., 2008).

3.8. Conclusion

In conclusion, this study shows that all three *L. catta* populations maintain a moderate level of genetic diversity, there is evidence for inbreeding among ring-tailed lemurs within the Tsaranoro population, and there is a greater level of structuring and thus genetic differentiation between Sakaviro and Tsaranoro and Anja and Tsaranoro.

While the populations that I examined retain a moderate level of diversity and show no sign of loss of genetic diversity (yet), we must consider the possibility that

deleterious genetic consequences have not been detected due to the possibility that the disturbance and fragmentation experienced by these populations is fairly recent (Whitlock and McCauly, 1999). In fact, the severe fragmentation has been suggested to have occurred within the past few decades (Andrianomena, pers. com.). Considering the remarkable behavioral and ecological flexibility of *Lemur catta*, combined with the variability of species' responses to environmental perturbations, such as fragmentation, it is possible that these populations are experiencing a time-lagged response before potentially dangerous losses of genetic variation. While *in situ* conservation efforts are already present in some of these particular areas (Gould and Gabriel, 2013; Gould et al., in prep) it is crucial that *L. catta* populations in south-central Madagascar remain a priority for conservation. One example of a recent conservation initiative includes the establishment of tree nurseries at Tsaranoro and Sakaviro, and a few other small fragments containing *L. catta* in this region, the goal of which is to expand the forest fragments and provide additional food trees for the ring-tailed lemurs (Gould, pers. comm). Fortunately, the majority of community associations in charge of managing conservation and ecotourism in the central highland region, such as Anja Miray, Sakaviro Miray, Samisorany, Andranobe, and the Association Tantely are motivated and eager to continue and expand their efforts (Gould, pers. comm). However, further education and funding for community conservation projects are crucial (See Chapter 6), especially considering that not all communities (i.e., Bedita and Ikomby) within the region are motivated to conserve what little ring-tailed lemur habitat remains (Gould, pers. comm).

Research focusing on the genetic and behavioral impacts of anthropogenic disturbance among wild primate populations are becoming more widespread (Grativol et al., 2001; Goncalves et al., 2003; Goossens et al., 2005; Bergl et al., 2008; Oklander and Corach, 2013; Martins et al., 2012), particularly since the majority of primate species now persist within anthropogenically-disturbed environments. In order to preserve genetically healthy wild primate populations and to establish proper *in situ* and *ex situ* conservation management plans, it is essential to understand the effects and consequences of habitat loss, fragmentation, and isolation on biodiversity.

To date, numerous studies on a variety of primate species, such as golden lion

tamarins (*L. rosalia*) (Grativol et al., 2001), black-faced lion tamarins (*Leontopithecus caissara*) (Martins et al., 2012), golden snub-nosed monkeys (*Rhinopithecus roxellana*) (Li et al., 2003), and black and gold howler monkeys (*A. caraya*) (Oklander and Corach, 2013) have indicated that habitat disturbance and loss have had devastating impacts on the genetic health of wild populations. In particular, Grativol et al (2001) concluded that their four isolated study populations of golden lion tamarins were bereft of genetic diversity. Despite the current climate that global biodiversity is facing, there is still reason for hope. Several studies, indicate that some primate species (e.g., ring-tailed lemur (*L. catta*) (this study), Bornean orangutan (Goossens et al., 2005), golden-crowned sifaka (*P. tattersalli*) (Quéméré et al., 2010), have been able to maintain a moderate to high level of genetic diversity despite intense habitat disturbance within their given geographic ranges. These data allow us to be cautiously optimistic for the future viability of wild primate populations, while at the same time prompt us to take immediate steps to prevent further deforestation, as well as to begin the process of reforestation where possible.

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Chapter 4. Population bottlenecks of *Lemur catta* populations in south-central Madagascar

4.1. Abstract

Madagascar has undergone a range of historical and contemporary landscape transformations, both natural and anthropogenic. These landscape transformations combined with additional human-induced disturbances have had devastating effects on the island's extant primate populations. *Lemur catta* represents one of the most well studied Malagasy strepsirhines; however, there has been a paucity of research regarding the population and conservation genetics of this endangered species. I examined three fragmented populations of *L. catta* inhabiting the central highlands of Madagascar for genetic bottleneck signals, specifically the Anja Reserve, Sakaviro, and the Tsaranoro Valley. A total of 30 individuals were genotyped at up to six polymorphic loci. I employed three approaches to examine evidence of genetic bottlenecks, including mode-shift and M-Ratio tests, as well as a test to detect heterozygosity excess using three mutation models: the two-phase model (TPM), step-wise mutation model (SMM), and the infinite allele model (IAM). Results were equivocal depending on the test that was applied; however, a mode-shift was detected for Anja, indicating this population suffered a historical bottleneck. Additionally, M-ratio tests indicated that all three populations underwent a historical bottleneck. Under the IAM, both Anja and Sakaviro showed significant heterozygosity excess. Despite the mixed results, habitat destruction and loss continue across Madagascar, including the central highlands; thus, all remaining *L. catta* populations should be considered a high conservation priority.

4.2. Introduction

The ability to detect population bottlenecks combined with additional molecular (e.g., genetic structuring) and landscape (e.g., presence of corridors and metapopulations) data can facilitate the identification of conservation priority areas and populations. These data can provide insight into the demographic history of populations in regards to how human-induced anthropogenic disturbances and natural environmental changes can influence and impact wild populations (e.g., Radespiel et al., 2008; Liu et al., 2009).

Effective population size (N_e) represents the number of *required* breeding individuals in a theoretically *ideal* population that can account for the observed level of variation in the actual population (Wright, 1931, 1938). A sudden decrease in effective population size (N_e) or a population bottleneck can have severe consequences for a population or species (Nei et al., 1975; Luikart and Cornuet, 1998; Reed, 2005). These can include increases in demographic stochasticity, inbreeding (leading to inbreeding depression), loss of genetic diversity, the fixation of deleterious recessive alleles, and a reduction in overall fitness which will diminish the ability to adapt to environmental changes, and ultimately increase the risk of extinction (Nei et al., 1975; Luikart and Cornuet, 1998; Reed, 2005). More specifically, when a population bottleneck occurs, there is a decline in the average heterozygosity per locus, and the rate of decline is dependent upon the effective population size (N_e), although if there is an increase in population size, average heterozygosity will increase via new mutations (Wright, 1931). Genetic bottlenecks produce an *excess of heterozygosity* due to alleles (rare alleles that are usually lost first and contribute little to expected heterozygosity) being lost more rapidly than heterozygosity (Wright, 1931). Furthermore, the size of a bottleneck and the population recovery or rate of regrowth, post-bottleneck, impacts the amount of reduction in heterozygosity, although the loss of alleles is contingent upon the bottleneck size (Nei et al., 1975).

Population bottlenecks have been documented across a range of taxonomic groups, including insects (Solbreck, 1991 (Black-and-red-bug)), birds (Glen et al., 1999 Whooping crane), reptiles (Kuo and Janzen, 2004 ornate box turtles), amphibians (Andersen et al., 2004 European tree frog), and mammals (Russello et al., 2004 Amur

tiger). Literature regarding population bottlenecks among non-human primates has begun to increase over the last decade and several of these studies have focused on the Malagasy strepsirhines (Louis et al., 2005; Olivieri et al., 2008; Lawler, 2008; Craul et al., 2009; Razakamaharavo et al., 2010; Brenneman et al., 2011; Parga et al., 2012).

Razakamaharavo et al. (2010) examined evidence of demographic bottlenecks in two populations of red ruffed lemurs (*Varecia rubra*) in Masoala National Park utilizing the program BOTTLENECK (Piry et al., 1999). A significant recent bottleneck signal (i.e., less than $4N_e$ generations ago) was detected under two mutation models, the infinite allele model (IAM) and the two-phase model (TPM) (see Methods below), for the DAMA population that resides in an area of the park that has suffered significant fragmentation (Razakamaharavo et al., 2010). In addition, a mode-shift test revealed a disruption in the allele distribution, suggesting the DAMA population also underwent a historical bottleneck (prior to $4N_e$). In contrast, the MAS population, which inhabits a pristine area of the park, showed no evidence of a bottleneck (Razakamaharavo et al., 2010). Despite a significant reduction in the DAMA's population size, a negative inbreeding coefficient (F_{IS}) indicates this population has not yet succumbed to inbreeding (Razakamaharavo et al., 2010).

Evidence of population bottlenecks were also found for four populations, Ranomafana, Manombo, Vatovavy, and Kianjavato, of black and white ruffed lemurs (*Varecia variegata*) (Louis et al., 2005). Not unlike the DAMA population, black and white ruffed lemurs from the Ranomafana population underwent a recent genetic bottleneck as evidenced by significant heterozygosity excess via IAM and TPM mutation models (Louis et al., 2005). Disruptions in allele distributions via mode-shift tests indicated that all four populations of *V. variegata* had experienced historical bottlenecks (Louis et al., 2005). However, the authors caution that these data may not be statistically supported due to smaller than the minimum required sample sizes needed for three (Manombo, Vatovavy, and Kianjavato) of the four populations (Louis et al., 2005). Regardless, other molecular data support evidence for historical bottlenecks in all populations, including significant allelic differentiation (each population is distinct), loss of heterozygosity, and high levels of inbreeding (Louis et al., 2005).

Parga et al. (2012) employed BOTTLENECK software and an M-ratio test (see Methods below) (Garza and Williamson, 2001) to investigate historical and recent bottlenecks in two populations of ring-tailed lemurs (*Lemur catta*) in southwestern Madagascar: Beza Mahafaly Special Reserve (BMSR) and Tsimanampetsotsa National Park (TNP). Similar to the DAMA (Razakamaharavo et al., 2010) and Ranomafana (Louis et al., 2005) populations, both *L. catta* populations revealed evidence of recent population bottlenecks under two of the three mutation models (IAM and TPM)(Parga et al., 2012). Similarly, M-ratio tests supported recent population declines (Parga et al., 2012). However, the mode-shift tests for *L. catta* did not indicate a historical bottleneck (Parga et al., 2012).

An examination of three rare mouse lemur species, *Microcebus ravelobensis*, *Microcebus danfossi*, and *Microcebus bongolavensis*, in northwestern Madagascar revealed evidence of both recent population declines, as well as population collapses (Olivieri et al., 2008). Specifically, population bottlenecks were found under the IAM and TPM models for seven out of eight sites for *M. ravelobensis* and one out of seven sites for *M. danfossi* (Olivieri et al., 2008). Results from MSVAR (Markov Switching VAR) (Beaumont, 1999; Storz and Beaumont, 2002), which utilizes a Bayesian likelihood method, suggest that all three *Microcebus* species had large effective population sizes (N_e) of between 10,000 to 20,000 individuals prior to undergoing a bottleneck (Olivieri et al., 2008). However, post-bottleneck N_e was reduced by two to three orders of magnitude (i.e., population collapse) (Olivieri et al., 2008). Evidence suggests that the population collapses (except one site for *M. ravelobensis*) coincided with rapid human population growth and expansion (i.e., within the last 500 years) (Olivieri et al., 2008). Despite population collapses, these mouse lemur populations are still genetically diverse, which could be due to fast reproductive rates (Olivieri et al., 2008). However, larger lemur species with slower reproductive rates could be more severely impacted (Olivieri et al., 2008), leading to slower recovery and possible local extinctions.

Although anthropogenic pressures, such as slash and burn agriculture and logging have had detrimental impacts since the arrival of humans to Madagascar, not all lemur species studied to date have shown evidence of population bottlenecks. For example,

Lawler (2008) found no historical bottleneck signal for a population of *Propithecus verreauxi* inhabiting the Beza Mahafaly Special Reserve (BMSR) in southwestern Madagascar, a region of the island that has been subjected to intense anthropogenic disturbance (Brinkmann et al., 2014).

Craul et al. (2009) utilized both BOTTLENECK and MSVAR to investigate evidence of demographic bottlenecks across 17 sites in northwestern Madagascar for *Lepilemur edwardsi*. Similar to Lawler (2008), the BOTTLENECK analysis provided no clear evidence for a population bottleneck for *L. edwardsi* under any of the three mutation models (i.e., SMM, IAM, and TPM)(Craul et al., 2009). In contrast, utilizing MSVAR revealed strong evidence of historical bottlenecks in two populations (i.e., Mariarano and Ankaririka) (Craul et al., 2009). Moreover, population collapses were found for both sites and a decline in effective population size (N_e) was about two orders of magnitude (Craul et al., 2009) larger than what was found for *Microcebus* spp. (Olivieri et al., 2008). Population collapses for *L. edwardsi* are estimated to have occurred later than that of *Microcebus* species (Olivieri et al., 2008), approximately 100 years ago (Craul et al., 2009).

In addition to the Malagasy strepsirhines, molecular examinations of population bottlenecks have been documented for a variety of other primate groups, including New and Old World monkeys (Storz et al., 2002 *Papio cynocephalus*; Ruiz-Garcia et al., 2007 *Alouatta spp.*; Kawamoto et al., 2008 *Macaca fuscata*; Milton et al., 2009, *Alouatta palliata*; Martins et al., 2012 *Leontopithecus caissara*), as well as the great apes (Goossens et al., 2005 *Pongo pygmaeus*; Bergl et al., 2008 *Gorilla spp.*).

Liu et al. (2009) employed BOTTLENECK and MSVAR to examine evidence of a bottleneck in a critically endangered colobine, the Yunnun snub-nosed monkey (*Rhinopithecus bieti*), a species that is now restricted to high-altitude forest fragments as a result of habitat fragmentation. Both methods failed to support demographic bottleneck signals for this species (Liu et al., 2009).

Milton and colleagues (2009) employed BOTTLENECK, MSVAR , and a M-ratio test to examine recent and historical bottlenecks in a population of mantled howler

monkeys (*Alouatta palliata*) on Barro Colorado Island, Panama. As with *P. verreauxi* (Lawler, 2008), *L. edwardsi* (Craul et al., 2009), *R. bieti* (Liu et al., 2009) recent population declines were not found for *A. palliata* under any of the three mutation models (i.e., IAM, SMM, TPM) (Milton et al., 2009). Similar to *L. catta* populations in southwestern Madagascar (Parga et al., 2012), a mode-shift test failed to support evidence of a recent bottleneck (Milton et al., 2009). In contrast to *L. catta*, M-ratio test results did not support a recent historical bottleneck for this population of howler monkeys (Milton et al., 2009). However, coalescent-based analyses (Beaumont, 1999; Storz and Beaumont, 2002) provided strong evidence of a substantial decline in N_e (~5,188 to ~14 individuals) over the last 141 years (Milton et al., 2009). This recent and marked reduction in effective population size of *A. palliata* (Milton et al., 2008) is comparable to that found for *L. edwardsi* (Craul et al., 2009).

In contrast to the more recent population declines that have been found for *A. palliata* (Milton et al., 2009), *L. edwardsi* (Craul et al., 2009), and *Microcebus spp.* (Olivieri et al., 2008), a population of *Papio cynocephalus* in East Africa was found to have a more ancient onset of population decline, estimated to have begun during the late Pleistocene or early Holocene (Storz et al., 2002). The current N_e for *P. cynocephalus* is estimated to be between 200 and 10,000 individuals, a substantial decline from the estimated ancestral (i.e., late Pleistocene/early Holocene) N_e of between 5,000 and 60,000 (Storz et al., 2002).

Finally, a comparative analysis via BOTTLENECK of genetic bottlenecks for three subspecies of gorilla, including the Cross River gorilla (*Gorilla gorilla diehli*), mountain gorilla (*Gorilla beringei beringei*), and Western lowland gorilla (*Gorilla gorilla gorilla*) indicated that gorilla populations inhabiting smaller fragmented and isolated forests experienced population declines. While both the Cross River gorilla and the mountain gorilla populations (Virgungas and Bwindi) were found to have undergone recent bottlenecks, the signal for the mountain gorilla populations were significantly weaker (Bergl et al., 2008). Conversely, the Western lowland population from Mondika, which is larger and more continuous, revealed no evidence of a population bottleneck (Bergl et al., 2008).

In this chapter, I report data regarding genetic bottlenecks in three populations of *Lemur catta* inhabiting forest fragments in south-central Madagascar. To understand the influence of historical and contemporary landscape transformations, including habitat fragmentation, loss, and isolation, I examine whether these populations experienced a reduction in effective population size (N_e) in the recent past or historically, evidenced by heterozygosity excess. I compare these data with that of Parga et al. (2012) in order to begin to piece together a more comprehensive picture of this species' demographic history in relation to anthropogenic landscape changes across its geographic range.

4.3. Methods

4.3.1. Study sites, sample collection, & laboratory protocols

I focused data collection on three fragmented populations of ring-tailed lemurs (*Lemur catta*) in Madagascar's central highlands (see Chapter 1 for site details). Fecal samples were collected opportunistically during July-October 2012. Immediately following collection, samples were stored in RNA*later* solution (Ambion, Austin, TX)(Di Fiore, 2003). Ten samples per site were utilized for this study (n=30) (see Chapter 2, Table 1).

DNA was extracted from fecal samples using QIAampDNA Stool Mini Kits (Hilden, Germany). Extraction procedures followed the manufacturer's protocols, with the following modifications: starting material was increased to 300mg; Proteinase K was increased to 35 µl; incubation was increased to 30 minutes (70°C for); and DNA was eluted twice in Buffer AE (75 µl and 50 µl, respectively).

Samples were amplified at six polymorphic loci utilizing Qiagen Multiplex PCR kits following (Burrell, 2009) using annealing temperatures following Parga et al. (2012). PCR products were run on an ABI 3730 DNA Analyzer with Gene Scan ROX size standard (ABI). Allele calls were performed by eye using Genemapper software *v. 4.0* (Applied Biosystems, Foster City, California). Genotypes were confirmed with multiple independent PCR amplifications, specifically using Taberlet and colleagues' (1999) 'multiple tubes' approach. Heterozygous genotypes were confirmed with at least two

independent reactions and homozygous genotypes were confirmed with 3-7 independent reactions (Taberlet et al., 1999).

Scoring errors (i.e., null alleles, allelic dropout) were assessed via Micro-Checker *v.2.2.3* (Van Oosterhout et al., 2003) and GENEPOP *v. 4.2* (Raymond and Rousset, 1995) was employed to confirm that loci were independent (i.e., test for linkage disequilibrium) (See Chapter 3 for results).

4.3.2. Statistical Analyses

In order to evaluate whether the three populations of *L. catta* underwent a historical and/or recent population bottleneck, I employed three approaches. The first two approaches utilized the software BOTTLENECK *v. 1.2* (Piry et al., 1998). This program evaluates genotypic data with as little as four polymorphic microsatellite loci and 20-30 individuals, making it appropriate for smaller genotypic data sets (Piry et al., 1999). BOTTLENECK is capable of detecting both historical (Luikart et al., 1998) and recent population bottlenecks ($<4N_e$) (Luikart and Cornuet, 1998).

Historical bottlenecks were assessed using the mode-shift test, which reveals disruptions in the distribution of allele frequencies and therefore, is able to distinguish between stable and bottlenecked populations (Luikart et al., 1998). Recent population bottlenecks can be detected via a sign test, standardized difference test, or a Wilcoxon's signed-rank test (Cornuet and Luikart, 1996; Luikart and Cornuet, 1998). More specifically, these tests identify heterozygosity excess in relation to what is expected under mutation-drift equilibrium (Piry et al., 1998). In addition, BOTTLENECK employs three mutation models with which to assess heterozygosity excess, including the infinite allele model (IAM), the stepwise mutation model (SMM), and the two-phase model (TPM) (Piry et al., 1998; Di Rienzo et al., 1994). IAM and SMM are considered more extreme than TPM, due to the assumptions that they make; for example, IAM assumes that every time a mutation occurs a brand new allele is created (Chakraborty and Jin, 1992). In contrast, SMM assumes that new alleles can only occur in a one step magnitude, either forward or backward, with equal probability (Chakraborty and Jin, 1992). In contrast, TPM is considered more intermediate, including addressing mutational changes that are greater than one unit (Di Rienzo et al., 1994).

All of the aforementioned mutation models were used in my analysis. Following Parga et al. (2012), TPM parameters were as follows: the proportion of mutations greater than one-step was set between 15 and 30%. In addition, one thousand iterations were used for all mutation models. I utilized the Wilcoxon's signed-rank test, as suggested by Piry et al. (1998), as it is the most powerful and robust test available for data sets with < 20 polymorphic loci. Finally, one-tailed tests were employed, with significance set at *P<0.05*. Significant p-values denote evidence of heterozygosity excess (i.e., a recent bottleneck) (Piry et al., 1998).

The second approach employed M-ratio tests via M_P_Val and Critical_M software (Garza and Williamson, 2001). This test is capable of identifying populations that have undergone a past reduction in population size by calculating *M,* the mean ratio of number of alleles to the range in allele sizes (Garza and Williamson, 2001). In order to identify a bottleneck, one must compare the calculated values of *M* to M_c (critical value of M) (Garza and Williamson, 2001). Three parameters are used when determining estimates of *M*: $\theta(=4N_e\mu$ where, N_e is the effective population size and μ is the mutation rate), p_s (proportion of one-step mutations) and Δ_g (the average size of non one-step mutation) (Garza and Williamson, 2001). Following Parga et al. (2012), *M* and M_c (critical value of *M*) were calculated under the two-phase model using parameters (p_s=0.9, Δ_g=3.5) suggested by Garza and Williamson (2001). The value for theta (θ) was set between 0.1 and 10, since the pre-bottleneck value of theta is unknown (Parga et al., 2012).

4.4. Results

L-shaped distributions were detected via mode-shift tests for two of the three populations, Sakaviro and Tsaranoro, indicating that these populations are at mutation-drift equilibrium. In contrast, a mode-shift (i.e., disruption to allele distribution) was detected for the Anja population, signifying that a historical bottleneck occurred. Under the SMM, none of the three populations showed evidence of heterozygosity excess (Wilcoxon, Tsaranoro: *P= 0.57*; Sakaviro: *P =0.57*; Anja: *P =0.78*) (Table 4.1), meaning no bottleneck occurred. However, under the IAM, both Anja (Wilcoxon, *P=0.054*) and Sakaviro (Wilcoxon, *P=0.054*) show values approaching significance, which likely

reveal an excess of heterozygosity, indicating that genetic bottlenecks have occurred. Conversely, Tsaranoro exhibited no significant heterozygosity excess (i.e., no bottleneck) under IAM (Wilcoxon, *P =0.21*). Regardless of a range in values for 'percentage of SMM' under the two-phase model (TPM), no heterozygosity excess was found for any of the three populations. Finally, M-ratio tests indicated significant evidence of past reductions in effective population size for all three populations under all values of theta (0.01-10) (Figure 4.2). Varying values of theta or pre-bottleneck effective population sizes (N_e) were employed, because I do not have knowledge of these populations' past N_e.

4.5. Discussion

In view of the extensive habitat fragmentation across the central highlands of Madagascar which has resulted in numerous scattered forest fragments, I predicted that ring-tailed lemurs inhabiting these fragments would show significant evidence of dramatic demographic declines. However, results were somewhat equivocal, depending on the test that was applied, which is what Parga et al. (2012) found in their analysis of *L. catta* in southwestern Madagascar (Table 4.3). Both BMSR and TNP populations revealed an L-shaped distribution indicating that no population decline had occurred (Parga et al., 2012).

In contrast, ring-tailed lemurs inhabiting the Anja fragment, like both red ruffed and black and white ruffed lemur populations in the northeast and southeast of the island (Razakamaharavo et al., 2010; Louis et al., 2005), showed evidence of suffering a historical population decline when mode-shift tests were applied. The Tsaranoro and Sakaviro populations did not show evidence of a mode-shift, which may be due to the small population sizes, and consequently low power in the test.

All three study populations in south-central Madagascar revealed a bottleneck signal under all values of theta when employing M-ratio tests. However, *L. catta* populations at BMSR and TNP only showed evidence of a bottleneck under the two lower values of theta (0.1 and 1) (Parga et al., 2012).

Examinations within the program BOTTLENECK indicated mixed results; under the TPM and SMM, no bottleneck signals were detected for any of the three populations. However, under IAM, a near-significant p-value indicated a likely bottleneck signal for both Sakaviro and Anja.

Table 4.1. Results of BOTTLENECK analyses for the three mutation models.

Forest	Mod	% in	Efr	Lc5	Lc7	L-2	Em	69HDZ	Wilcox
Tsaranoro	IAM	-	0.72	0.76	0.70	0.73	0.77	0.866	0.218
	TPM	70	0.75	0.77	0.73	0.77	0.79	0.874	0.281
	TPM	75	0.75	0.77	0.73	0.77	0.79	0.877	0.281
	TPM	80	0.76	0.78	0.74	0.77	0.79	0.879	0.281
	TPM	85	0.76	0.78	0.74	0.78	0.80	0.879	0.343
	SM	-	0.77	0.79	0.75	0.79	0.81	0.885	0.578
Sakaviro	IAM	-	0.58	0.61	0.31	0.69	0.48	0.617	**0.054**
	TPM	70	0.62	0.64	0.90	0.72	0.52	0.657	0.343
	TPM	75	0.62	0.65	0.90	0.72	0.52	0.65	0.343
	TPM	80	0.63	0.65	0.90	0.72	0.53	0.656	0.421
	TPM	85	0.62	0.66	0.90	0.73	0.54	0.657	0.421
	SM	-	0.66	0.67	0.90	0.74	0.55	0.676	0.578
Anja	IAM	-	0.51	0.06	0.44	0.22	0.35	0.594	**0.054**
	TPM	70	0.58	0.12	0.30	0.32	0.22	0.387	0.578
	TPM	75	0.59	0.11	0.26	0.39	0.23	0.342	0.578
	TPM	80	0.59	0.14	0.27	0.36	0.25	0.307	0.578
	TPM	85	0.61	0.13	0.26	0.37	0.19	0.328	0.578
	SM	-	0.67	0.21	0.16	0.46	0.17	0.223	0.781

NOTES: Values approaching significance, $P<0.05$, indicated in bold

Table 4.2. Results of M-Ratio tests with values of theta (θ).

Forest	Theta	Average	M_C
Tsaranoro	10	**0.2**	**0.56**
	4	**0.2**	**0.62**
	1	**0.2**	**0.72**
	0.1	**0.2**	**0.82**
Sakaviro	10	**0.16**	**0.56**
	4	**0.16**	**0.62**
	1	**0.16**	**0.72**
	0.1	**0.16**	**0.82**
Anja	10	**0.15**	**0.56**
	4	**0.15**	**0.62**
	1	**0.15**	**0.72**
	0.1	**0.15**	**0.82**

NOTES: Average M was calculated for all loci across all three populations. Population declines (*where* $M < M_c$) are indicated in bold.

Table 4.3. Comparison of genetic bottleneck results of *Lemur catta* in southwestern and south-central Madagascar.

Population	Mutation Models[1] (showing significance)	Mode-shift[1]	M-ratio[2]	Reference
Tsaranoro	-	L-shaped distribution	Bottleneck detected	This study
Sakaviro	IAM	L-shaped distribution	Bottleneck detected	This study
Anja	IAM	Mode-shift detected	Bottleneck detected	This study
Beza Mahafaly Special Reserve	TPM, IAM	L-shaped distribution	Bottleneck detected	Parga et. al., 2012
Tsimanampetsotsa National Park	TPM, IAM	L-shaped distribution	Bottleneck detected	Parga et. al., 2012

1. Piry et al., 1999
2. Garza and Williamson, 2001

Similar to ring-tailed populations in south-central Madagascar, *L. catta* inhabiting TNP and BMSR showed no evidence of heterozygosity excess under the SMM (Parga et al., 2012). Likewise, populations of *V. variegata*, *V. rubra*, *M. ravelobensis*, and *M. danfossi* revealed no heterozygosity excess under the SMM (Louis et al., 2005; Olivieri et al., 2008; Razakamaharavo et al., 2010). Unlike ring-tailed lemur populations at Anja, Sakaviro, and Tsaranoro the DAMA population of *V. rubra*, *V. variegata* within Ranomafana, *L. catta* populations from BMSR and TNP, *M. ravelobensis* (seven of eight sites), and *M. danfossi* (one of seven sites*)* were found to have suffered population bottlenecks under the TPM (Louis et al., 2005; Olivieri et al., 2008; Razakamaharavo et al., 2010; Parga et al., 2012). Under the IAM, all of the above mentioned populations revealed evidence of genetic bottlenecks (Louis et al., 2005; Olivieri et al., 2008; Razakamaharavo et al., 2010; Parga et al., 2012), including the Anja and Sakaviro populations.

Several variables may account for the mixed results and the unexpected level of genetic diversity maintained within these fragmented populations (i.e., Sakaviro, Anja, and Tsaranoro). Firstly, there is the possibility that these populations are experiencing a time-lagged response to the fragmentation and isolation and have yet to shed their genetic diversity (Tilman et al., 1994) and do not yet show consistent evidence of population bottlenecks. Another possibility is that these three populations had not been fragmented for an extended period of time before the population sizes decreased, thus they underwent a more slow and steady decline. As pointed out by Lawler (2008), this type of population reduction is unlikely to produce a statistically significant bottleneck signal and therefore would not be identified through this analysis. Moreover, Cornuet and Luikart (1996) have demonstrated that depending on the conditions (e.g., number of loci, sample size), the statistical power of the mutation model analyses can be impacted. For example, when a larger number of loci are used versus a larger overall sampling of individuals, statistical power increases. In addition, when microsatellite loci adhere to the SMM versus the TPM or IAM model, statistical power is reduced (Cornuet and Luikart, 1996). Finally, failure to consistently detect bottleneck signals amongst these populations may be a result of low

power and statistical limitations (e.g., Bonhomme et al., 2008; Lawler, 2008). As recommended by Lawler (2008) and demonstrated by Milton et al (2009), utilizing more powerful and complex statistical methods, such as Bayesian coalescent models (Beaumont, 1999; Storz and Beaumont, 2002), it is possible to obtain signatures of past demographic declines that otherwise may have been obscured. Due to the inherent requirements regarding one's data set for Bayesian coalescent methods, such as MSVAR, my sample was not large enough in terms of individuals and number of loci.

Considering that the two-phase model (TPM) is regarded as the most appropriate model in which to assess microsatellite loci undergoing mutational processes (Di Rienzo et al., 1994; Estoup et al., 2002; Schlotterer, 2000), it is evident that that none of the three *L. catta* populations in south-central Madagascar have undergone a genetic bottleneck in regards to the mutation model analyses. However, bearing in mind the historical and contemporary anthropogenic threats to this region, it is not improbable to think that future investigations with larger sample sizes and more robust analyses (e.g., Bayesian) may better reflect the reality of these populations' history.

This study shows that Anja is the most likely population among the three studied to have undergone a historical genetic bottleneck. In contrast, the results were more ambiguous for Sakaviro and Tsaranoro. Taking into account the level of degradation, likely isolation, and small size of the Anja fragment these results are not unexpected. Surprisingly, despite the smaller sizes of the three fragments and potentially compromised dispersal ability due to forest degradation, these populations maintain a moderate level of genetic diversity (Clarke, Chapter 3), which may suggest they had similar historical effective population sizes. In view of the available data regarding genetic bottlenecks for *L. catta* - it appears that populations in both the southwestern (Parga et al., 2012) and south-central regions of the island underwent a historical demographic decline. Future research integrating genetic sampling from additional *L. catta* sites covering more of the species' range are needed in order to piece together a more comprehensive picture of the interplay between landscape transformations and demographic declines. It is important to note that these data serve as a baseline for future studies of ring-tailed lemur population genetics within in these forest fragments.

Specifically, it will allow for future researchers to monitor and sample these populations and document any changes to their genetic health.

To date, we do not have any definitive time line as to when the fragmentation occurred in this area. Perhaps, ring-tailed lemurs, a species known for its behavioral and ecologically flexibility, are persisting better in fragments than we would have expected. Despite the mixed bottleneck results, habitat destruction and loss continue across Madagascar, including the central highlands; thus all remaining *L. catta* populations should be considered a high conservation priority.

The island of Madagascar has suffered extensive habitat loss and fragmentation, which has had widespread adverse impacts on the local biodiversity (Myers et al., 2000; Harper et al., 2007; Schwitzer et al., 2013). However, Madagascar is not unique in this instance; human-induced disturbances have and are impacting biodiversity all around the globe. Like the Malagasy lemurs, many other taxa have suffered deleterious effects to their genetic health as a result of destruction and degradation of their natural habitats.

The black-faced lion tamarin (*L. caissara*), endemic to Brazil, is restricted to a small distribution within the Atlantic Coastal Forest, including Superagüi Island and Ariri and Patus River Valleys (Martins et al., 2012). This critically endangered primate (Kierulff et al., 2008) is threatened by habitat destruction and hunting, as well as the construction of highways and/or reservoirs (Martins et al., 2012). The original population of *L. caissara* was split due to the widening and extending of a channel in the 1950s, which now acts as a barrier to gene flow and has resulted in two small populations of black-faced lion tamarins versus one larger population (Martins et al., 2012).

The ornate box turtle (*Terrapenne ornate*) has also experienced extensive habitat loss and degradation throughout its natural range, which has been attributed to historical and contemporary anthropogenic impacts, including the arrival of European settlers, agricultural and industrial activities, and more recently, a surge in the pet trade (Kuo and Janzen, 2004). Yet, in different regions, the populations evidenced different effects. An isolated population in Illinois was found to have undergone a recent genetic bottleneck (Kuo and Janzen, 2004). In contrast, a population in Nebraska revealed no significant

signature of a bottleneck (Kuo and Janzen, 2004). Both populations were found to be genetically healthy and exhibited similar levels of genetic diversity (Kuo and Janzen, 2004). Considering the level of disturbance and isolation of the Illinois population, these results are somewhat surprising. Kuo and Janzen (2004) propose that this could be due to the long lifespan of the species (estimates in the wild range from 22-30 years) and/or the occurrence of a less severe bottleneck.

Finally, populations of European tree frogs (*Hyla arborea*) inhabiting Jutland (located on the mainland) and Lolland Island, Denmark, were also found to have undergone genetic bottlenecks as a result of anthropogenic disturbances (Anderson et al., 2004). Tree frog populations on the mainland (Jutland) recovered more quickly and even expanded, whereas populations on Lolland were reduced to half the size (Anderson et al., 20044). Moreover, the majority of Lolland populations suffered significant losses of genetic variation, as well as reduced survival due to inbreeding depression. The authors suggest that these differences are a result of the greater levels of fragmentation that occurred on the Island of Lolland (Anderson et al., 2004).

The majority of examples and taxa discussed throughout this chapter, have three central variables in common, 1) the populations have suffered deleterious impacts to their genetic health, whether it be demographic declines (i.e., genetic bottlenecks), decreased genetic diversity, inbreeding depression, and/or reduced survivability, 2) these negative impacts are the result of historical and/or contemporary anthropogenic disturbances, such as habitat loss, fragmentation, and isolation, and 3) most, if not all, of these species are still under threat due to continued human-induced disturbances across the globe. In order to ensure the viability of these populations, continued scientific research examining how natural populations respond to anthropogenic disturbances are crucial. Moreover, understanding how to help populations recover and thrive is also essential. Finally, conservation education and the implementation of well-developed management plans, both *in situ* and *ex situ*, must be applied in concert with scientific endeavors, if global biodiversity is to persist.

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Chapter 5. Genetic population assignment of *Lemur catta* in south-central Madagascar

5.1. Abstract

The genetic structuring of a population or the distribution of genetic variation is linked to a variety of characteristics for a given species, including sex-biased dispersal, social organization, and mating systems. Moreover, natural and anthropogenic disturbances can also have a prominent effect on the genetic variation and distribution of wild populations by creating disruptions or barriers to gene flow. Because of these connections, molecular data allow scientists to gain insights into a species' mating system, social structure, dispersal patterns, and patterns of gene flow. These types of data have important and valuable real-world applications, such as aiding in the development of conservation management plans for threatened species. I used microsatellite data to explore genetic structuring among three currently isolated populations of ring-tailed lemurs in south-central Madagascar: Anja Reserve, Sakaviro, and Tsaranoro Valley. I employed *GenAlEx* and STRUCTURE software to investigate each individual's *population of origin*, as well as genetic structuring amongst the three fragments. Population assignment analyses suffered from a likely 'lack of signal'. Therefore, individuals were unable to be reliably assigned to their *population of origin*. In addition, genetic population structure was ambiguous. It is possible that three study populations are not genetically differentiated enough for proper population assignment, or perhaps the sample is not robust enough for population assignment analyses to produce unequivocal results.

5.2. Introduction

The genetic structure of a population, or the pattern of genetic variation within and among populations, is influenced by a various factors, such as sex-biased dispersal, social organization, and mating systems (Wright, 1978; Storz, 1999; Ross, 2001). Moreover, both natural barriers (e.g., rivers, mountains) and anthropogenic disturbances (e.g., roads, habitat fragmentation) have a prominent effect on the genetic structuring of wild populations by creating disruptions or barriers to gene flow (e.g., Fünfstück et al., 2014 Western lowland gorilla (*Gorilla gorilla gorilla*); Paquette et al., 2007 radiated tortoise (*Geochelone radiate*); Radespiel et al., 2008 golden-brown mouse lemur (*Microcebus ravelobensis*); van Vuuren et al., 2012 boky-boky (*Mungotictis decemlineata*).

Studies that examine the genetic structuring of populations provide researchers with valuable data which aid in a more comprehensive understanding of a species' biology, including social structure, dispersal patterns, and mating systems (Di Fiore, 2003; Vigilant and Guschanski, 2009). Interpretations of these data play a key role in assessing the conservation status of a species, as well as aiding in the establishment of appropriate conservation management strategies (Di Fiore, 2003; Vigilant and Guschanski, 2009). More specifically, they can determine whether or not a population is viable, as well as identify distinct populations, and indicate what parts of the landscape should be preserved as corridors (Di Fiore, 2003; Manel et al., 2005; Vigilant and Guschanski, 2009).

Handley-Lawson and Perrin (2007) note that investigations and interpretations of sex-biased dispersal in the wild are extremely challenging. For example, dispersal events may be difficult to observe depending on several variables, such as activity pattern (e.g., nocturnal, cathemeral), whether a species engages in cryptic behaviors, keeping up with highly mobile species (e.g., flying-foxes, sperm whales, grey wolf) (Handley- Lawson and Perrin, 2007), and/or difficulty in navigating the landscape. Therefore, a combination of direct observations, molecular genetic protocols, and/or GPS technology would be ideal for the identification of dispersal behavior (Handley-Lawson and Perrin, 2007; Tomkiewicz et al., 2010). Moreover, by applying a molecular protocol to dispersal

studies we will be able to reveal how dispersal 'translates' into gene flow (Handley-Lawson and Perrin, 2007).

During the past two decades, molecular approaches to the examination of sex-biased dispersal have become more widely used for a variety of species. For example, Di Fiore and colleagues (2009) examined dispersal patterns in two ateline primates, lowland woolly monkeys (*Lagothrix poeppigii*) and white-bellied spider monkeys (*Ateles belzebuth),* by employing both field observations and multi-locus genotyping. Both invasive and non-invasive samples were utilized (i.e., muscle tissue and fecal samples) (Di Fiore et al., 2009). Data collection took place within Yasuní National Park and Biosphere Reserve, specifically, Tiputini Biodiversity Station (TBS) and Proyecto Primates Research Area (PPRA), which lie approximately 35km apart. Eight and 16 microsatellite loci were employed for the genotyping of *Lagothrix* and *Ateles*, respectively. Similar to that of Di Fiore and colleagues (2009), Zhan et al. (2007) focused on giant panda (*Ailuropoda melanoleuca*) dispersal patterns and spatial distribution of related individuals by utilizing non-invasive fecal samples (n=254) for microsatellite genotyping. Zhan and colleagues (2007) used nine microsatellite loci to compare the spatial distribution of giant pandas in two populations (Wanglang and Baima Tibetan Community, China), and the spatial proximity of relatives in another (Huanglong) in order to deduce dispersal patterns. This study represents a key example of how non-invasive sampling and molecular techniques can reveal information about a species that has traditionally been difficult to observe in the wild (Zhan et al., 2007). Studies of both woolly and spider monkeys revealed that female dispersal is widespread, but evidence for male gene flow has been more equivocal (Di Fiore et al., 2009). A considerable amount of male gene flow was detected for male woolly monkeys (Di Fiore et al., 2009). These data were supported by assignment indices (i.e., assignment tests) (AIs), statistics that summarize the likelihood that a sampled individual's genotype originated in the population in which it was sampled, and revealed no evidence of a strong sex bias for *Lagothrix* dispersal patterns (Di Fiore et al., 2009). In contrast, one group of spider monkeys located within TBS revealed greater relatedness among males versus females, which is consistent with data from assignment indices suggesting female dispersal for this species (Di Fiore et al., 2009). Observations of male social behavior, such as close

affiliative and cooperative behaviors, and group defense, further confirm molecular data for the spider monkey group inhabiting TBS (Di Fiore et al., 2009). Interestingly, a second study group (*A. belzebuth)* located within PPRA revealed a different pattern, where average relatedness between both sexes within the group was close to zero (i.e., no difference in relatedness among the adult males vs. the adult females within this group) (Di Fiore et al., 2009). Differences in average relatedness, dispersal patterns, and social behavior between the two *Ateles* groups is attributed to the PPRA group being heavily impacted by anthropogenic disturbance (Di Fiore et al., 2009).

Assignment indices (AIs) were also employed by Zhan et al. (2007) and revealed that *A. melanoleuca* (giant pandas) exhibit female-biased dispersal. However, as with woolly monkeys (Di Fiore et al., 2009), some male dispersal was detected. The overall rate of dispersal is low for giant pandas, < 10%, which could be related to habitat fragmentation throughout this species' range (Zhan et al., 2007). Molecular evidence suggests that female giant pandas disperse farther than males, and males typically establish new territories adjacent to their natal home range (Zhan et al., 2007). Furthermore, the average dyadic spatial distance of related female pandas was significantly larger than males, which corroborates results from AIs (Zhan et al., 2007). Lastly, Zhan et al. (2007) note that a recent macro-ecological study of giant pandas (Pan et al., 2001 as cited in Zhan et al., 2007) substantiates their molecular evidence of female biased dispersal, and that males establish new home ranges near their places of birth. The authors note that the determination of dispersal patterns for the giant panda will be essential in identifying areas in which forest corridors are needed for successful gene flow between isolated populations (Zhan et al., 2007).

These two studies, Di Fiore et al. (2009) and Zhan et al. (2007), illustrate the suggestion by Handley-Lawson and Perrin (2007) that a combination of field observation methods and molecular genetic techniques are valuable in examinations of sex-biased dispersal, because they aid in advancing our understanding of this important life history characteristic. Additionally these methods allow one to make inferences about the proximate and ultimate causes for this behavior. Moreover, they help to emphasize the dramatic and potentially deleterious impact that habitat fragmentation can have on

important life-history traits, such as dispersal (Zhan et al., 2007; Di Fiore, 2009; Di Fiore et al., 2009).

Cegelski et al. (2003) examined the population structure and gene flow of the Montana wolverine (*Gulo gulo*), a highly mobile species, which has the ability to disperse up to 300km within a given year (Cegelski et al., 2003). The populations of wolverines found in Montana are the largest and most stable in North America, due to their close proximity to wolverine populations in Canada (Cegelski et al., 2003). Results detected at least three subpopulations of wolverines within Montana (Cegelski et al., 2003). These results were unexpected due to the geographical proximity of the subpopulations, approximately 300km apart, the same distance that this species is capable of dispersing in a given year (Cegelski et al., 2003). Such high genetic structure and low level of gene flow, which are absent in Canadian and Alaskan wolverine populations, are explained by a greater degree of habitat fragmentation and anthropogenic disturbance across Montana, such as roads and agricultural land (Cegelski et al., 2003). Assignment tests revealed low levels of female dispersal and a strong male-biased dispersal for all three subpopulations of wolverines (Cegelski et al., 2003). This is consistent with previous genetic analyses on Canadian wolverine populations (Wilson et al., 2000 as cited in Cegelski et al., 2003). Due to the low levels of female dispersal, the authors concluded that male wolverines are the predominant dispersers and females represent the philopatric sex (Cegelski et al., 2003).

Likewise, Piggott and colleagues (2006) discovered a non-panmictic population (i.e., non-random mating) with distinct genetic differentiation between their four study colonies of wallabies. In addition, there was high genetic similarity among individuals within each of the four rock-wallaby colonies (Piggott et al., 2006). These results are best explained by the presence of the Wolgan River valley and river, which split the four colonies into two groups (Piggott et al., 2006). Unlike the wolverine subpopulations, natural landscape features are assumed to act as barriers to rock-wallaby gene flow and may represent one of the main reasons for the significant genetic differentiation between colonies (Piggott et al., 2006). Brush-tailed rock-wallabies were found to have low dispersal rates, as only ~ 5% of individuals were identified as potential dispersers

(Piggott et al., 2006). By employing the statistical program GENECLASS, Piggott and colleagues were able to determine that three of the four dispersers were immigrants from unknown populations (Piggott et al., 2006). Thus, only one individual was detected as a potential disperser between two of the sampled colonies, which are situated on the same side of the Wolgan river valley (Piggott et al., 2006). These data support previous results that suggest the river valley represents a dispersal barrier for *P. penicillata* (Piggott et al., 2006). Due to the low levels of dispersal detected in this study, it was not possible to determine whether wallabies are male or female philopatric (Piggott et al., 2006)

From a conservation standpoint, these molecular data have uncovered potential unknown and unsampled rock-wallaby colonies within the Wolgan river valley in New South Wales, Australia. Such data could indicate conservation priority areas with which the endangered rock-wallaby populations could exploit, in order to maintain gene flow, and thus maintain overall genetic diversity (Piggott et al., 2006).

Similar to above mentioned studies, Blundell and colleagues (2002) employed a range of methods, including assignment tests, isolation by distance (assesses correlation between genetic and geographic distances), Bayesian analyses, and radio telemetry to examine gene flow and patterns of space use among wild river otters (*Lontra canadensis*) in Prince William Sound, Alaska. Molecular data revealed genetically distinct subpopulations with intermediate levels of genetic differentiation among microsatellite loci (Blundell et al., 2002), which are analogous to the population structure found among rock-wallaby and wolverine populations (Cegelski et al., 2003; Piggott et al., 2006). Blundell and colleagues (2002) noted that evidence of gene flow among the seven river otter populations could indicate either sink-source population or metapopulation dynamics. Similar to woolly monkeys and pandas (Zhan et al., 2007; Di Fiore et al., 2009) both male and female river otters were found to disperse (Blundell et al., 2002). Furthermore, rates of natal dispersal were low for both sexes, as was found with pandas (Blundell et al., 2002; Zhan et al., 2007). The river otter results were further substantiated by telemetry observations (Blundell et al., 2002). Despite similarities of male and female natal dispersal rates, telemetry tracking revealed differences in movement patterns between the sexes that were indicative of a male-biased dispersal,

prompted by breeding dispersal. Thirty percent of the 40 radio-tracked male river otters displayed some form of dispersal (i.e., natal or breeding), where more than half (22.5%) was due to breeding dispersal events. Additionally, male river otters were found to expand their home ranges during the mating season, which still allowed opportunities for gene flow and potential reproductive success. The assignment tests revealed that the majority of male river otters disperse over shorter distances, as do male pandas (Zhan et al., 2007); and in contrast, when females dispersed they dispersed further (60-90 km). Due to the short dispersal distances, male river otters showed significant isolation by distance, thus genetic differentiation increased with geographical distance and males were found to have higher rates of gene flow over shorter distances, whereas females had higher genetic differentiation, which is indicative of female philopatry.

These studies represent diverse examples of combinations of invasive and/or non-invasive sampling, molecular applications, and field observations (e.g., radio telemetry) that enhance our understanding of wild animal population genetics. Furthermore, these multi-disciplinary approaches have played a valuable role in providing data on species (e.g., pandas) that are traditionally difficult to observe in the wild. Finally, there is great potential for the development of improved conservation strategies and management of wild populations based on these data. Multi-disciplinary approaches have the unique ability to enhance our knowledge in ways that traditional methods (i.e., strict behavioral observations) could not. Knowledge of wild animal population mating systems, dispersal patterns, population structure, gene flow, and the identification barriers to gene flow will be vital to the future viability of global biodiversity, especially during times of increased global warming and continued habitat destruction.

In this chapter, I report data regarding the population assignment (i.e., the likelihood that a particular individual's genotype originated in a given population) of thirty individual ring-tailed lemurs from three fragmented forests in south-central Madagascar. To understand the impact of natural (e.g., mountains) and anthropogenic disturbances (e.g., roads, habitat fragmentation) on male reproductive strategies (dispersal) and population structuring, I employed genotypic data to determine the population of origin for these individuals; that is, whether they are natal or migrants to a

given population. Data regarding gene frequencies of the populations are necessary; therefore all sampled individuals (males and females) must be included in at least the initial analysis (Pritchard et al., 2000; Falush et al., 2003, 2007).

5.3. Methods

5.3.1. Sample Collection and Genetic Analyses

Non-invasive fecal samples were collected (July-October of 2012) opportunistically from three fragmented populations of ring-tailed lemurs (*Lemur catta*) in the central highlands of Madagascar (see Chapter 1 for site details). Samples were immediately stored in RNA*later* solution (Ambion, Austin, TX) (Di Fiore, 2003). Ten samples per site were utilized for this study (n=30) (see Chapter 2, Table 1).

DNA extraction, PCR (polymerase chain reaction), and microsatellite genotyping were performed at New York University's Molecular Anthropology lab (for details on laboratory protocols see Chapter 3, Methods). Allele calls were performed by eye via Genemapper software *v. 4.0* (Applied Biosystems). Genotypes were confirmed with multiple independent PCR amplifications, employing Taberlet et al. (1999) 'multiple tubes' approach. Heterozygous genotypes were confirmed with at least two independent reactions and homozygous genotypes were confirmed with 3-7 independent reactions (Taberlet et al., 1999).

Micro-Checker *v.2.2.3* (van Oosterhout et al., 2004) was utilized to assess scoring errors (i.e., null alleles, allelic dropout). Linkage disequilibrium (LD) was examined via GENEPOP *v. 4.2* (Raymond and Rousset, 1995). For scoring error and LD results please see the *Results* section in Chapter three.

I expect that male ring-tailed lemurs in the study fragments will have compromised dispersal and thus, gene flow will be adversely affected. Considering the geographic distances between Tsaranoro and Sakaviro (34 km) and Tsaranoro and Anja (27 km) combined with severe forest disturbance, and the presence of a highway, roads, savannah, and villages, male dispersal is impossible. Therefore, I expect that individuals from the Tsaranoro population will be genetically assigned to the population they were sampled within (i.e., Tsaranoro). In contrast, it is more probable that individuals from the

Anja and Sakaviro populations may be genetically assigned to a population other than the population they were sampled in, as a result of closer geographic proximity between the fragments (12 km). Ultimately, I predict that the three study populations will be genetically assigned to two clusters or populations (i.e., *K*=2, [Anja+Sakaviro] and [Tsaranovo]).

5.3.2. Statistical Analyses

To determine the *populations of origin* for my sample of 30 individual ring-tailed lemurs from the three study fragments, I employed two different assignment methods (AM), both of which use nuclear genotypic data to assign individual membership to a given population. The first, conducted via *GenAlEx v. 6.5*, assigns individuals to predefined populations and utilizes estimated allele frequencies, which assume Hardy-Weinberg Equilibrium (HWE) and no linkage disequilibrium (LD) (Peakall and Smouse 2006, 2012). In order to deal with potential biases, Peakall and Smouse (2006, 2012) suggest utilizing the 'Leave one out' option, which removes a given individual's genotype prior to the calculation of the expected frequency of that genotype; this option was employed here. It should be noted that this assignment test has several limitations. For example, statistical power depends on the degree of polymorphism, allele frequencies, number of loci, and the extent of genetic differentiation (Peakall and Smouse, 2006, 2012). Despite its limitations, this test serves as a useful and informative initial data exploration tool for codominant genotypic data (Peakall and Smouse, 2006, 2012).

The second assignment method was performed in STRUCTURE *v. 2.3.4* (Pritchard et al., 2000; Falush et al., 2003, 2007), one of the three most widely used Bayesian clustering programs available (Latch et al, 2006). Unlike *GenAlEx*, this software does not rely on predefined populations and employs a Bayesian clustering approach to identify the number of populations or clusters (*K*) and the likelihood of membership for each individual within a population (i.e., the likelihood that a particular genotype originated in a given population) (Pritchard et al., 2000). The user inputs the number of *K* (i.e., populations) and STRUCTURE produces a range of probability scores of *K* based on the available data (Pritchard et al., 2000). The range of probability scores

which correspond to the range of *K* values are then evaluated by the user to determine which number of populations (*K*) or clusters are the most probable (Pritchard et al., 2000). STRUCTURE minimizes Hardy-Weinberg and linkage disequilibrium (HWD and LD), which enables individuals to be more accurately grouped into populations/clusters (Pritchard et al., 2000).

One widespread criticism of STRUCTURE is that *K* can be underestimated when populations do not have significant genetic differentiation (Manel et al., 2005; Latch et al., 2006). Additionally, it has been argued that when small sample sizes (<50) and a low number of loci (<20) are employed, the program can have difficulty deducing *K* (i.e., number of populations), as well as decreased accuracy in assigning individuals to populations or clusters (Waples and Gaggiotti, 2006). Despite these criticisms, empirical tests have revealed that STRUCTURE performs well when populations have low levels of differentiation, specifically standardized F_{ST} values (i.e., values that account for within-population heterozygosity) ranging between 0.03 and 0.05 (97% of individuals are correctly assigned) (Hendrick, 2005). Thus, Latch and colleagues (2006) recommend the STRUCTURE Bayesian clustering method for inferring the number of *K* and the assignment of individuals when levels of differentiation among populations are low.

A Kruskal-Wallis one-way analysis of variance test was performed via JMP® Pro, *version 11* (SAS Institute Inc., Cary, NC, 1989-2007) to test for significant differences among the distributions of posterior probability scores of inferred ancestry for the three study populations. Pairwise comparisons for each population's posterior probability scores were examined with a Wilcoxon rank sum test in JMP® Pro, *version 11* to identify which groups significantly differ from one another. The Kruskal-Wallis and Wilcoxon tests are non-parametric tests analogous to the one-way ANOVA and t-tests, respectively. The non-parametric tests are more statistically conservative (i.e., less likely to incorrectly reject the null hypothesis) than their parametric equivalents, and do not assume a normal distribution of the data (Sokal and Rohlf, 2003).

5.3.2.1. Program Settings: STRUCTURE v.2.3.4

I used the recommended *admixture model*, which assumes an individual may have mixed ancestry (i.e., each individual obtains a proportion of his/her genome from each of the *K* populations meaning not from a single population) (Pritchard et al., 2000). The admixture model produces *posterior mean estimates* of the proportion that a given individual shares genetic ancestry with ancestors from a given population (Pritchard et al., 2000). The *allele frequency model*, which incorporates correlated allele frequencies, was also implemented into the population assignment analysis (Falush et al., 2003a). This model assumes that allele frequencies in the various populations are analogous due to migration or shared ancestry (Falush et al., 2003a). The program parameters were as follows: *K* was set to between 1-4 (to infer the number of genetically differentiated clusters), the length of the burnin period was set at 50,000 steps (i.e., length of time the simulation runs before collecting data) with 10 iterations or runs, followed by a run of 100,000 Markov chain Monte Carlo (MCMC) repetitions.

5.4. Results

Population assignment tests performed in *GenAlEx* did not result in clearly differentiated populations, and therefore individuals were not reliably assigned to their population of origin (Table 1)(Peakall and Smouse 2006, 2012) (Figure 1, a population assignment graph for all sampled individuals, depicts that the individuals do not clearly cluster or group into two populations). Bayesian program STRUCTURE (Pritchard et al., 2000; Falush et al., 2003, 2007) was also unable to partition the populations or assign individuals (i.e., individual genotypes) into their *likely* population of origin. Posterior probability scores for individual assigned ancestry are presented in Table 2. Kruskal-Wallis tests revealed no significant differences among posterior probability scores for the Anja (*P=0.99*) and Sakaviro (*P=0.71*) populations (Figures 2 and 3). However, there was a significant difference found among the probability scores within the Tsaranoro population (*P=0.00*) (Figure 4). Pairwise comparisons were only found to be significantly different between the Tsaranoro and Sakaviro populations (Wilcoxon *P=0.00*). Mean F_{ST} values (values representing mean population differentiation) for the ten runs of *K*=2 and *K*=3, two and three inferred clusters or populations, respectively, are reported in *Appendix 1*.

Table 5.1. Population Assignment Values.

Animal	Site of	Sakaviro	Tsaranoro	Anja	Assigned
11	Sakaviro	9.442	14.875	7.319	Anja
12	Sakaviro	6.743	13.127	9.937	Sakaviro
13	Sakaviro	6.539	11.093	7.676	Sakaviro
14	Sakaviro	10.202	13.252	8.889	Anja
23	Sakaviro	6.190	12.127	10.937	Sakaviro
24	Sakaviro	11.804	7.336	8.208	Tsaranoro
35	Sakaviro	2.436	7.988	5.968	Sakaviro
36	Sakaviro	9.253	10.340	10.435	Sakaviro
51	Sakaviro	14.880	14.389	11.002	Anja
52	Sakaviro	1.630	6.766	5.366	Sakaviro
3	Tsaranoro	8.653	7.164	8.088	Tsaranoro
9	Tsaranoro	15.452	6.438	11.319	Tsaranoro
15	Tsaranoro	9.518	8.212	7.952	Anja
17	Tsaranoro	12.749	8.930	8.736	Anja
27	Tsaranoro	16.771	7.355	14.541	Tsaranoro
33	Tsaranoro	13.114	9.470	7.907	Anja
39	Tsaranoro	17.414	8.931	9.590	Tsaranoro
28	Tsaranoro	13.679	8.249	12.289	Tsaranoro
25	Tsaranoro	9.945	7.973	10.296	Tsaranoro
8	Tsaranoro	7.197	7.485	7.199	Sakaviro
1	Anja	5.814	12.641	7.775	Sakaviro
4	Anja	6.234	10.306	8.169	Sakaviro
6	Anja	6.419	4.084	6.044	Tsaranoro
21	Anja	7.021	6.850	7.491	Tsaranoro
22	Anja	10.891	8.336	8.012	Anja
29	Anja	11.457	13.088	6.836	Anja
30	Anja	10.842	11.644	13.447	Sakaviro
46	Anja	11.424	12.224	7.785	Anja
68	Anja	10.345	7.470	9.489	Tsaranoro
100	Anja	7.582	6.127	4.711	Anja

NOTES: Population Assignment was performed using *GenAlEx* 6.5. Log-likelihood values have been converted to positive values, with the lowest value indicating the most likely population of origin.

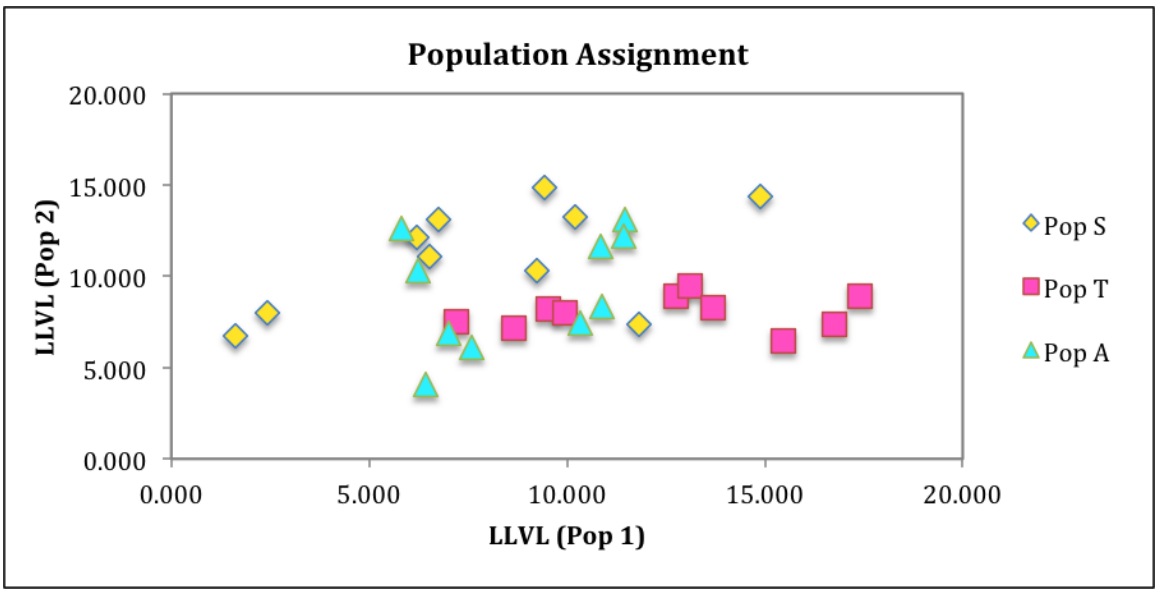

Figure 5.1. Population Assignment was performed using *GenAlEx* 6.5.

NOTES: Log-likelihood values (LLVL) have been converted to positive values (x and y axes), with the lowest value indicating the most likely population. Population Sakaviro or Pop S (yellow), Population Tsaranoro or Pop T (pink), and Population Anja or Pop A (blue).

Table 5.2. Posterior Probability Scores of Inferred Ancestry.

			Posterior Probability		
Animal ID	**Site of Sample Collection**	**Assigned Population of Origin**	**Assigned Population**	**Sakaviro**	**Tsaranoro**
1	Anja	Anja	0.342	0.329	0.329
4	Anja	Anja	0.325	0.337	0.338
6	Anja	Anja	0.341	0.329	0.330
21	Anja	Anja	0.346	0.326	0.328
22	Anja	Anja	0.332	0.333	0.335
29	Anja	Anja	0.338	0.332	0.330
30	Anja	Anja	0.324	0.337	0.339
46	Anja	Anja	0.318	0.339	0.342
68	Anja	Anja	0.337	0.333	0.330
100	Anja	Anja	0.329	0.338	0.333
			Assigned Population	**Sakaviro**	**Anja**
3	Tsaranoro	Tsaranoro	0.316	0.340	0.344
8	Tsaranoro	Tsaranoro	0.338	0.331	0.332
9	Tsaranoro	Tsaranoro	0.325	0.339	0.337
15	Tsaranoro	Tsaranoro	0.330	0.336	0.333
17	Tsaranoro	Tsaranoro	0.338	0.329	0.333
25	Tsaranoro	Tsaranoro	0.319	0.339	0.343
27	Tsaranoro	Tsaranoro	0.313	0.342	0.345
28	Tsaranoro	Tsaranoro	0.313	0.343	0.343
33	Tsaranoro	Tsaranoro	0.322	0.338	0.340
39	Tsaranoro	Tsaranoro	0.311	0.340	0.349
			Assigned Population	**Tsaranoro**	**Anja**
11	Sakaviro	Sakaviro	0.330	0.334	0.336
12	Sakaviro	Sakaviro	0.327	0.337	0.336
13	Sakaviro	Sakaviro	0.342	0.330	0.329
14	Sakaviro	Sakaviro	0.351	0.322	0.326
23	Sakaviro	Sakaviro	0.327	0.337	0.336
24	Sakaviro	Sakaviro	0.335	0.333	0.333
35	Sakaviro	Sakaviro	0.350	0.326	0.323
36	Sakaviro	Sakaviro	0.331	0.333	0.336

NOTES: Posterior probability scores were determined via STRUCTURE *v.2.3.4*. Orange boxes indicate individual males.

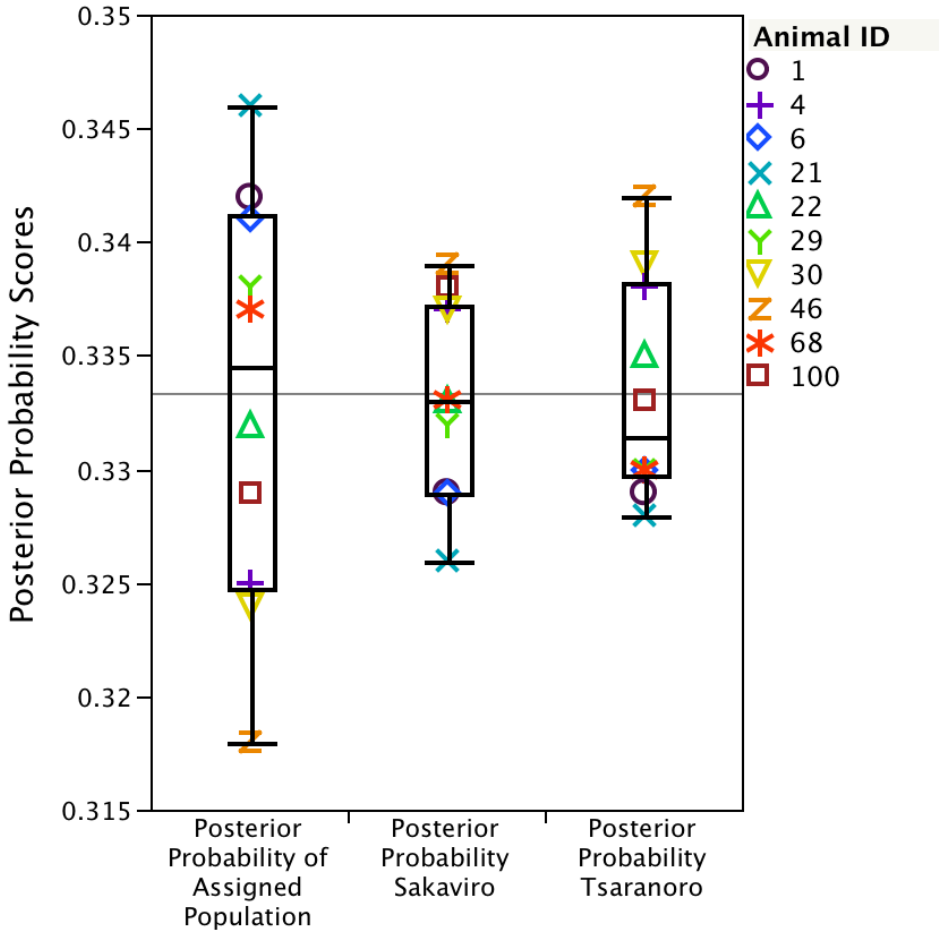

Figure 5.2. Box plots for posterior probabilities of Anja Population.

NOTES: Box plot generated in JMP® Pro, *version 11*. All individuals from the Anja population are represented by a different color/symbol. The line through the center of the plot represents the grand mean (i.e., data for all groups pooled together).

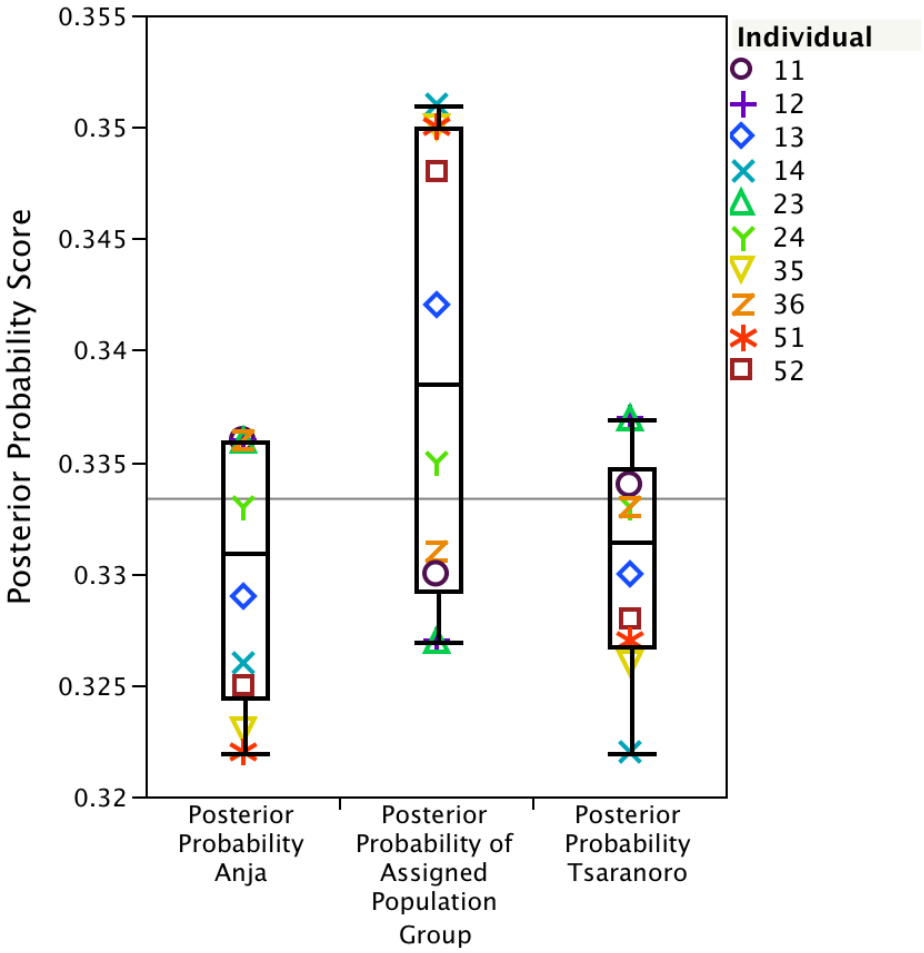

Figure 5.3. Box plots for posterior probabilities of Sakaviro Population.

NOTES: Box plot generated in JMP® Pro, *version 11*. All individuals from the Sakaviro population are represented by a different color/symbol. The line through the center of the plot represents the grand mean (i.e., data for all groups pooled together).

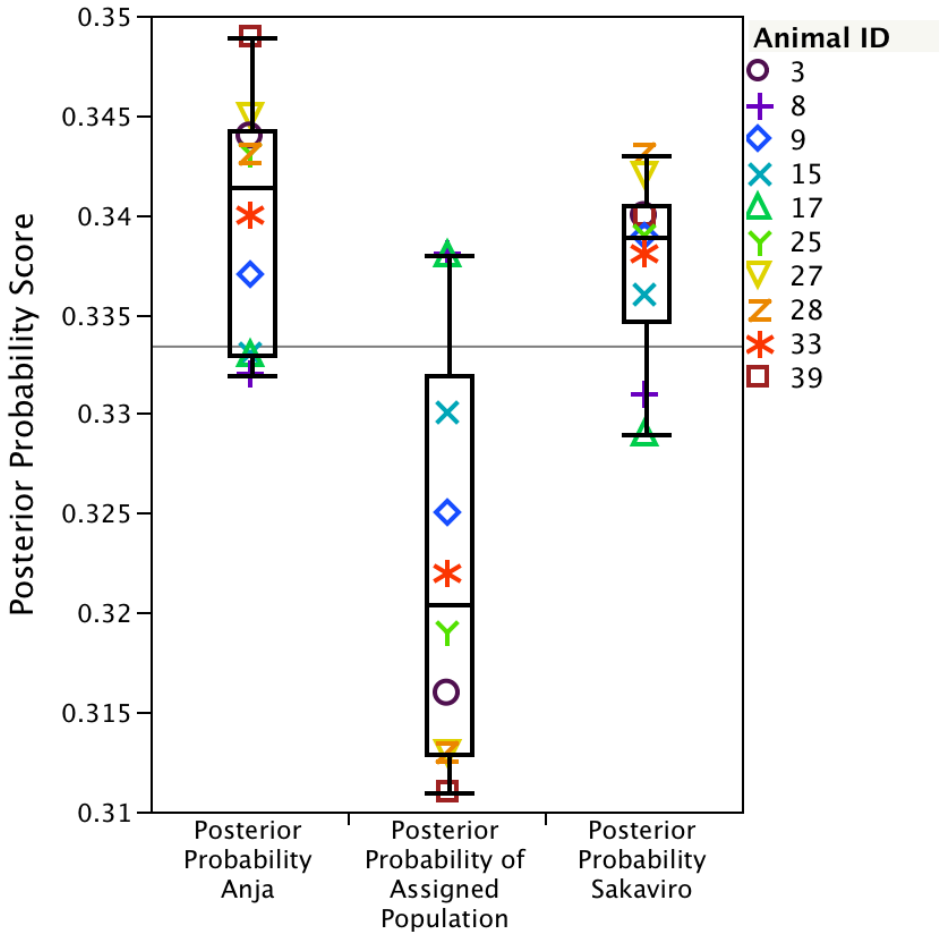

Figure 5.4. Box plots for posterior probabilities of Tsaranoro Population.

NOTES: Tsaranoro Population: Box plot generated in JMP® Pro, *version 11*. All individuals from the Tsaranoro population are represented by a different color/symbol. The line through the center of the plot represents the grand mean (i.e., data for all groups pooled together).

5.5. Discussion

The determination of an individual's natal origin or *population of origin* through the use of molecular data has been used by scientists to gain knowledge about mating systems, social structures, and dispersal patterns (Di Fiore, 2003; Vigilant and Guschanski, 2009). These types of data have important and valuable real-world applications, such as aiding in the development of conservation management plans for threatened species (Di Fiore, 2003; Vigilant and Guschanski, 2009).

While we know that ring-tailed lemurs are a female philopatric-male dispersing species (Jolly, 1966; Sussman, 1991, 1992, Gould, 1994, 1997), we do not yet have an understanding of *if* and *how* male reproductive strategies (e.g., the timing and distance of male dispersal events) have been impacted by extensive habitat loss and fragmentation. My three study populations inhabit highly fragmented forests and in the cases of Anja and Sakaviro, are isolated from nearby conspecifics (Cameron and Gould, 2013; Gabriel, 2013).

Population assignment tests for the three study fragments were ambiguous, both *GenAlEx* and the Bayesian clustering analysis suffered from a likely 'lack of signal'. STRUCTURE (i.e., Bayesian clustering) assigned all individuals, males and females, to the population from which they were sampled, but at relatively low probability. Posterior probability scores of inferred ancestry for all sampled individuals to each of the three populations (Anja, Sakaviro, and Tsaranoro) ranged from 30-35%, meaning there is an almost equal chance that a given individual could have originated from one of the three populations (see Table 1). While pairwise comparisons indicated a significant difference between the Tsaranoro and Sakaviro populations ($p=0.00$), signifying these two population's inferred ancestry scores differ significantly from each other, the range of difference is only 2%.

This particular analysis was incapable of determining whether or not male dispersal was impacted by natural or anthropogenic barriers, or reliably inferring the genetic assignment and structure among these three study fragments. There are two possible explanations for these results: 1) these populations may not yet be genetically differentiated enough for reliable population assignments, or 2) the sample is not robust

enough for population assignment analyses to produce unequivocal results. Due to the challenges I encountered working with non-invasive samples and microsatellites in the laboratory, it is most likely that the sample size (n=30), number of loci (n=6), and missing data may have been an issue for this specific analysis. While I adopted all of the suggested protocols when collecting my samples and when conducting laboratory and statistical analyses (See Chapter 1), challenges were still encountered. Future studies regarding *L. catta* dispersal and population assignment will benefit from larger overall samples incorporating a greater number of microsatellite loci. In addition, one could also consider the option of utilizing higher-quality samples, such as blood or tissue. However, invasive sampling has logistical and financial drawbacks. For example, the darting of wild animals to obtain blood or tissue samples requires detailed planning, a proficient darting team, and a veterinarian− all of which increase research expenses (Taberlet et al., 1999; Vigilant and Guschanski, 2009).

Similar results were found for a population of mantled howler monkeys (*Aloutta palliata*) on Barro Colorado Island, Panama where posterior probability scores were equal for all individuals across all specified clusters (or populations) (Milton et al., 2009). In addition, STRUCTURE was unable to assign *A. palliata* into more than one cluster or population, indicating weak genetic structuring (Milton et al., 2009). Likewise, *GenAlEx* and STRUCTURE results for *L. catta* population assignment and structure failed to assign individuals to more than one cluster or population, signifying weak genetic structure. However, pairwise F_{ST} values (See Table 5, Chapter 3) between Tsaranoro and Sakaviro and Anja and Tsaranoro suggest a moderate level of population structuring indicating genetic differentiation.

Ring-tailed lemurs are the most terrestrial of the Malagasy strepsirhines, spending approximately 30-40% of their time on the ground (Jolly, 1966; Sussman, 1972; Cameron and Gould, 2013), making for easier data collection; yet, dispersal distances for male *L. catta* have yet to be documented. However, males inhabiting the Beza Mahafaly Special Reserve (BMSR) have been observed migrating into groups outside the reserve boundaries several kilometers away (Sussman, 1991, 1992; Gould et al., 2003, Gould, 2006). Anthropogenic barriers to dispersal, such as roads and agricultural land, have been

found to compromise dispersal ability and gene flow for other extremely mobile species, including the Montana wolverine, which can disperse up to 300km in a year (Cegelski et al., 2003). Habitat fragmentation has also led to low dispersal rates for giant pandas (Zhan et al., 2007). Furthermore, anthropogenic impacts (i.e., road, a petroleum well pad) that characterize one of two groups of spider monkey home ranges in Proyecto Primate Research Area (PPRA) were discovered to have created differences in the patterns of genetic relatedness, population structure, and social behavior among male spider monkeys (primarily the philopatric sex) (Di Fiore et al., 2009). Considering the dramatic level of historical and contemporary anthropogenic disturbances across the central highlands of Madagascar, and in particular the likely barriers to gene flow (i.e., highway, anthropogenic savannah, villages) that are present near my three study fragments, male dispersal is likely to be difficult and potentially dangerous.

Natural barriers to gene flow can also impact an organisms' ability to successfully disperse (Di Fiore, 2003; Vigilant and Guschanski, 2009). For example, the Menarandra and Manambovo rivers in southern Madagascar were found to represent major barriers to dispersal for several populations of endangered radiated tortoises (Paquette et al., 2007). These natural barriers have resulted in moderate to strong genetic structuring (i.e., genetic differentiation) amongst populations (Paquette et al., 2007). Rivers were also found to be significant barriers to populations of western lowland gorillas inhabiting the Central African Republic, and the Republics of Congo and Cameroon (Fünfstück et al., 2014). Depending on the analysis employed, population assignment varied, STRUCTURE and BAPS software distinguished two clusters, while GENELAND determined three (Fünfstück et al., 2014). Analyses that divided western lowland gorillas into two clusters identified the Sangha River as a significant barrier to gene flow between the two populations (Fünfstück et al., 2014). GENELAND analyses, which identified a third gorilla population, determined that the Ngoko River acts as a barrier between the Odzala and Lobéké populations (Fünfstück et al., 2014). While both rivers represent barriers to gorilla dispersal and gene flow, the Sangha River, which is 1,300km in length and hundreds of kilometers wide versus the 700km long and 120km wide Ngoko River, plays a larger role in directing western lowland gorilla gene flow (Fünfstück et al., 2014).

The topography of my three study fragments is extremely rocky and mountainous, making for a challenging environment in which to be mobile. However, *Lemur catta* at these sites are quite adept at traversing the rocky-outcrop habitat (Clarke, pers. observ.). Moreover, ring-tailed lemurs inhabiting Tsinjoriake Protected Area and Tsimanampetsotsa National Park in southwestern Madagascar habitually use the limestone cliff-faces and caves which characterize these two study sites (Sauther et al., 2013). Thus, this species is extremely adaptable and proficient at moving within rocky and mountainous habitats (Goodman and Langrand, 1996), which may aid in successful male dispersal within these types of environments.

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Chapter 6. Conclusions

6.1. Summary

The loss and fragmentation of natural habitats as well as global climate change represent the most critical threats to global biodiversity and ecological processes worldwide (Fahrig, 2003; Travis, 2003). Anthropogenic landscape alterations have been documented to have a myriad of impacts and pose significant challenges for wildlife, including compromised dispersal, reduced or absent gene flow (Dietz et al., 2000), reduced reproductive fitness (Charpentier et al., 2008), increased vulnerability to predation, hunting, and disease (Fahrig, 2003), and an inability to deal with or respond to environmental changes and/or disease (Oklander et al., 2010).

The island of Madagascar, which is characterized by high species diversity and endemism, has been severely affected by a variety of historical and contemporary anthropogenic impacts, such as hunting, mining, and the illegal logging of precious hardwoods (Schwitzer et al., 2013a). In particular, slash and burn agriculture has led to acute forest loss and fragmentation, resulting in the loss of more than 90% of original forest cover (Harper et al., 2007). If current rates of deforestation are maintained, Madagascar is predicted to lose countless species to extinction in the near future (Brooks et al., 2002; Irwin et al., 2010; Schwitzer et al., 2013a, 2014). Thus, Madagascar's lemurs now represent the most endangered group of mammals in the world (Schwitzer et al., 2013a; 2014).

Lemur catta, the island's flagship species (Mittermeier et al., 1992), is endangered and is showing a declining population trend (Andriaholinirina et al., 2014). Despite being Madagascar's most well studied primate, little research has focused on how this ecologically and behaviorally flexible species has fared in regards to habitat loss across its geographic range (but see Cameron and Gould, 2013; Gabriel, 2013a,b; Gould and Gabriel, 2014). Moreover, only two population genetic studies have been conducted to date, both of which examine ring-tailed lemur populations in southwestern Madagascar (Parga et al., 2012; Pastorini et al., under review). Hence, my study represents the first to

examine the impacts of habitat fragmentation and isolation on the population and conservation genetics of three ring-tailed lemur populations inhabiting the central highlands of Madagascar.

Considering the severe level of fragmentation, isolation, and relatively small size of the three study fragments, Anja, Sakaviro, and Tsaranoro (Chapter 2), some of my results were unexpected. Knowing that diversity is lost more rapidly amongst smaller populations and is intensified when occupying a fragmented or isolated habitat (Lawes et al., 2000; Bergl et al., 2008), I expected that my study populations would be bereft of genetic diversity. However, at the time of study, these three populations appear to maintain a moderate level of genetic diversity (Chapter 3). As predicted, the smallest fragment, Sakaviro, revealed the lowest level of allelic richness, whereas the largest fragment, Tsaranoro, displayed the greatest. Evidence of possible past admixture between the Anja and Sakaviro populations was supported by a strong pattern of isolation of distance and low levels of genetic differentiation (Chapter 3). These findings are encouraging and valuable with respect to providing specific information for local conservation associations and the local government on areas that should remain and/or be included as high conservation priorities. By maintaining, expanding, and potentially connecting (where possible) these fragmented habitats, we can preserve the genetic diversity of these populations, as well as safeguard their long-term viability.

In order to begin to piece together a more comprehensive picture of this species' demographic history in relation to anthropogenic landscape changes across its geographic range, I tested the three populations for evidence of past and/or recent genetic bottlenecks (Chapter 4). Due to the historical and on-going habitat destruction across Madagascar, I expected to find evidence of population declines or genetic bottlenecks for all three populations. Three statistical approaches were utilized and the results were somewhat equivocal (Chapter 4). For example, M-Ratio analyses revealed heterozygosity excess (i.e., bottlenecks) in all three populations (Chapter 4). Conversely, mode-shift tests suggested that only the Anja population underwent a significant demographic decline (Chapter 4). This is similar to what Parga et al. (2012) found when examining two populations of ring-tailed lemurs in southwestern Madagascar. However, overall

evidence suggests that the Anja population suffered a significant historical demographic decline or bottleneck (Chapter 4). Since there is no definitive timeline as to when these populations became fragmented, it is possible that the demographic declines have occurred slowly and steadily, which would be unlikely to produce a significant bottleneck signal. Additionally, the ambiguous results may be a result of these populations experiencing a time-lagged response to habitat fragmentation, wherein they have yet to lose their genetic diversity (Whitlock and McCauley, 1999). These data, when considered with the genetic diversity results (Chapter 3), lend support to the suggestion that these fragments represent areas in which concerted conservation efforts are necessary if genetic diversity is to be maintained and future demographic declines are to be prevented.

Population assignment analyses are valuable for scientists because they can provide data on the genetic structuring of populations, while also being informative about a particular species' dispersal patterns, social organization, and mating system (Chapter 5). Moreover, they provide us with a way in which to determine areas of high or low gene flow between populations. The identification of these areas can help to ensure proper conservation management strategies for endangered species.

To address the question of whether or not male dispersal and thus gene flow has been negatively impacted by either natural and/or anthropogenic barriers, I conducted two population assignment analyses (Chapter 5). Since the three forest fragments have been severely impacted by human-induced disturbances, I predicted that male dispersal would be compromised. Results revealed a likely 'lack of signal' and therefore, individuals were unable to be partitioned into their likely populations of origin (Chapter 5). Furthermore, both *GenAlEx* and STRUCTURE failed to determine more than one cluster or population, indicating weak genetic structuring (Chapter 5). However, pairwise F_{ST} values suggest moderate genetic structuring between Anja and Tsaranoro and Sakaviro and Tsaranoro (Chapter 3). It is possible that these populations are not yet genetically differentiated enough for reliable population assignment or perhaps the sample was not large enough to produce clear results.

To gain a coherent picture of these three fragmented populations' genetic health and future viability, all data must be considered concomitantly. Evidence of moderate levels of genetic variation (Chapter 3), combined with comparable levels of diversity of larger ring-tailed lemurs populations in southwestern Madagascar (Parga et al., 2012) (Chapter 3), and the near absence of historical and/or recent demographic declines (except for a historical bottleneck at Anja) (Chapter 4), suggest that these populations, as of now, are genetically healthy. Due to the lack of signal for population assignment analyses, the extent of impact from the fragmentation and isolation on male dispersal cannot be clearly identified (Chapter 5). However, inbreeding coefficient results indicate the possibility of compromised gene flow and potential inbreeding for the Tsaranoro population (Chapter 3). Thus, it appears, at this time, there is no definite conclusion that can be drawn regarding the long-term effects of habitat fragmentation and isolation on these populations. To obtain a more unambiguous conclusion, a larger sample per fragment and a larger number of genotyped loci are ideal, as well as incorporating a multidisciplinary approach, which could include utilizing habitat structure, composition, and quality data with landscape data (GIS and satellite imagery). Detailed knowledge of landscape characteristics will be vital for elucidating our understanding of micro-evolutionary processes. Furthermore, the identification of spatial-genetic patterns combined with ecological data will aid in the discovery of correlations between genetics and environmental variables. Finally, my results are informative for the local community conservation associations working within south-central Madagascar. Specifically, these data can be now applied to determine areas of conservation priority and where forest corridors will be the most beneficial for maintaining gene flow. For example, the development of forest corridors between the small fragments within the Tsaranoro Valley will promote safe dispersal and increase gene flow. This may be of particular importance for this population, considering there are already signs of possible inbreeding and compromised gene flow (Chapter 3).

To summarize, this study shows that the three fragmented and isolated populations of ring-tailed lemurs within the central highlands of Madagascar currently maintain a moderate level of genetic diversity, and that one population, Tsaranoro, shows evidence of inbreeding. Greater levels of genetic structuring and thus, greater genetic

differentiation were found between the Sakaviro and Tsaranoro populations and the Anja and Tsaranoro populations. Moreover, Anja, the most islolated of the three populations shows strong evidence of suffering a historical bottleneck. To conclude, while some of these data are encouraging and may suggest that *Lemur catta* is persisting well in these fragments, it is imperative that all of these populations remain a conservation priority, particularly since anthropogenic threats continue to plague the island and ring-tailed lemur populations are declining.

6.2. Community-Based Conservation: Successes in Primate Habitat Countries

Community-based conservation (CBC), including integrated conservation and development projects (ICDPs), ecotourism, and a variety of *in situ* conservation and education programs, have come to the forefront of conservation and in many ways have replaced traditional conservation methods (Adams and Hulme, 2001; Adams et. al., 2004; Berkes, 2007; Gavin et al., 2007). The popularity of CBCs may be due to their ability to "work on multiple fronts" (i.e. tackling biological and anthropogenic factors), which Bettinger and colleagues (2010) argue is essential if we are to successfully promote positive biological change. The goal of CBC is to incorporate conservation goals (e.g. long-term protection of a particular ecosystem and the species that it hosts) with the needs of local communities. Ultimately, CBC aims to create an atmosphere in which conservation goals are met in addition to increasing knowledge via education programs, implementation of a more sustainable way of living, development of infrastructure (e.g. building of schools), creating lasting economic opportunities (e.g. ecotourism), and helping to establish a feeling of pride and empowerment (Savage et al., 2009; Adams and Hulme, 2001; Berkes, 2007; Gubbi et al., 2009). The CBC model has been demonstrated to be enormously successful in a variety of primate habitat countries, for example, Belize (Horwich and Lyon, 2007 Community Baboon Sanctuary), Columbia (Savage et al., 2009 Proyecto Titi Project), Vietnam (http://www.catbalangur.org Cat Ba Langur Conservation Project), the Philippines (http://www.tarsiusproject.org/home-page/ The Tarsius Project), Africa (http://www.pasaprimates.org Pan African Sanctuary Alliance/PASA), and Madagascar (Wright and Andriamihaja, 2002 Ranomafana National Park; Gould and Gabriel, 2013 Anja Community Reserve). The following examples highlight some of the

successes and future conservation goals from existing community-based conservation programs and organizations that will be imperative to the successful conservation and protection of a range of biodiverse areas, many of which host some of the world's most threatened primates.

In 1985, Robert Horwich and Fallet Young established the Community Baboon Sanctuary (CBS) in Belize, which now serves as a model for primate sanctuaries around the globe (http://www.howlermonkeys.org/about-the-cbs/). The primary goals of the sanctuary include conservation, education, research, and tourism (http://www.howlermonkeys.org/about-the-cbs/). Through community- based conservation initiatives, CBS now has more than 200 private landowners across seven villages who voluntarily vowed to conserve their land for the preservation of *Alouatta pigra* (black howler monkey, locally referred to as baboons) habitat (http://www.howlermonkeys.org/about-the-cbs/). The sanctuary has been financially and socially sustainable since 1990, and in 1998 CBS was taken over by a local women's conservation group and became an officially registered sanctuary, which allowed it to acquire funding to develop an education center (Horwich and Lyon, 2007). The profits earned by CBS are used to employ sanctuary management, as well as to develop and present education programs (Horwich and Lyon, 2007). In addition, a portion of the income is given to landowners and participating villages (Horwich and Lyon, 2007).

Proyecto Titi, a CBC program based in Colombia, has been remarkably successful in a variety of contexts (Gubbi et al., 2009; Savage et al., 2009). Proyecto Titi developed out of Dr. Anne Savage's field research on the reproductive biology of the critically endangered cotton-top tamarin (*Saguinus oedipus*) (Savage et al., 2009). Prior to outlining the aims and conservation and developmental strategies for Proyecto Titi, Savage and her Colombian colleagues conducted evaluations and surveys within the local communities (Savage et al., 2009). They found that more than 90% of Colombians surveyed had no knowledge that cotton-top tamarins were endemic to Colombia (Savage et al., 1997). In addition, their evaluations revealed that locals had several 'myths and misconceptions' regarding the forest and the local wildlife (Savage et al., 1997 cited in Savage et al., 2009). As a result of these surveys and evaluations, a community education

strategy was developed that incorporated formal and informal programs aiming to increase knowledge regarding cotton-top tamarins and the local biodiversity, address unsustainable farming practices, increase scientific literacy by developing teacher training programs, address the illegal pet trade of *S. oedipus,* and affiliate with local educational entities to integrate this information into the existing school curriculum (Savage et al., 2009). Evaluations conducted after the integration of the community education programs revealed numerous positive results (Savage et al., 2009). For example, evaluations of more than 300 students across fifteen schools revealed an 81% increased level of accuracy for correctly identifying a cotton-top tamarin (Savage et al., 2009). In addition to community education programs, Proyecto Titi focused on developing economic alternatives with and for the local communities, such as the creation of an artisans group in 2004, ASOARTESANAS (Savage et al., 2009). ASOARTESANAS creates 'eco-mochilas' bags, which are crocheted from plastic bags and then sold on national and international markets (Savage et al., 2009). Local women received training via workshops to create eco-mochilas, as well as business training classes, which helped them turn their artisan group into a registered business in Colombia (Savage et al., 2009). The creation of this artisan group has provided local women with a means to achieve economic stability, in addition to providing a sense of empowerment and pride (Savage et al., 2009). Another example of an economic alternative that had positive conservation impacts was the development and use of *new* bindes, traditional Colombian cooking stoves, which had previously been constructed from termite mounds (Savage et al., 2009). The new and improved bindes were constructed from clay and were more durable and longer lasting (Savage et al., 2009). One of the major benefits to using the bindes was the dramatic reduction in harvesting of firewood from cotton-top tamarin habitat (Savage et al., 2009). Moreover, local community members were trained to construct the new and improved bindes and were then able to sell them to other community members or people in neighboring communities (Savage et al., 2009).

CBC projects have also seen notable successes throughout various regions of Madagascar (Schwitzer et al., 2013a). For example, in 2009 the Antanetiambo Nature Reserve was created in northern Madagascar by Malagasy wildlife guide Desiré Rabary (http://antanetiambo.marojejy.com/Intro_e.htm). This 14 ha rainforest reserve is the only

reserve in Madagascar to be established by a local Malagasy. Rabary has joined forces with the Duke Lemur Center (DLC) and its SAVA Conservation Project, a non-profit group that focuses on the conservation and research of the critically endangered silky sifaka (*Propithecus candidus*) to develop ecotourism and conservation initiatives, including fish farming, tree nurseries, and monitoring and long-term research of the northern bamboo lemur (*Hapalemur occidentalis*) (http://antanetiambo.marojejy.com/Intro_e.htm). Since collaborations began, the reserve has expanded (>40%) through the purchasing of additional parcels of forest, *H. occidentalis* groups have been habituated, a new toilet and showers have been built as well as a community library, almost 7,000 trees have been planted, and more than 11,000 seedlings, of mostly native species, have been grown in a tree nursery (http://antanetiambo.marojejy.com/Intro_e.htm).

These examples represent a small sampling of successful CBCs; however, the CBC model is now becoming the standard model of conservation and is being employed in many areas of the world for a variety of endangered primate species (Louis et al., 2013 Madagascar; Vietnam http://www.catbalangur.org Cat Ba Langur Conservation Project; the Philippines http://www.tarsiusproject.org/home-page/ The Tarsius Project; Africa http://www.pasaprimates.org Pan African Sanctuary Alliance/PASA). These community-based conservation programs demonstrate the positive impact that CBCs can have within a particular region, specifically, with respect to how particular initiatives and strategies benefited indigenous communities with economic development and advancement, as well as safeguarding the forests and biodiversity they contain. Each of these programs have had their own degree of successes and have identified and outlined specific conservation initiatives that will need to be addressed and carried out for continued success in the future. While these programs can serve as models for future community-based initiatives, one must keep in mind the importance of designing programs with measurable objectives, which will help in making sure program goals are being met. Moreover, conservationists need to be cognizant that particular efforts that have been successful in one region may not work in another (Bettinger et. al., 2010). Therefore, it is imperative to research and understand local cultures, traditions, and languages in order to ensure that CBCs are culturally sensitive, relevant, and ultimately, successful (Bettinger et al., 2010).

6.2.1. Community-Based Conservation: A conservation plan for the ring-tailed lemur

While aspects of the CBC model are being employed by some of the community associations in the central highland region of Madagascar (Gould pers. comm.; Gould and Gabriel, 2013), the need for constant presence and support, perhaps in the form of an international NGO, would be beneficial to the region, the local people, and conservation of the local biodiversity. An NGO to oversee all forest fragments containing *L. catta*− in addition to collaborating with the existing community associations− would allow for consistent funding opportunities for various initiatives, such as building schools, providing medical care to local people, creating conservation education centers, establishing new protected areas, further development of ecotourism, and training of local people for jobs in ecotourism. Other important feature that would aid in the overall success of CBC efforts are pre-and-post evaluations (see Savage et al., 2009 Proyecto Titi Project). These are crucial to the identification of concepts, strategies and teaching methods that are capable of increasing knowledge and awareness of conservation issues, in addition to adjusting ones that do not. Long-term monitoring and evaluations will be vital in terms of documenting genuine changes in behavior over time, as well as overall effectiveness of the program(s) in relation to the local community and the local biodiversity. Furthermore, *ex situ* conservation initiatives will also play an important role in ring-tailed lemur conservation and should include fundraising, education and outreach to the general public (local conservation groups, zoos, schools, summer camps), and the utilization of social media (e.g., Twitter, Facebook) to promote conservation messages and initiatives. In addition, the IUCN-SSP Primate Specialist Group for Madagascar has developed and outlined a number of *priority actions* that need to be implemented if we are to maintain the long-term viability of wild lemur populations. The IUCN-SSP Primate Specialist Group highlights the integration of *ex situ* and *in situ* conservation, which will be essential to combating lemur extinctions (Schwitzer et al., 2013ab). The 'One Plan' approach, developed by this committee recommends that conservation planning incorporate all populations of a species, both inside and outside its natural habitat (Schwitzer et al., 2013b). Thus, managing captive and wild populations as a single metapopulation will aid in protecting against extinctions by creating 'reserves for future restocking' of wild lemur populations (Schwitzer et al., 2013b). Furthermore, in order to

achieve and maintain genetically healthy populations (i.e., captive and wild) the exchange and translocation of animals should include individuals from both inside and outside Madagascar (i.e., zoos and captive breeding facilities)(Schwitzer et al., 2013b). Finally, these initiatives should work in concert with continued genetic and behavioral monitoring and research which will be imperative to understanding, maintaining, and ensuring the future viability of the ring-tailed lemur throughout its geographic range.

In conclusion, non-human primates inhabit much of the globe's mega-diverse regions and are therefore considered icons for conservation (Laurance, 2003). According to the IUCN-SSP Primate Specialist Group, almost half (48%) the world's primate species are now threatened with extinction (http://www.primate-sg.org/red_list_threat_status/). Predicting extinctions and understanding how organisms respond to the synergistic effects of deforestation, hunting, or climate warming poses a great challenge for biologists, primatologists, and conservationists (Travis, 2003; Opdam and Wascher, 2004). By understanding how various anthropogenic disturbances are affecting wild primate populations, better informed conservation decisions can be made to protect and manage the endangered ecosystems that host our primate relatives as well as other threatened species (Marsh, 2003, 2013). This can be achieved through the application of a multi-disciplinary research approach to empirical studies; for example, utilizing landscape genetic techniques in concert with climate change modeling. However, to ensure long-term success for endangered primate populations, community-based conservation efforts should also be incorporated. This approach will not only enhance our scientific knowledge, but will be advantageous to global ecosystems and the biodiversity they contain, as well as benefiting local indigenous communities.

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APPENDIX A

Mean F_{ST} value for K=2

Values represent the mean population differentiation (F_{ST}) for the ten runs of K=2 performed in STRUCTURE.

Run Number	Mean F_{ST} value for *K=2*
1	Cluster 1: 0.0044 Cluster 2: 0.0078
2	Cluster 1: 0.0089 Cluster 2: 0.0004
3	Cluster 1: 0.0053 Cluster 2: 0.0059
4	Cluster 1: 0.0086 Cluster 2: 0.0358
5	Cluster 1: 0.0020 Cluster 2: 0.0063
6	Cluster 1: 0.0011 Cluster 2: 0.0060
7	Cluster 1: 0.0198 Cluster 2: 0.0021
8	Cluster 1: 0.0125 Cluster 2: 0.0209
9	Cluster 1: 0.0099 Cluster 2: 0.0216
10	Cluster 1: 0.0053 Cluster 2: 0.0074

APPENDIX B

Mean F_{ST} values for *K*=3

Values represent the mean population differentiation (F_{ST}) for the ten runs of *K*=3 performed in STRUCTURE.

Run Number	Mean FST value for *K*=3
1	Cluster 1: 0.0204 Cluster 2: 0.0150 Cluster 3: 0.0071
2	Cluster 1: 0.0220 Cluster 2: 0.0171 Cluster 3: 0.0089
3	Cluster 1: 0.0053 Cluster 2: 0.0132 Cluster 3: 0.0027
4	Cluster 1: 0.0014 Cluster 2: 0.0153 Cluster 3: 0.0272
5	Cluster 1: 0.0053 Cluster 2: 0.0129 Cluster 3: 0.0084
6	Cluster 1: 0.0153 Cluster 2: 0.0295 Cluster 3: 0.0420
7	Cluster 1: 0.0186 Cluster 2: 0.0252 Cluster 3: 0.0117
8	Cluster 1: 0.0346 Cluster 2: 0.0343 Cluster 3: 0.0351
9	Cluster 1: 0.0056 Cluster 2: 0.0046 Cluster 3: 0.0076
10	Cluster 1: 0.0266 Cluster 2: 0.0061 Cluster 3: 0.0007

APPENDIX C

Glossary of Genetic Terms:

'Allele call': The visual examination and interpretation of an electropherogram that allows for the determination of an individuals genotype for a given locus.

Allelic dropout: Allelic dropout occurs when an allele fails to amplify; if this happens to one allele of a heterozygous individual, the individual will falsely appear to be a homozygote. Therefore, allelic dropout can result in incorrect genotypes being obtained, resulting in an overall reduction of observed heterozygosity -leading to inaccurate data regarding a population's genetic diversity (Taberlet et al., 1999).

Allelic richness (AR): One way in which to measure genetic diversity in a population (Grativol et al., 2001; Olivieri et al., 2008). Typically represented by mean # of alleles per locus (MNA).

Assignment index (AI): AIs are statistics that summarize the likelihood that a sampled individual's genotype originated in the population in which it was sampled (Di Fiore et al., 2009; Manel et al., 2005).

Bottleneck: When a population bottleneck occurs, there is a decline in the average heterozygosity per locus, and the rate of decline is dependent upon the effective population size (N_e), though if there is an increase in population size, average heterozygosity will increase via new mutations. Thus, genetic bottlenecks produce an *excess of heterozygosity* due to alleles (rare alleles usually lost first and contribute little to expected heterozygosity) being lost more rapidly than heterozygosity (Wright, 1931).

Effective population size: Effective population size (N_e) represents the number of *required* breeding individuals in a theoretically *ideal* population that can account for the observed level of variation in the actual population (Wright, 1931, 1938).

Expected heterozygosity (H_E) & observed heterozygosity (H_O): The expected proportion of heterozygote individuals (ranging from 0 to 1.0) per locus (Hartl and Clark, 2007).

H_O (observed heterozygosity) is the observed proportion of heterozygote individuals per locus (Hartl and Clark, 2007).

Heterozygosity Excess: Genetic bottlenecks produce an ***excess of heterozygosity*** due to alleles (rare alleles that are usually lost first and contribute little to expected heterozygosity) being lost more rapidly than heterozygosity (Wright, 1931).

Wright's F-statistic or fixation index: F_{ST} is the proportion of the total genetic variance contained in a subpopulation (*S*) relative to the total genetic variance (*T*) (Wright, 1969, 1978). Fixation index values can range from 0 (signifying no differentiation between the overall population and its subpopulations) to a theoretical maximum of 1. Higher F_{ST} values imply a substantial degree of differentiation among populations (Wright, 1969,

1978; Hartl and Clark, 2007).

F_{IS} (inbreeding coefficient): F_{IS} measures the proportion of deviation of genotypic frequencies in regards to heterozygosity deficiency or excess. F_{IS} values range between -1 and +1, where positive values indicate a heterozygous deficiency (inbreeding) and negative values signify heterozygous excess (outbreeding) when compared to HWE (Wright, 1931, 1938, 1969; Hartl and Clark, 2007).

Genetic distance: Genetic distance between two populations, Nei's distance (*Nei's D*) is one way to assess and measure genetic distance (Nei, 1972, 1973).

Isolation by distance (IBD): Measurement of genetic distance in regards to geographic distance. Genetic differentiation will increase with increased geographic distance (Wright, 1943; Slatkin, 1993).

Linkage equalibrium/disequalibrium: Measures the degree to which alleles at two loci are associated. Alleles that are in random association (inherited completely independent in each generation) with each other are considered to be in **linkage equilibrium.** When two alleles are in **linkage disequilibrium** (non-random association of alleles at different loci) it indicates that particular alleles of each gene are inherited together more often than would be expected by chance (Manel et al., 2005).

Microsatellite: Microsatellites, also referred to as simple sequence repeats (SSR) and short tandem repeats (STR), consist of a variable number of tandem repeats of a 1-6 base pair nucleotide motif distributed randomly within a given species genome (Di Fiore, 2003; Selkoe and Toonen, 2006).

Mutation-drift equilibrium: The quantification of the equilibrium or balance between the introduction of new alleles via mutation and the reduction of variation via genetic drift (Hartl and Clark, 2007).

Null alleles: Alleles that are present but fail to amplify, due to polymorphism or mutations in one of the primer binding sites. If an individual is heterozygous and a primer binding mutation occurs for one of the alleles, the individual would incorrectly be identified as a homozygote (Taberlet et al., 1999).

Population structure or structuring: Pattern of genetic diversity across populations. This can be estimated by Pairwise F_{ST} (Wright, 1978).

Rule of thumb for interpretation of F_{ST}- 0-0.05=low levels of genetic differentiation, 0.05-0.15=moderate level, 0.15-0.25=great level, and anything above 0.25= very great levels of differentiation (Hartl and Clark, 2007).

Population Assignment Test: Statistical test that examines the assumption that an individuals multilocus genotypes should be assigned to a particular locale or population (Manel et al., 2005).

Primer: Short (typically ~20 base pairs long) synthetic chains of nucleotides use as

starting points for enzymatic replication by PCR (http://seqcore.brcf.med.umich.edu/doc/educ/dnapr/mbglossary/mbgloss.html).

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