City University of New York (CUNY)
CUNY Academic Works

School of Arts & Sciences Theses | Hunter College

Spring 5-19-2016

Population genetic analysis of the critically endangered black-and-white ruffed lemur (Varecia variegata) in Ranomafana National Park, Madagascar

Amanda Mancini
CUNY Hunter College

How does access to this work benefit you? Let us know!

More information about this work at: https://academicworks.cuny.edu/hc_sas_etds/73
Discover additional works at: https://academicworks.cuny.edu

This work is made publicly available by the City University of New York (CUNY).
Contact: AcademicWorks@cuny.edu

Population genetic analysis of the critically endangered black-and-white ruffed lemur (*Varecia variegata*) in Ranomafana National Park, Madagascar

By

Amanda N. Mancini

Submitted in partial fulfillment
of the requirements for the degree of
Master of Arts in Anthropology, Hunter College
The City University of New York

2016

Thesis Sponsor:

May 19th, 2016	Dr. Andrea L. Baden
Date	Signature
May 19th, 2016	Dr. Jessica M. Rothman
Date	Signature of Second Reader

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Andrea Baden, for her unending support, guidance, and encouragement throughout this project. She has been a critical resource while designing and implementing my research, as well as in obtaining funding. Additionally, I would like to thank my second reader, Dr. Jessica Rothman, for contributing her comments, revisions, and expertise during the writing of this thesis. Research in Madagascar was made possible through assistance from Madagascar Institut pour la Conservation des Ecosystèmes Tropicaux (MICET), Madagascar National Parks (MNP), and the Centre ValBio Research Station (CVB). Laboratory support was provided by Dr. Andrea Baden at the Primate Molecular Ecology Laboratory at Hunter College (City University of New York) and the DNA Analysis Facility at Yale University on Science Hill. Funding for this research was provided by: the American Society of Primatology, the Hunter College Master Thesis Support Grant, the Department of Anthropology Research and Training program at Hunter College, and the Professional Staff Congress at the City University of New York (Dr. Andrea Baden).

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Madagascar is among the world's leading biodiversity hotspots. It is also a conservation priority, as the country has lost more than 90% of its primary vegetation. Ongoing deforestation threatens many of the island's endemic taxa, including the Critically Endangered black-and-white ruffed lemur (*Varecia variegata*). Individuals of this taxon are sensitive to habitat degradation and have experienced extensive hunting pressure, leading to rapid population declines throughout the species' remaining habitat. Furthermore, recent studies have indicated low levels of genetic diversity in *V. variegata*, particularly within the southern portion of its' range. With continuing habitat loss and fragmentation, tracts of continuous forest- such as those found in national parks- may be critical for preserving genetic diversity of *V. variegata* groups and ensuring their proliferation into the future. The aim of this study was to determine the efficacy of a national park in preserving genetic diversity and gene flow in and between *V. variegata* groups, as well as assess any evidence of population decline. To achieve this, I sampled the DNA from feces of 19 adult *V. variegata* individuals from four sites in Ranomafana National Park. A suite of seven microsatellite markers was used to evaluate genetic diversity and population genetic structure, as well as to test for evidence of population bottleneck signals, which are useful for detecting recent population declines. Both a Bayesian cluster analysis and a multivariate clustering method (Discriminate Analysis of Principle Components) provided evidence for one genetic cluster within Ranomafana National Park, suggesting that RNP harbors one continuous *V. variegata* genetic population. Mean number of alleles per locus within the park was 3.57, and observed and expected heterozygosity were 0.666 and 0.553, respectively. These levels of genetic variability are similar to previous studies performed within the park, albeit lower than in several other lemur taxa. Additionally, evidence for a recent population

bottleneck was found under all three mutation models assessed. Together, these results suggest that Ranomafana National Park has been successful in maintaining gene flow in *V. variegata* throughout the southern parcel, although this population may have suffered a recent decline in population size and genetic diversity. Finally, the presence of a *V. variegata* resident group within a section of the park subject to historic logging suggests positive implications for the potential recolonization of recovering forests.

INTRODUCTION

Madagascar is categorized as a leading biodiversity hotspot due to its high levels of endemism in most taxonomic groups and severe habitat depletion (Myers *et al.,* 2000). The enormous endemic diversity on the island is being threatened by ongoing deforestation and habitat fragmentation, with less than 10 percent of the primary vegetation remaining on the island. This extreme habitat loss has occurred within the last two thousand years and was catalyzed by the arrival of humans to the island. Over the last 65 years, deforestation rates have remained between 1 to 2.5% of habitat lost per year, particularly in the humid rainforests in the east (Green and Sussman, 1990; Harper *et al.,* 2007; Grinand *et al.,* 2013).

Forest loss and associated habitat fragmentation pose critical threats to species worldwide and are among the primary drivers of species extinction (Cowlishaw and Dunbar, 2000; Di Marco *et al.,* 2014). Habitat loss and fragmentation can severely impede species' ability to thrive in affected areas due to their inability to disperse between adjacent populations. When dispersal is restricted, this can lead to smaller effective population sizes, increased inbreeding, decreased genetic variability, and reduced gene flow (Olivieri *et al.,* 2008; Radespiel *et al.,* 2008; Craul *et al.,* 2009; Dixo *et al.,* 2009; Holmes *et al.,* 2013). Increases in inbreeding and the loss of genetic diversity can lead to an increased susceptibility to disease (Spielman *et al.,* 2004) and a lowered

immunocompetence (Hale and Briskie, 2007). Together, these processes have severe consequences for endemic Malagasy species, particularly lemurs which have been characterized the world's most endangered mammalian taxa (Schwitzer *et al.*, 2013).

Given the myriad negative consequences associated with fragmented and degraded habitats, pristine and healthy contiguous forest- such as that found in many national parks- are crucial for most species' persistence. Determining the degree to which a tract of continuous forest supports a cohesive and genetically diverse population is a baseline step in developing future conservation initiatives in that species. In this study, I evaluate the efficacy of a tract of continuous forest (Ranomafana National Park) in maintaining genetic variability and stability in the Critically Endangered black-and-white ruffed lemur (*Varecia variegata*; Andriaholinirina *et al.*, 2010). Population estimates are difficult in this species as they are patchily distributed throughout their remaining habitat (Irwin *et al.*, 2005). Ganzhorn *et al*. (2001) and Schwitzer *et al.* (2013) estimate that fewer than 10,000 *V. variegata* individuals remain, although this may be a generous estimate. In the most heavily logged sections of forest *V. variegata* is absent, and this is likely related to their locomotive abilities. *Varecia variegata* are arboreal quadrupeds and require a continuous canopy to move effectively, therefore disruptions in the canopy cover as a result of logging likely make dispersal and movement difficult in this species (White *et al.*, 1995). Moreover, *V. variegata* are obligate frugivores with more than 75% of their diet comprising fruit (White *et al.*, 1995; Balko, 1998). The disturbance caused by logging may lead to a lowered abundance and diversity of available fruit, making these regions unsuitable to support *V. variegata* groups (Ratsimbazafy, 2002; Balko and Underwood, 2005). Together, this evidence suggests that *V. variegata* are particularly sensitive to habitat degradation and forest loss. As logging and other forms of habitat degradation continue we will likely see an extreme

reduction in genetic diversity in this species and a loss of resident groups entirely. Already studies found relatively low levels of genetic diversity, little to no haplotype diversity, and high levels of population differentiation in the southern portion of *V. variegata*'s range south of the Mangoro River (Holmes *et al.*, 2013; Baden *et al.*, 2014; but see Perry *et al.*, 2013).

Here, I assess the genetic diversity, population genetic structure, and indications of recent genetic bottleneck events in black-and-white-ruffed lemurs (*Varecia variegata*) in Ranomafana National Park (RNP). I looked at four localities within Ranomafana: Talatakely, Vatorahanana, Valohoaka, and Mangevo, which represent a gradient from high levels of historical disturbance (Talatakely) to a pristine rainforest environment (Mangevo). Results generated from this study are useful to provide insight into the relative efficiency of a protected, contiguous forest site in promoting gene flow and genetic variability in a Critically Endangered primate species.

BACKGROUND

Conservation status in Madagascar

Habitat degradation and forest loss pose a critical threat to species worldwide and are among the primary drivers of species extinction (Cowlishaw and Dunbar, 2000; Di Marco *et al.*, 2014). Habitat loss is particularly rampant in the most biodiverse regions of the globe (i.e. biodiversity hotspots), which creates a significant problem in maintaining worldwide biodiversity. Although many habitats are naturally heterogeneous and fragmentation can occur without anthropogenic influence (Haig *et al*., 2000), the primary drivers of both loss and degradation of ecosystems are human activities. Given limited conservation funds, it has become necessary for conservationists to devise parameters through which they can identify areas in need of immediate conservation intervention. Myers *et al*. (2000) sought to achieve this in biodiversity hotspots by assessing two primary factors, namely the percentage of species endemism found in

a region and the amount of primary habitat loss. Through this assessment five hotspot conservation priorities were identified: the Tropical Andes, Sundaland, Brazil's Atlantic Forest, the Caribbean, and Madagascar. Combined these five hotspots constitute over fifteen percent of all known flora and fauna, but occupy less than 0.4% of land surface worldwide. Myers *et al*. (2000) expanded on this analysis to identify the "hottest hotspots" by dividing species endemism into four specific factors- total number of endemics and the number of endemic species/area ratio in both vertebrae and plants- and assessing these in conjunction with primary habitat loss. The "hottest hotspot" identified using these five parameters was the island country of Madagascar.

Madagascar is host to over 9,000 species of endemic plants and over 700 species of endemic vertebrates (Myers *et al*., 2000). The enormous diversity on the island is being threaten by ongoing deforestation and habitat degradation, with less than 10 percent of the primary vegetation remaining. This extreme habitat loss has occurred within the last two thousand years and was catalyzed by the arrival of humans to the island. Over the last 65 years, rates of deforestation have remained between approximately 1 to 2.5% of habitat lost per year, particularly in the humid rainforests in the east (Green and Sussman, 1990; Harper *et al.,* 2007; Grinand *et al*., 2013). As forests are cleared, the underlying soil is exposed and a high degree of erosion occurs. This exposure makes the landscape susceptible to extreme environmental events such as cyclones, which are known to cause massive damage to an area in the form of flooding or landslides (Wright, 1999).

Deforestation in Madagascar, or "*tavy*," is primarily driven by the shifting agricultural practices, whereby individuals or small groups of farmer fell and burn sections of forest to plant crops, typically rice paddies (Mittermeier *et al.,* 2010). The practice of *tavy* has decimated the landscape in Madagascar because soils are stripped of their nutrients within a few years of

cultivation, leading locals to abandon their fields and clear additional forest for new agricultural land. The unproductive fields that remain may not recover for years as the soil can no longer support the colonization of most forest tree species (Mittermeier *et al.*, 2010). Furthermore, because of *tavy* is practiced by individuals or small groups the sections of forest that are cleared are not coordinated between parties, leading to a patchwork of agriculture in the remaining habitat. This fragmentation of the forest may have disastrous consequences for the species residing within it, such as smaller effective population sizes, increased inbreeding, decreased genetic variability, and reduced gene flow (Olivieri *et al.*, 2008; Radespiel *et al.*, 2008; Craul *et al.*, 2009; Dixo *et al.*, 2009; Holmes *et al.*, 2013).

In addition to the threat of *tavy*, much of Madagascar's remaining habitat is subject to degradation due to forestry and selective logging. Illegal logging of rosewood has caused immense disturbance, particularly in northern regions of the country (Allnut *et al*., 2013), and selective logging of other hard wood species such as *nato* (*Sideroxylon*) and *ramy* (*Canarium sp.*) (Green and Sussman, 1990; White *at al.*, 1995) contribute the degradation of remaining habitat in the eastern escarpment. Logging disrupts canopy cover (White *et al*., 1995), leading to a loss of crucial food resource trees and other plant species, facilitating hunting, and/or leading to the introduction of invasive flora, fauna, or diseases (Mittermeier *et al.*, 2010; Brown and Gurevitch, 2004), all of which pose a significant threat to species inhabiting the forests in which these practices occur. Finally, other practices such as mining exacerbate the intensive deforestation that threaten the myriad endemic species that rely on the remaining Malagasy forests.

One endemic Malagasy taxon that is at particular risk due to ongoing deforestation and habitat degradation are the lemuriform primates. Historically, this taxon has witnessed 17

extinction events in the recent past (the last 2,000 years), all which have been attributed to the arrival of humans on the island (Burney *et al.,* 2004; Crowley 2010). Members of the Lemuroidea were recently categorized as the world's most endangered mammalian group (Schwitzer *et al.*, 2013), therefore conservation efforts and research are needed in order to prevent the extinction of many species on that list. Currently 103 species of lemurs have been identified, of which more than 90% are classified as at least Vulnerable and 24 species are classified as Critically Endangered (Andriaholinirina *et al.,* 2010; Schwitzer *et al.,* 2013).

Lemurs are crucial seeds dispersers in Malagasy rainforests, particularly for larger-seeded tree species. Unlike many other rainforest environments- such as in the Neotropics- Madagascar is host to few frugivorous avian and chiroptera species, and large-bodied fruit eating ungulates, squirrels, rodents, and tortoises are entirely absent (Dew and Wright, 1998). It is therefore likely that the vast diversity of primate species on the island serve as primary seed dispersers for many of the fruiting tree species on the island (Wright *et al.*, 2011). Additionally, studies have shown that seed germination is significantly improved following passage through the digestive tract of frugivorous strepsirrhines as this process aids in scarifying the seed's exocarp (Shaefer and Ruxton 2011; Dew and Wright 1998). Successful seed dispersal is critical for maintaining forest structure and stability, both of which are threatened by the potential loss of numerous lemur species. Already, several studies have hypothesized that with the recent extinction of 17 lemurid taxa many species of rainforest trees have been "orphaned" (Godfrey *et al.,* 2008; Crowley *et al* 2011; Federman *et al* 2016), suggesting an inevitable extinction of these taxa as well. This foreboding hypothesis has severe implications for the viability of Madagascar's rainforests and suggests a critical need for the conservation of the remaining lemur taxa, particularly those that play key roles in seed dispersal. One lemur taxon in particular, the black-and-white ruffed lemur

(*Varecia variegata*), has been suggested as one of the most efficient seed dispersers in Malagasy rainforests (Dew and Wright, 1998). Furthermore, this taxon is one of the only remaining species that is physiologically capable of ingesting and passing large seeds (such as *Canarium*) that were likely dispersed by subfossil lemur species prior to their extinction (Federman *et al.*, 2016). Together, this evidence makes *V. variegata* a prime target for conservation research.

Black-and-white ruffed lemurs (*Varecia variegata*)

Black-and-white ruffed lemurs (*Varecia variegata*) are the largest remaining taxon of the Lemuridae (body size 3.5-4.5 kg; Baden *et al.*, 2008), show no evidence of sexual dimorphism, and are found throughout the remaining mid-to-low altitude eastern rainforest corridor. Current taxonomy identifies three subspecies of black-and-white ruffed lemurs, namely *Vareica variegata editorum, V. v. subcincta,* and *V. v. variegata* (Andriaholinirina *et al.*, 2010). This taxonomic categorization of *V. variegata* is based primarily on pelage variation in regards to coloration and saddle patterning, but investigations by Vasey and Tattersal (2002) suggest that this variation is not exclusively driven by geographic location nor genetic types; additionally a recent species-wide population genetic study of *V. variegata* found support for only two genetic clusters throughout its' remaining range in the eastern escarpment, bringing into question current taxonomy (Baden *et al*., 2014).

Species-level population estimates of *V. variegata* range from 1,000 to 10,000 individuals (Ganzhorn *et al*., 2001; Schwitzer *et al.*, 2013), although the true population size is likely closer to the lower end of this range. Population estimations in *V. variegata* are challenging using current methodologies (e.g. distance sampling; Buckland *et al.*, 1993) because this species is patchily distributed it's throughout range (Irwin *et al.*, 2005). It is unclear what landscape variables drive their distribution, as much of the remaining habitat in the eastern corridor seems

suitable from an objective standpoint. This patchiness has implications for the degree of gene flow between groups, particularly in regions where deforestation and fragmentation are high, and factors into a relatively low overall species abundance and density (estimated at 11.12 ind/km^2; Irwin *et al.,* 2005).

As stated previously, rates of deforestation in Madagascar are relatively high (1 to 2.5% of habitat lost per year) and have been sustained at a comparable level over the past 65 years (Green and Sussman, 1990; Harper *et al.,* 2007; Grinand *et al.,* 2013); this is especially true in the eastern escarpment containing *V. variegata*'s last remaining habitat. Over the last 30 years, *V. variegata* has suffered extreme losses in population size due to by habitat loss, with estimates of up to 80% declines (Andriaholinirina *et al.,* 2010). Furthermore, selective logging of two fruiting tree taxa that are preferentially exploited by *V. variegata*, namely *nato* (*Sideroxylon*) and *ramy* (*Canarium sp.*), occurs frequently because of their hard wood (White *et al.,* 1995), depleting a critical resource for this species.

Past studies have shown that *V. variegata* are absent from heavily disturbed sections of forest (White *et al.,* 1995; Herrera *et al.,* 2011; Wright *et al.,* 2012), which is likely a result of limitations in their locomotive abilities and/ or dietary requirements. Black-and-white ruffed lemurs are arboreal quadrupeds and therefore require a continuous patchwork of branches to traverse through the canopy (White *et al.,* 1995). Heavily logged and degraded forest regions exhibit decrease canopy cover and connectively compared to intact forest (White *et al.,* 1995), likely proving a significant locomotive challenge for *V. variegata* and hindering this species ability to move and forage in the canopy. Additionally, *V. variegata* are obligate frugivores, with their diet comprising more than 75% fruit (Balko, 1998; White *et al.,* 1995). As their habitat is degraded through selective logging, with preferentially exploited trees such as *nato* and *ramy*

being among those to disappear, there may be a decrease in fruit availability and therefore inadequate resources to support *V. variegata* groups. Ratsimbazafy (2002) found that natural environmental events, such as cyclones, can cause immense habitat disturbance in an area and a significant decrease in the amount of available fruit. Logging decreases forest biodiversity and the abundance of large crowned fruiting tress and may have a similar impact on fruit abundance as natural environmental events, particularly when damage to residual tree stands occurs (Johns, 1988; Tavankar *et al.,* 2013).

Due to their obligate frugivory, adequate fruit availability is particularly important for *V. variegata* as this impacts reproductive potential and fecundity. With their "boom-bust" reproductive strategy (characterized by synchronous bouts of reproduction separated by long inter-birth intervals; Baden *et al.,* 2013), *V. variegata* exhibit a relatively low fecundity, perhaps a consequence of the harsh and unpredictable climate that is characteristic of Malagasy rainforests (Bollen and Donati, 2005; Baden *et al.,* 2013). In disturbed forests Balko (1998) and Ratsimbazafy (2002) observed that *V. variegata* individuals had a less variable diet compared to individuals in intact forests, which may cause nutritional stress and, therefore, lowered reproductive potential of affected individuals. Following a cyclone event in the Manombo Special Reserve, Ratsimbazafy (2002) and Louis *et al.* (2005) found that *V. variegata* inter-birth intervals were as long as a decade, and this was attributed to the destruction of most mature fruiting tree species in the area and with that much of the available ripe fruit. Together, evidence of the dietary requirements, life history, and reproductive patterns of *V. variegata* indicate a high sensitivity to habitat degradation in this species.

In addition to anthropogenic habitat loss and degradation hunting presents a significant pressure to *V. variegata* populations (Golden *et al.,* 2009). Hunting is often associated with

decreased population size, disruption of population genetic structure, and changes in age-sex class composition, which together can make populations vulnerable to local extinctions (Cowlishaw and Dunbar, 2000). Coupled with their slow reproductive replacement and irregular reproductive cycles, disruptions to age-sex class composition and population genetic structure could very likely lead to population instability and collapse in *V. variegata*. Because of their large body size *V. variegata* are preferentially and unsustainably targeted by hunters (Golden, 2009), both of which may severely impact the affected populations' ability to thrive. Their loud alarm calls that can be heard up to approximately one kilometer in distance, contributing to hunter's ability to locate groups of this species. Through their assessment of call distributions throughout the day, Geissmann and Mutschler (2005) suggest that these loud calls are related to spatial organization between groups and/ or serve to alarm other individuals of potential threats. It is unclear if *V. variegata* adapt their call patterns in locations subject to high levels of hunting, but it is a possibility as individuals appear more cryptic in these areas (Mancini, personal observation).

One behavioral characteristic of *V. variegata* that may offset some of the deleterious effects of anthropogenic activities is their fission-fusion social system (Baden *et al.*, 2016). Fission-fusion dynamics allow species to cope with changes in the abundance and quality of their food resources by participating in a temporally and spatially fluid social system (Aureli *et al.*, 2008). This social system enables groups to adjust to dynamic changes in resource availability without compromising the benefits of large group living (such as territory defense). By adjusting group size and structure relative to resource abundance and composition, *V. variegata* may persist longer than expected in an area even as resources become less reliable due to environmental stochasticity and anthropogenic pressures. Additionally, *V. variegata* participate

in a form of allomaternal care known as créching, in which mothers park their infants in communal nests to share the burden of infant care following parturition (Baden, 2011; Baden *et al.*, 2013). These communal nests, or crèches, allow females to spend more time foraging and increase the chance of offspring survival (Baden *et al*., 2013). Maximizing resource acquisition and reproductive success by participating in créching behavior may promote population persistence in areas subject to resource depression. Although fission-fusion dynamics and créching may buffer the effects of habitat degradation, both traits are likely evolved in response to environmental stochasticity and therefore their potential to buffer anthropogenic influences may be limited when environmental impacts are large (as in Ratsimbazafy, 2002).

Conservation and population genetics of black-and-white ruffed lemurs

As anthropogenic habitat disturbance in Madagascar continues (Green and Sussman, 1990; Harper *et al.*, 2007; Grinand *et al.*, 2013) the landscape becomes increasingly heterogeneous and fragmented, which likely impedes species' ability to thrive in affected areas as a result of decreased habitat quality or an inability to adequately disperse between adjacent populations. When dispersal between two populations is restricted or eliminated, it can lead to smaller effective population sizes, increased inbreeding, decreased genetic variability, and reduced gene flow (Olivieri *et al.*, 2008; Radespiel *et al.*, 2008; Craul *et al.*, 2009; Dixo *et al.*, 2009; Holmes *et al.*, 2013). Increases in inbreeding and the loss of genetic diversity can lead to an increased susceptibility to disease (Spielman *et al*., 2004) and a lowered immunocompetence (Hale and Briskie, 2007). Furthermore, habitat degradation can cause a reduction in species abundance in highly disturbed regions (Herrera *et al*., 2011). Together, the negative impacts that habitat degradation, fragmentation, and loss have on species diversity, abundance, genetic variability, and health highlight the need for conservation work to help deter these effects in

endangered taxa.

Effective conservation management relies on a clear and agreed upon definition of 'species,' as well as the identification of the unit within a species in which conservation activities will occur. The biological species concept (BSC)- in which species exhibit reproductive isolation, have independent evolutionary fates, and show no serious signs of outbreeding depression- is arguably the most appropriate definition of 'species' for conservation management purposes (Frankham, 2010). Within a species, however, there is still the need to identify specific units within which to properly and effectively manage said species. These management units (MUs) are best defined as entities whose population dynamics are largely influenced by local birth and death rates, as opposed to immigration, making them demographically independent from other groups within the same species (Palsbøll *et al.,* 2006). The identification of MUs is crucial for successful conservation projects as they typically delineate the appropriate size and composition of groups for effectively regulating the impacts of anthropogenic activities on a population, as well as aid in developing monitoring programs (Schwartz *et al.,* 2006).

One promising conservation tool is the use of genetic techniques to monitor and address the genetic factors that influence a species' extinction risk, as well as help optimize management regimes to mitigate these risks (Frankham, 2003; Schwartz *et al.,* 2006). There are several issues within conservation biology that deal directly with genetic factors, including inbreeding depression, population fragmentation, decreased ability to evolve in response to an environmental change due to loss of genetic variability, genetic drift overriding natural selection, and the accumulation of deleterious alleles (Frankham, 2003). The monitoring of a species or MUs for conservation purposes requires managers to quantify the changes in population genetic parameters and data overtime, with an emphasis on this temporal dimension (Schwartz *et al.,*

2006). This differs from assessments, which only capture these genetic parameters at a single point in time and are generally considered less effective for conservation of natural populations in the long-term compared to continual monitoring. Nevertheless, population genetic assessments are a crucial starting point for initiating monitoring programs as they are a useful measure for determining the appropriate MUs or initial state of an MU (Schwartz *et al.*, 2006), although are not necessarily the only measure that should be taken into account (Taylor and Dizon, 1999). Statistical analyses like Bayesian methods and multivariate techniques can be utilize to identify distinct genetic clusters (Pritchard *et al.*, 2000; Jombart *et al.*, 2010), as well as identify and model the impacts that environmental and anthropogenic stressors have on populations (Ellison, 1996). These inferences can be used to streamline and clarify results for policy makers and generate sound ecological and environmental management.

Because of the critical role *V. variegata* plays in seed dispersal and maintenance of rainforest structure, coupled with their sensitivity to habitat degradation, several studies have attempted to quantify the genetic effect of habitat degradation and fragmentation on this species, as well as identify areas of conservation priority. Previous studies of the genetic diversity and structure in *V. variegata* have found evidence for low haplotype diversity, low genetic variation, genetic isolation, and recent population declines (Wyner *et al.*, 1999; Louis *et al.*, 2005; Holmes *et al.*, 2013; Baden *et al.*, 2014). However, using a comparative genomic framework Perry *et al.* (2013) found some of the highest levels of genetic diversity in *V. variegata* compared to other primate species.

Recent studies by Baden *et al*. (2014) and Holmes *et al*. (2013) utilized population genetic analyses to identify variability and structure at a species-wide scale and in regards to regional fragmentation, respectively. In their species level analysis, Baden *et al*. (2014) used data

collected from nineteen sites throughout the remaining *V. variegata* habitat range in the eastern forest corridor to assess genetic variability, population differentiation, and possibility of genetic bottlenecks in this species. Through a Bayesian cluster analysis, the authors found significant evidence of population differentiation between *V. variegata* groups located north and south of the Mangoro River (a large river that bisects the eastern escarpment near the center), indicating that this river was a significant barrier to gene flow. In addition, the southern cluster could be further sub-structured into two more clusters insinuating that a significant level of population differentiation may be occurring in the southern portion of *V. variegata*'s range. Assessments of genetic variability revealed that the northern cluster exhibited greater genetic diversity (higher levels of allelic diversity, greater genetic and haplotype diversity, and higher levels of gene flow) than the southern cluster, suggesting that greater historic connectivity in the northern region compared to the south enabled increased gene flow in the region. Taken with the structure data, the lower levels of genetic diversity south of the Mangoro River indicate that the southern portion of the *V. variegata* range may be a conservation target for future work. It should be noted that the higher levels of genetic diversity in the northern cluster are seen despite the greater levels of illegal hunting and logging in the region (Jenkins *et al*., 2011; Allnut *et al*., 2013). This could imply that 1) hunting and selective logging have a lesser impact on observed genetic variability and structuring than fragmentation or 2) results are indicative of historic structure and variability due to a delayed genetic response to environmental changes (Loehle and Li 1996). Finally, Baden *et al*. (2014) did not witness any compelling evidence for population declines through their bottleneck analyses.

To assess the implications that habitat fragment and isolation have on genetic signatures within *V. variegata*, Holmes *et al*. (2013) assessed genetic variability, population differentiation,

and the presence of genetic bottleneck events at a regional scale. Through their analysis of the Ranomafana and Kianjavato regions, the authors found support for three genetic clusters that consisted of a continuous forest (Ranomafana National Park; RNP), a large forest fragment (Kianjavato), and a small forest fragment (Vatovavy). The clustering of individuals at RNP into one genetic population differs from the findings of Radespiel *et al*. (2008), who identified significant structuring of another lemur taxon (golden-brown mouse lemur; *Microcebus ravelobensis*) within a continuous forest region. Holmes *et al*. (2013) concluded that this discrepancy may be a result of either the patchy distribution exhibited by *V. variegata* or the limited number of sampling sites (two) used within Ranomafana National Park. The authors found significant genetic differentiation between Vatovavy and both Kianjavato and RNP, as well as higher genetic diversity in both the contiguous tract of forest and the larger fragment compared to the smallest fragment. Similar patterns of differentiation between isolated forest fragments have been seen in other primate species (Olivieri *et al* 2008; Radespiel *et al* 2008; Quéméré *et al* 2010). Finally, Holmes *et al*. (2013) identified evidence of a recent population decline through a bottleneck analysis in the smallest and most isolated fragment. Their results suggest that with increasing habitat fragmentation and isolation a reduction in genetic diversity of *V. variegata* populations is likely to occur as effective population size diminishes and inbreeding increases.

Much of the conservation genetic work done today focuses on fragmented landscapes and its implications for species viability, but to ensure the persistence of species well into the future identifying the viability of populations within continuous habitat is equally important. Given the high levels of deforestation in Madagascar, identifying the degree to which the remaining contiguous habitat is sufficient, especially when that habitat is or historically was subject to

degradation, will elucidate the efficiency with which conservation work can be implemented in more fragmented forests. If remaining continuous forest proves insufficient to support species diversity, genetic diversity, etc., then conservation intervention may prove more useful in these regions compared to fragmented forests, as populations residing in the continuous forest have a better chance for ensuring the long-term persistence of a species compared to those found in highly fragmented regions. Therefore, this study investigates at the population genetics of *V. variegata* within a national park (RNP; one the largest remaining in its range) to determine the efficacy with which the park is maintaining gene flow and genetic diversity.

Specific Aims

This study has three specific aims: 1) identify levels of genetic diversity among black-and-white-ruffed lemur (*Varecia variegata*) individuals within Ranomafana National Park (RNP); 2) assess the population genetic structure within RNP; and 3) test for evidence of recent bottleneck events. To achieve these aims I collected genetic fecal samples from 19 adult *V. variegata* individuals at four locations in RNP: Talatakely, Vatorahanana, Valohoaka, and Mangevo and genotyped the samples at nine polymorphic microsatellite loci (Louis *et al*., 2005). These four sites represent a gradient from high levels of historical disturbance (Talatakely) to a pristine rainforest environment (Mangevo), allowing for an assessment of the effects that habitat disturbance have on the genetic variability and structure of *V. variegata* within a continuous, albeit heterogeneous, forest. Results generated from this study are useful to provide insight into the relative efficiency of a protected, contiguous forest site in promoting gene flow and genetic variability in a critically endangered primate species.

METHODS

Study site

Sample and data collection took place at four sites in the southern parcel of Ranomafana National Park (RNP; Figure 1; Table I), located in southeastern Madagascar. RNP is a 41,000 ha tract of contiguous mid-to-low altitude montane rainforest (47°18’ to 47°37’E, 21°02’ to 22°25’S; Wright *et al.*, 2012). Approximately 50% of the national park is characterized as primary forest with the remainder having been subject to some degree of anthropogenic disturbance (e.g. logging and mining). From 1986 to 1989, intensive and selective logging occurred near the main road that bisects the park in what is known locally as Talatakely and Vatorahanana; this logging ceased following the establishment of the park in 1991 (Wright *et al.*, 2012). In addition to this historic anthropogenic disturbance, the forests in this region are affected annually by cyclones that cause intense, natural habitat degradation (Ratsimbazafy, 2002). Until recently, *V. variegata* populations have been absent or found in extremely low densities in the most disturbed regions of RNP (White *et al.*, 1995; Balko and Underwood, 2005; Herrera *et al.*, 2011; Wright *et al.*, 2012). Along with Marotandrano (42,200 ha), Ambatovaky (60,050 ha), and Zahamena (42,300 ha), RNP serves as one the largest remaining protected areas within *V. variegata*’s range, making this tract of forest crucial for the long-term conservation of this species.

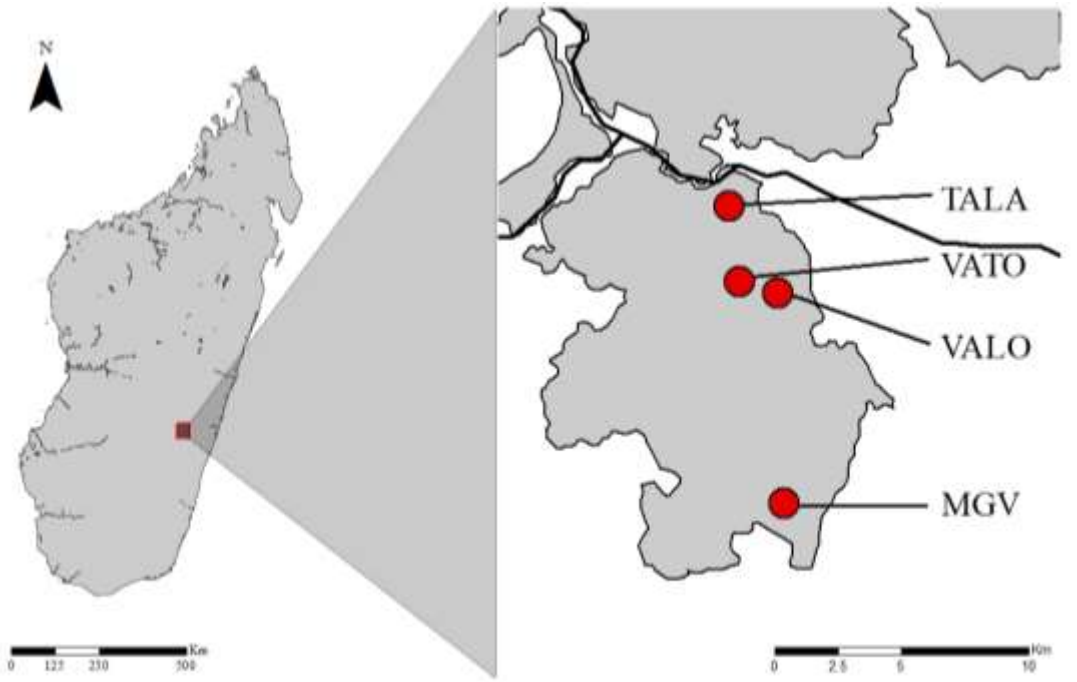

Figure 1. Map of sampling localities in Ranomafana National Park: Talatakely (TALA), Vatorahanana (VATO), Valohoaka (VALO), and Mangevo (MGV). GIS layers courtesy of Conservation International, Gerber (2010), Kremen *et al.* (2008), and Hijmans *et al*. (2004).

Table I. Sampling localities, geographic coordinates, and sample sized used in this study.

Site name	**Site code**	**Latitude**	**Longitude**	***n***
Talatakely	TALA	S 21.2662 °	E 047.4255 °	3
Vatorahanana	VATO	S 21.2932 °	E 047.4282 °	5
Valohoaka	VALO	S 21.2969 °	E 047.4426 °	5
Mangevo	MVG	S 21.3722 °	E 047.4449 °	6
Overall				19

Sample collection

I sampled from 19 *V. variegata* adult individuals from four localities (distance between sites range from 1.55 to 11.91 km; Figure 1; Tables I and II) from June to August 2015 in the southern parcel of Ranomafana National Park (RNP), Madagascar. All samples were collected noninvasively with the assistance of field technicians from the Centre ValBio Research Station (CVB). We conducted targeted sampling of *V. variegata* by walking existing trails on foot and

listening for vocalizations/ detecting movements in the canopy. Following detection, individuals were localized and 1 to 4 samples were collected from all *V. variegata* individuals present. Fecal samples were collected directly following defecation and preserved in silica crystals. Samples were kept at room temperature for 15-85 days until transport to the Primate Molecular Ecology Lab (PMEL; Hunter College of the City University of New York, New York, NY), where they were stored at 4°C.

Table II. Euclidean distance (kilometers) and genetic differentiation (F_{ST}) between sampling localities. Distances are shown above the diagonal and F_{ST} values are shown below. Significant comparisons (those below the Bonferroni-adjusted 5% significance level for multiple comparisons at $p = 0.0083$) are in bold.

	Talatakely	Vatorahanana	Valohoaka	Mangevo
Talatakely	-	3.00	3.83	11.91
Vatorahanana	0.083	-	1.55	8.92
Valohoaka	0.16	0.013	-	8.34
Mangevo	0.009	0.032	**0.127**	-

No direct contact or interaction was made with any *V. variegata* individuals during the course of this study. Sample collection and export/ import protocols adhered to and were approved by the Hunter College of the City University of New York Institutional Animal Care and Use Committee (IACUC # 4/18-01) and Madagascar National Parks (Permit: 2015 N° 126/15/MEEMF/SG/DGF/DCB.SAPT/SCBT).

DNA extraction and microsatellite genotyping

Total genomic DNA was extracted using QIAamp DNA Stool Mini Kits (QIAGEN, Valencia, CA) following the manufacturer's protocol with one modification (an extended 65 hour incubation period). Extracts were quantified and tested for impurities using nuclear DNA

quantitation and melt analysis on a Rotor-Gene Q real-time PCR system with the Rotor- Gene SYBR Green PCR Kit (QIAGEN, Valencia, CA). Extracts were run in duplicate using 2.5 μL in a total reaction volume of 25 μL following the manufacture's protocol. To estimate concentration in extracts, a standard curve was generated using Male Human Genomic DNA at known quantities (15ng, 5ng, 500pg, 50pg, and 5pg). Primers utilized were from the 81 base pair portion of the single copy *c-myc* gene that allows for specific targeting of primate DNA (Morin *et al.*, 2001). Cycling profile were as follows: initial denaturing at 95°C for 5 minutes, 50 cycles of 95°C for 5 seconds (denaturing step) and 60°C for 10 seconds (combined extension and annealing step), and ramping from 75°C to 90°C increasing 0.2°C every cycle with a 5 second hold (melt-curve step).

A suite of nine microsatellite markers that have been shown to reliably amplify fecal DNA (Appendix I; Louis *et al.*, 2005; Baden *et al.*, 2014) were used to genotype all individuals. All extraction products were amplified in a 13 μL volume reaction using Hot Star Taq DNA polymerase Master Mix, 20 mg/ mL BSA, 10 μM primer pairs, and 3 μL (0.25-1 ng) of DNA template. DNA fragments were analyzed on a ABI 3730xl Genetic Analyzer (Applied Biosystems) where all amplification products were separated and alleles sized through comparison to an internal standard (ROX-500) using GENE Marker Software v.4.0 (SoftGenetics). All allele calls were made by eye and checked for consistency across plates using the same sample or individual. Final genotype assignment was based on multiple independent reactions depending on concentration in which two replicates were used to confirm heterozygotes and five were used to confirm homozygotes. (as in Morin *et al.*, 2001).

Population genetic analyses

Genetic diversity

To test for the presence of null alleles, I used the program MICRO-CHECKER (van Oosterhout *et al.,* 2004). Two microsatellite markers were found to have evidence of null alleles (51HDZ20 and 51HDZ691) and were therefore discarded from further analyses; the remaining seven loci were used for the following analyses. To assess the efficacy of the remaining markers to adequately distinguish individuals, I calculated $P_{(ID)}$ and $P_{(ID)}$sibs using the program Cervus v.3.0.7 (Kalinowski *et al.,* 2007). $P_{(ID)}$ estimates the power of a suite a markers in differentiating between randomly selected individuals, while $P_{(ID)}$sibs represents the power of differentiating between sibling pairs and is therefore a more conservative measure (Waits *et al.,* 2001). All loci pairs were tested for linkage disequilibrium in the program GENEPOP v.4.2 using 100 batches of 10,000 iterations following a 10,000 iteration dememorization phase (Raymond and Rousset, 1995).

I used the program GENODIVE (Meirmans and van Tienderen, 2004) to calculate measures of genetic diversity, including number of alleles per locus (n_A), mean number of alleles per locus (MNA), allelic richness (AR), and observed (H_O) and expected (H_S) heterozygosities, at each of the four sampling locations as well as with all data pooled together. Because of uneven sampling between sites, allelic richness (AR) was standardized to the smallest sample size using rarefaction implemented in the program HP-Rare 1.1 (Kalinowski, 2005). Lastly, deviations from Hardy-Weinberg equilibrium were assessed by estimating Wright's F_{IS}, according to Weir Cockerham (1984), and tested for significant deviations using 10,000 permutations in the program FSTAT v.2.9.3 (Goudet, 1995; Goudet 2001).

Genetic differentiation and population genetic structure

To assess the relationship among the four sampling localities I used the program GENODIVE (Meirmans and van Tienderen, 2004) to generate a pairwise matrix of genetic distances using Wright's fixation index, F_{ST} (Weir and Cockerham, 1984). F_{ST} is a measure of genetic differentiation and is useful in determining whether and to what extent two populations are considered distinct genetic populations. One of the underlying assumptions of F-statistics is that populations are in migration-drift equilibrium, although a violation of this assumption may lead to patterns indicative of historic rather than contemporary equilibria (Whitlock and McCauley, 1999).

To infer population genetic structure among individuals included in my sample, I first used a model-based Bayesian clustering method performed in STRUCTURE v2.3.4 (Pritchard *et al.*, 2000). Using a Markov Chain Monte Carlo (MCMC) approach, this method groups individuals into an optimal number of populations (*K*) based solely on their multilocus genotypes. No *a priori* information regarding geographic sampling locations is used in this analysis, therefore the identified populations represent purely genetic clusters. Following, a burn-in of 50,000 iterations, I ran 100,000 iterations of MCMC to evaluate the hypotheses K = 1-7, the number of sampling localities plus 3 (Evanno *et al.*, 2005); these parameters were use based on successes in Holmes *et al.* (2013) and Baden *et al.* (2014). For each value of *K*, I performed 20 runs assuming admixture and correlated allele frequencies. The admixture model allows for the estimation of the number of natural genetic clusters and detection of historical population admixture (Falush *et al.*, 2003; Ostrowski *et al.*, 2006). Additionally, fractional membership of individuals within each population (*Q*) was also calculated. I used the program STRUCTURE HARVESTER v.0.6.93 (Earl and vonHoldt, 2012) to identify the most likely number of genetic populations (*K*). This program

calculates the second order rate of change of the likelihood distribution (ΔK), which corresponds to the most pronounced subdivision within the data. The value of K that produced that highest ΔK is inferred as the optimal number of genetic clusters.

I corroborated my STRUCTURE analysis using a Discriminant Analysis of Principle Components (DAPC; Jombart *et al*., 2010) performed using the package *adegenet* in R Studio (Jombart, 2008; RStudio Team, 2015). DAPC is a multivariate approach that was explicitly developed for use with genetic data in order to identify and describe clusters of genetically related individuals. This method uses a set of retained principle components (determined by the user to optimize variance explained) to segregate the data into clusters that maximize between-group variability while also minimizing within-group variability. DAPC is not sensitive to the underlying population genetic model (i.e. the model driving the population structure), and is therefore more flexible than Bayesian clustering methods. The DAPC uses a Bayesian Information Criterion (BIC) to assess the optimal number of clusters in the dataset, with the optimal cluster number exhibiting the smallest BIC value.

Bottleneck analysis

The results obtained from the Bayesian clustering assignments and the DAPC were used to guide a bottleneck analysis. In order to test for signatures of a recent declines in effective population size (N_e), I performed a Wilcoxon signed-rank test comparing observed and expected heterozygosity at mutation-drift equilibrium in the software BOTTLENECK (Cornuet and Luikart 1996; Piry *et al*., 1999). This test was chosen because of its robusticity at small sample sizes (<30) and small number of loci (<20) (Piry *et al.,* 1999). I calculated this test under three mutation models (IAM: infinite alleles model; TPM: two-phase mutation model; SMM: stepwise mutation mode) to account for uncertainty in the underlying mutation model and assess

sensitivity to the model selected. Finally, to qualitatively assess the distribution of allele frequencies in the population I used the mode-shift indicator in BOTTLENECK (Piry *et al.,* 1999). This parameter is used to detect a shift in allele frequencies that often occurs following a genetic bottleneck, during which low frequency alleles are lost at disproportional rate to intermediate-frequency alleles (Luikart *et al.,* 1998).

RESULTS

Genetic diversity

The observed probability of identity ($P_{(ID)}$) was 1.57×10^{-5} and the value for $P_{(ID)}$sibs was 8.37×10^{-3} for the seven loci retained in this study. These values indicate that the 19 individuals identified here can be confidently differentiated from one another using the suite of seven loci described above (Table III). Six of these loci were polymorphic with 3-6 alleles, while the remaining locus (51HDZ204) was dimorphic (Table III). After pooling individuals across the four sampling localities, no evidence for linkage disequilibrium between marker pairs was found. One locus was found to deviate significantly from Hardy-Weinberg equilibrium (51HDZ560; Table IV), although it did not deviate from HWE in any one site specifically and was therefore retained in further analyses. Corrected allelic richness varied from 1.96-2.36 across the sampling localities, while mean corrected allelic richness was 2.31 ± 1.78 when individuals were pooled across sampling sites (Table IV). Evidence of private alleles was low across all four sampling locations (≤ 0.21). The mean observed heterozygosity was 0.666 (± 0.086; mean ± S.E.) and the mean expected heterozygosity was 0.553 (± 0.054). Overall F_{IS} was -0.199 (lower/ upper 95% confidence interval (CI) = -0.329 to – 0.060) and was significantly different than zero. F_{IS} values for the four sampling localities ranged from -0.351 at Talatakely to -0.044 at Vatorahanana (Table IV); only F_{IS} at Mangevo was found to deviate significantly from zero. Finally, mean F_{ST}

over all sampling localities was 0.067 (lower/ upper CI = 0.20 to 0.121) and differed significantly from zero. Only one pairwise comparison (Valohoaka-Mangevo) yielded a significant F_{ST} value, suggesting that most locations were not genetically differentiated (Table II).

Table III. Characteristics of seven polymorphic microsatellite loci amplified for 19 *Varecia variegata* individuals, including number of alleles (n_A), observed (H_O) and expected (H_S) heterozygosity, and deviations from Hardy-Weinberg Equilibrium (HWE) calculated using 10,000 permutations. Significant values ($p < 0.05$) are shown in bold. Markers from Louis *et al.* (2005).

Marker	Size Range	Annealing Temp.	n_A	H_O	H_S	HWE	GenBank
51HDZ25	169-175	54	3	0.375	0.358	0.563	AF468500
51HDZ204	129-139	60	2	0.633	0.465	0.116	AF468503
51HDZ247	249-265	50	6	1.000	0.759	0.055	AF468504
51HDZ560	253-259	52	3	0.875	0.607	**0.016**	AF468508
51HDZ598	201-217	51	3	0.396	0.451	0.423	AF468509
51HDZ790	211-215	50	3	0.533	0.481	0.306	AF468513
51HDZ816	280-292	54	5	0.850	0.751	0.291	AF468514

Table IV. Allelic diversity at each of the four sampling locations, including mean number of alleles (MNA), observed (H_O) and expected (H_S) heterozygosity, inbreeding coefficient (F_{IS}), and deviations from Hardy-Weinberg Equilibrium (HWE) calculated using 10,000 permutations. Significant values ($p < 0.05$) are shown in bold.

Site	N	MNA	AR (SE)	H_O	H_S	F_{IS}	HWE
Talatakely	3	2.143	1.96 (0.229)	0.595	0.440	-0.351	0.147
Vatorahanana	5	3.143	2.36 (0.195)	0.650	0.623	-0.044	0.418
Valohoaka	5	3.000	2.16 (0.207)	0.657	0.536	-0.227	0.063
Mangevo	6	3.143	2.35 (0.216)	0.762	0.596	-0.277	**0.007**
Overall	19	3.571	2.31 (0.178)	0.666	0.553	-	-

Population genetic structure

Using STRUCTURE, I identified only one genetic cluster within Ranomafana National Park. For all values of *K* (1-7), each of the 19 individuals was assigned a 1/*K* probability of

membership to each cluster yielding a "stripy" pattern when visualizing assignment probabilities on a bar graph (Figure 2). Because the STRUCTURE program was unable to assign individuals to one cluster with a greater probability than to the others regardless of the value of *K*, the results suggest that $K = 1$ is the true number of genetic clusters present in the data.

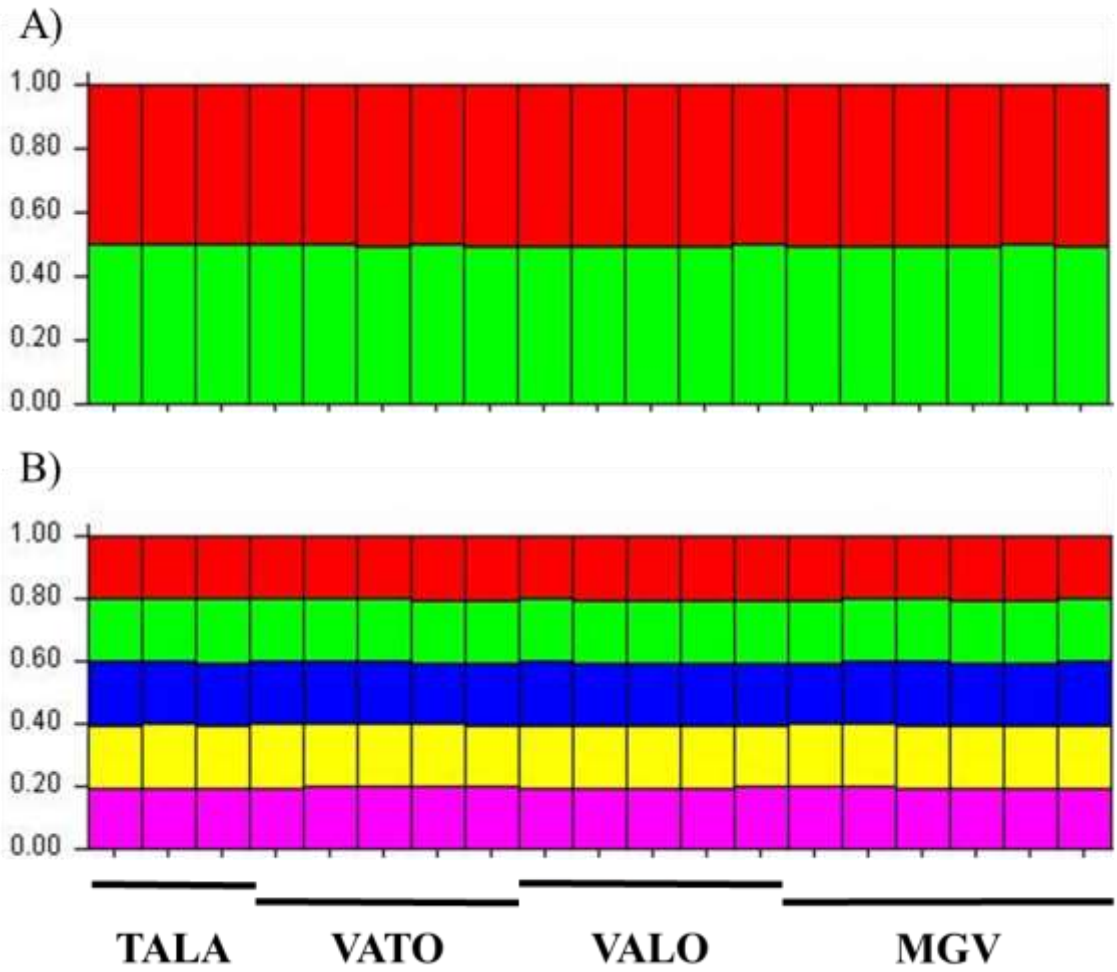

Figure 2. Examples of "stripy" bar plots from STRUCTURE analysis. Bar plots show graphical representation of fractional membership (*Q*) of individuals in A) two clusters and B) five clusters.

Results from the Discriminate Analysis of Principle Components (DAPC) suggest that two genetic clusters may be present in the data (Figure 3), however there was no definitive

geographic association between individuals assigned to Cluster 1 versus Cluster 2. Furthermore, the difference in the Bayesian Information Criterion (ΔBIC) between the $K = 1$ and $K = 2$ was ΔBIC = 2 (Figure 4) which suggests only weak evidence for classifying the data into two versus one clusters (Stylianou *et al*,. 2013).

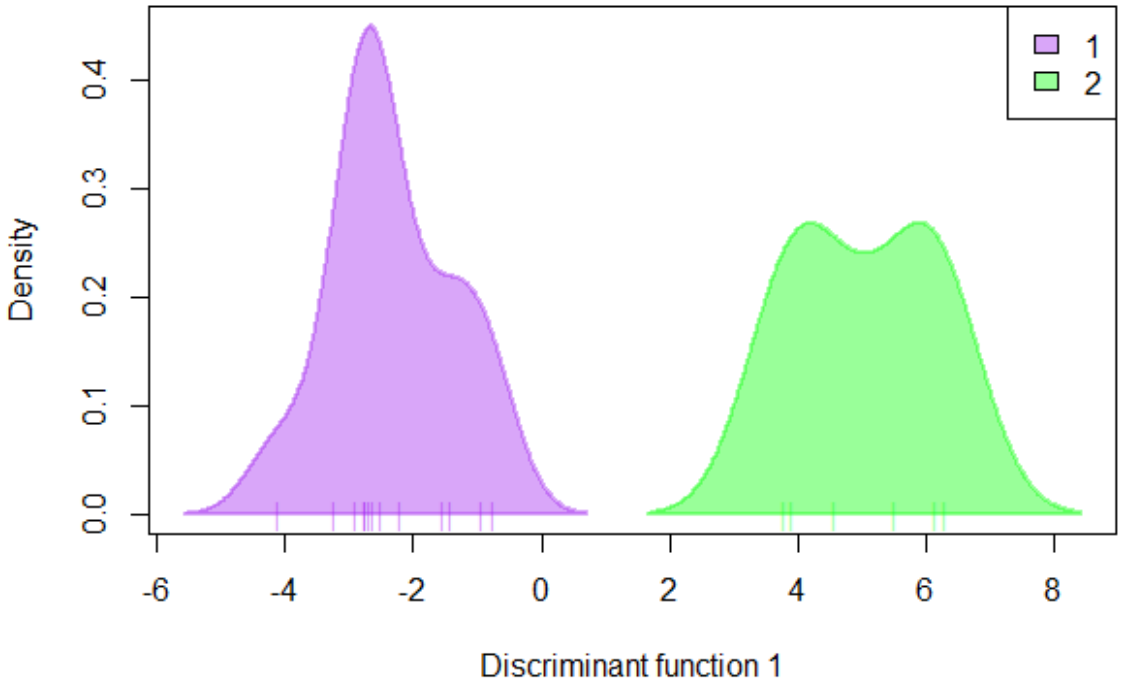

Figure 3. Discriminant Analysis of Principle Components. The graph represents a visualization of the between-group difference from cluster one (purple) to cluster two (green). Cluster one is comprised of all individuals from Talatakely and Mangevo, three individuals from Vatorahanana, and one individual from Valohoaka; cluster two is comprised of two individuals from Vatorahanana and four individuals from Valohoaka.

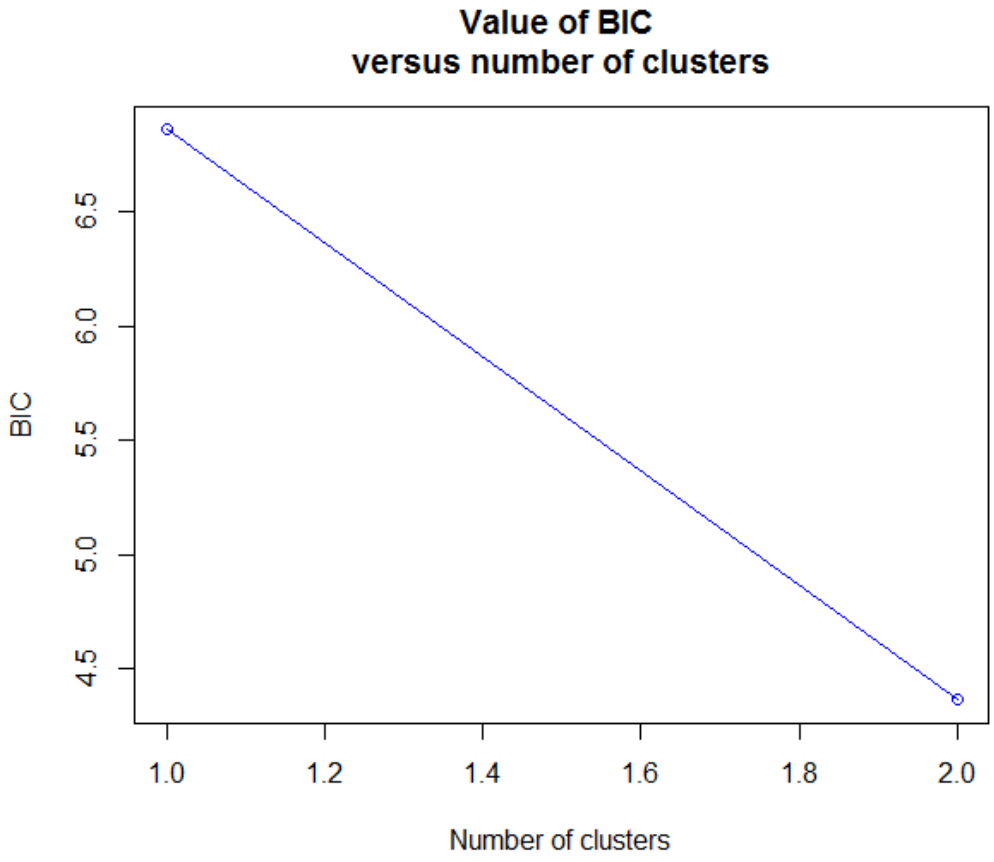

Figure 4. BIC index from the Discriminate Analysis of Principle Components showing the support for one versus two genetic clusters present in the data.

Bottleneck analysis

Following results from the STRUCTURE analysis, I performed a bottleneck analysis using data from all 19 genotypes as one genetic cluster. Analyzing the data as one genetic population yielded significant evidence for heterozygosity excess under all three mutation models (IAM, TPM, and SMM). The probability of heterozygosity excess within Ranomafana National Park was 0.00391, 0.00781, and 0.01953 for the IAM, TPM, and SMM models, respectively. Additionally, the genetic population in RNP displayed a mode-shift which indicates that there is a significant deficit in the abundance of low frequency alleles with respect to the intermediate-frequency alleles.

DISCUSSION

Genetic Differentiation

Together, my analyses suggest that black-and-white ruffed lemur individuals present within Ranomafana National Park comprise one continuous genetic cluster, despite the spatial scale of this study extending nearly 12 km. The results from the STRUCTURE analysis clearly suggest that no genetic substructure is present within Ranomafana National Park. Results obtained from the Discriminant Analysis of Principle Components were less clear with weak evidence for two genetic clusters (Figure 4), although this substructure was not consistent with the geographic clustering of the sampling localities. Given the weak support for two clusters, lack of geographic association of individuals within the clusters, and conflicting results from STRUCTURE analyses, the best supported hypothesis at this time is that Ranomafana National Park consists of only one genetic population.

Pairwise F_{ST} comparisons supported this hypothesis by yielding non-significant results in all but one case, indicating that most sampling localities were not genetically differentiated. Curiously, the one pairwise comparison that did suggest significant differentiation between localities was not between the most distant sampling localities (i.e., Talatakely and Mangevo; 11.91 km apart), but instead was between two more proximally located sites, Valohoaka and Mangevo (8.34 km apart; Table II). Furthermore, Valohoaka was also the most differentiated locality of those compared to Talatakely, albeit not significantly after Bonferroni adjustment. Collectively, these results suggest that although gene flow is occurring across the full extent of the study area there may be a weak dispersal barrier hindering gene flow between Valohoaka and the remaining sites, albeit not effectively isolating the location at this time. However, results should be interpreted with caution as all sample sizes within this study are small, and therefore

may suffer for a lack of robusticity and/or incorrect assumptions of migration-drift equilibrium. Further investigations (e.g., landscape genetic analyses) would be useful in identifying of any potential barriers to gene flow within Ranomafana.

The clustering of all sampling localities into one genetic population within RNP is consistent with the results found in Holmes *et al*. (2013), where individuals from Vatorahanana and Mangevo constituted one genetic population. These results, however, contrast patterns found in Radespiel *et al.* (2008), where significant substructure was found in golden-brown mouse lemurs (*Microcebus ravelobensis*) within contiguous forest in northwestern Madagascar. It is important to note, however, that the spatial scales assessed within this study and in Radespiel *et al.* (2008) differed greatly, as did the life history characteristics of the species assessed. The spatial scale of this study spanned approximately 12 km, while in Radespiel *et al.* (2008) the study area spanned approximately 70 km. Additionally, *V. variegata* are much larger bodied than mouse lemurs (3.5-4.5 kg versus 56-87 g, respectively; Rasoloarison *et al.*, 2000; Andrianntompohavana *et al.*, 2006; Baden *et al.*, 2008), and therefore occupy significantly larger home ranges and likely disperse much greater distances (Radespiel *et al.*, 2008; Baden, 2011). The spatial extent in Radespiel *et al.* (2008) was up to 35 times that of the maximum dispersal distance of *M. ravelobensis*, generating both patterns of isolation-by-distance along with differentiation due to landscape characteristics. Conversely, the spatial scale used within this study may be only minimally larger than the maximum dispersal distance of black-and-white ruffed lemurs. Past censuses in Mangevo identified one collared individual originating from Vatorahanana (Balko, 1998), indicating that *V. variegata* may feasibly disperse across nearly 9 km of forest in one or more dispersal bouts. This ability to disperse among all or the majority of the study area aids in the functional connectivity of this landscape, a pattern that was absent in

Radespiel *et al.* (2008).

The lack of sub-structure found within Ranomafana in this study suggests that higher orders of clustering in black-and-white ruffed lemurs are only observable at a larger spatial scale. Species-level assessments of *V. variegata* population genetic structure suggest that Ranomafana may be a part of a larger genetic cluster comprising localities both north (Fandriana) and south (Manombo) of the park (Baden *et al.,* 2014), despite substantial fragmentation between localities. Baden *et al.* (2014) infer that this may be reflective of historic rather than contemporary structuring due to time lags that often occur between fragmentation and increased genetic differentiation. A clearer delineation of how Ranomafana clusters within the southern portion of the *V. variegata* range requires a larger and more continuous sample size within the park, as well as more continuous sampling both north and south of this region (e.g. in the Andringitra Corridor; Mittermeier *et al.*, 2005).

Genetic diversity

Levels of genetic variability within this study were similar to that found in the Ranomafana region in Baden *et al.* (2014) both prior to and following the rarefaction correction for differences in sample sizes. The uncorrected mean number of alleles was also similar to that found in the Ranomafana region in Holmes *et al*., (2013), although allelic richness was corrected to a sample size of 13 individuals in that study so a direct comparison may not be valid. Furthermore, allelic diversity (i.e. mean number of alleles per locus or MNA) in this study was comparatively low when assessed in reference to that found in other lemur species (Table V). This comparison contradicts Perry *et al.* (2013), which found that *V. variegata* had one of the highest levels of genetic diversity compared to other primate species. These conflicting results may be due to differences in molecular techniques [i.e. microsatellite markers in this study

versus SNPs from RNA sequence data in Perry *et al.* (2013)] utilized in each study.

Additionally, the significant F_{IS} value detected in the Ranomafana cluster suggests that this population contains an excess in heterozygosity compared to allelic richness. Two potential processes that may have resulted in this observed pattern are significant outbreeding of the population or a past genetic bottleneck. Black-and-white ruffed lemurs are absent from the northern parcel of Ranomafana National Park (Wright *et al.,* 2012; Larney, pers. comm.; Herrera, pers. comm.), limiting immigration only from the Andringitra Corridor south of the park. Therefore, given this somewhat limited distribution of *V. variegata* adjacent to the park combined with the results from the bottleneck analysis (see "Bottleneck analysis" discussion below), the latter seems to be a more parsimonious explanation.

Table V. Mean number of alleles assessed from microsatellite data across seven lemur taxa. Minimum and maximum MNA values were calculated within each sampling locality unless otherwise noted. Mean MNA was calculated among all sampling localities. Parentheses indicate MNA was calculated with *K* clusters.

					Sampling locality			
Family	Species	Sample	Marker	*N*	*N*	Min MNA	Max MNA	Mean MNA
Cheirogaleidea	*Microcebus bongolavensis*[1]	Tissue	8 microsats	45	3	3.63	5.00	-
	Microcebus danfossi[1]	Tissue	8 microsats	78	7	2.75	6.63	-
	Microcebus murinus[2]	Tissue	10 microsats	167	3	-	-	18.2
	Microcebus ravelobensis[1]	Tissue	8 microsats	205	8	4.38	6.50	-
	Microcebus ravelobensis[3]	Tissue	8 microsats	187	12	3.83	4.73	-
Indriidae	*Propithicus tattersali*[4]	Feces	13 microsats	82	(3)	(2.69)	(3.99)	6.30
	Propithicus tattersali[5]	Feces	13 microsats	230	9	3.00	6.00	4.92
Lemuridae	*Varecia variegata*[6]	Blood, feces	10 microsats	209	19	2.20	4.70	10.20
	Varecia variegata[7]	Feces	7 microsats	19	4	2.14	3.14	3.57
Lepilemuridae	*Lepilemur edwarsi*[8]	Tissue	14 microsats	20	2	3.86	4.00	-

[1]Olivieri *et al.* (2008)
[2]Fredsted *et al.* (2005)
[3]Radespiel *et al.* (2008)
[4]Quéméré *et al.* (2009)
[5]Quéméré *et al.* (2009)
[6]Baden *et al.* (2014)
[7]This study
[8]Craul *et al.* (2009)

Bottleneck analysis

The bottleneck analysis performed in this study generated significant evidence for a deviation from mutation-drift equilibrium under all three models tested (IAM, TPM, and SMM). One criticism of the approach utilized in the BOTTLENECK software is its use of summary statistics to infer deviations from mutation-drift equilibrium, resulting in a lack of power in the analysis (Felsenstein, 1992). However, when significant signals of population bottlenecks are observed across all three mutation models (as in Gossens *et al.*, 2006), the signal is considered strong enough to be detected by this summary approach. Therefore, evidence from the bottleneck analysis combined with the significantly negative F_{IS} value found in this population suggest that the genetic cluster in Ranomafana National Park has undergone a past genetic bottleneck.

In their study, Holmes *et al*. (2013) found weak evidence for a population bottleneck in the Ranomafana cluster (consisting of individuals from Vatorahanana and Mangevo) under the IAM (an unconstrained model), although no signals were detected under the TPM or SMM. Sample sizes between the two studies were comparable [22 individuals in Holmes *et al.* (2013) and 19 individuals in this study], albeit sample collection between the two studies occurred nearly one decade apart. Therefore, it is possible that the results presented in Holmes *et al.* (2013) are more representative of heterozygosity levels prior to habitat disturbance in the Talatakely-Vatorahanana region due to a time lag in detection of genetic consequences following degradation; samples collected in this study may represent levels of heterozygosity following that disturbance.

Conservation implications

Results from this study indicate that the establishment of Ranomafana National Park has been effective in maintaining continuity of gene flow within the southern parcel. This has

significant implications for the success of future conservation initiatives within and surrounding the national park. Already, reforestation programs are being organized and implemented by the Centre ValBio Research Station near the Ranomafana village on the eastern boundary of the park. Ruffed lemurs are not only one of the most successful seed dispersers in Malagasy rainforests, but are also one of the only lemur species capable of dispersing larger-seeded tree species such as *ramy* (*Canarium sp.*; Martinez and Razafindratsima, 2014; Federman *et al.*, 2016). Therefore, successful gene flow and dispersal capabilities in *V. variegata* is crucial for the proliferation of regenerating forests and forest corridors.

Despite low levels of genetic diversity and evidence of a population bottleneck found in this study, a black-and-white ruffed lemur resident group was found in Talatakely for the first time since logging ceased in 1989 (White *et al*., 1995; Balko and Underwood, 2005; Herrera *et al*., 2011; Wright *et al.*, 2012). Ruffed lemurs are obligate frugivores (Balko, 1998) and therefore are absent from highly disturbed forests due to a decrease in canopy cover, and with that fruit density (White *et al.*, 1995). The presence of a *V. variegata* resident group in Talatakely suggests that this region has recovered sufficiently from past logging to support this obligate frugivore. As *V. variegata* groups continue to colonize the regenerating forests of Talatakely, we may see migration to the neighboring northern parcel of RNP. Since the inception of the park in 1991 no *V. variegata* resident groups have been identified within its' northern parcel, although one individual was spotted during transects near the site of Miaranony (Herrera, pers. comm.). Finally, the expansion of *V. variegata* resident groups into regenerating forests suggests that ongoing and future reforestation initiatives may experience similar successes.

One caveat to this possible success is that ongoing *tavy* (slash-and-burn agriculture) is occurring at unprecedented rates near the eastern boundary of the park, near Mangevo, and in the

Andringitra Corridor south of the park (Mancini, pers. obs.). Furthermore, illegal hunting is ongoing within the Andringitra Corridor (Mancini, pers. obs.), but whether it is occurring at a sustainable rate is unknown. Combined, the deforestation and hunting occurring around the park may have negative implications for maintaining genetic diversity within and hinder immigration of individuals to RNP, ultimately compromising the stability of the *V. variegata* population present. Considering levels of genetic diversity are already low and evidence of a genetic bottleneck was observed in Ranomafana, it is crucial that these anthropogenic activities be deterred in the near future.

Conclusions

In summary, *V. variegata* individuals within Ranomafana National Park comprise one cohesive genetic cluster. Evidence of relatively low genetic diversity and a population bottleneck may reflect historic logging and habitat degradation in the Talatakely and Vatorahanana regions and/or be driven by continued deforestation around the eastern and southern boundaries of the park. Despite these genetic signatures, a resident *V. variegata* group has been established in the most intensively logged region of the park (Talatakely) for the first time in nearly 25 years. As reforestation projects in the Ranomafana region progress, similar expansions of black-and-white ruffed lemur groups into these regenerating areas seem promising.

LITERATURE CITED

Allnutt TF, Asner GP, Golden CD, and Powell GVN. 2013. Mapping recent deforestation and forest disturbance in northeastern Madagascar. *Tropical Conservation Science* 6:1–15.

Andriaholinirina N, Baden AL, Blanco M, Chikhi L, Cooke A, Davies N, Dolch R, Donati G, Ganzhorn J, Golden C, Groeneveld LF, Hapke A, Irwin M, Johnson S, Kappeler P, King T, Lewis R, Louis EE, Markolf M, Mass V, Mittermeier RA, Nichols R, Patel E, Rabarivola CJ, Raharivololona B, Rajaobelina S, Rakotoarisoa G, Rakotomanga B, Rakotonanahary J, Rakotondrainibe H, Rakotondratsimba G, Rakotondratsimba M, Rakotonirina L, Ralainasolo FB, Ralison J, Ramahaleo T, Ranaivoarisoa JF, Randrianahaleo SI, Randrianambinina B, Randrianarimanana L, Randrianasolo H, Randriatahina G, Rasamimananana H, Rasolofoharivelo T, Rasoloharijaona S, Ratelolahy F, Ratsimbazafy J, Ratsimbazafy N, Razafindraibe H, Razafindramanana J, Rowe N, Salmona J, Seiler M, Volampeno S, Wright PC, Youssouf J, Zaonarivelo J, and Zaramody A. 2010. *Varecia variegata*. The IUCN Red List of Threatened Species. Version 2014.3. <www.iucnredlist.org>. Downloaded on 05 December 2014.

Andrianntompohavana R, Zaonarivelo J, Enberg S, Randriamampionona R, McGuire S, Shore G, Rakotonomenjanahary R, Brenneman R, and Louis EE Jr. 2006. Mouse lemurs of northwestern Madagascar with a description of a new species at Lokobe Special Reserve. *Occasional Papers, Museum of TexasTech University* (259): 1-24.

Aureli F, Schaffner CM, Boesch C, Bearder SK, Call J, Chapman CA, Connor R, Di Fiore A, Cundar RIM, Henzi SP, Holekamp K, Korstjens AH, Layton R, Lee P, Lehman J, Manson JH, Ramos-Fernandez G, Strier KB, and van Schaik CP. 2008. Fission-fusion dynamics: New research frameworks. *Current Anthropology* 49 (4): 627-54.

Baden AL, Brenneman RA, Louis EE Jr. 2008. Morphometrics of wild black-and-white ruffed lemurs (*Varecia variegata*; Kerr, 1972). *American Journal of Primatology* 70: 13-926.

Baden AL. 2011. Communal infant care in black-and-white ruffed lemurs (*Varecia variegata*). PhD Dissertation. Stony Brook University, Stony Brook, NY.

Baden AL, Wright PC, Louis EE Jr, Bradley BJ. 2013. Communal nesting, kinship, and maternal success in a social primate. *Behavioral Ecology and Sociobiology* 67: 1939-50.

Baden AL, Holmes SM, Johnson SE, Engberg SE, Louis EE Jr, Bradley BJ. 2014. Species-level view of population structure and gene flow for a critically endangered primate (*Varecia variegata*). *Ecology and Evolution*. doi:10.1002/ece3.1119.

Baden AL, Webster TH, and Kamilar JM. 2016. Resource seasonality and reproduction predict fission-fusion dynamics in a Malagasy strepsirrhine, *Varecia variegata*. *American Journal of Primatology* 78 (2): 256-79.

Balko EA. 1998. A behaviorally plastic response to forest composition and logging disturbance by *Varecia variegata variegata* in Ranomafana National Park, Madagascar. PhD Dissertation, State University of New York, Syracuse, NY

Balko EA and Underwood HB. 2005. Effects of forest structure and composition on food availability for *Varecia variegata* at Ranomafana National Park, Madagascar. *American Journal of Primatology.* 66: 45-70.

Bollen A and Donati G. 2005. Phenology of the littoral forest of Sainte Luce, southeastern Madagascar. *Biotropica* 37 (1): 32-43.

Brown KA and Gurevitvh J. 2004. Long-term impacts of logging on forest diversity in Madagascar. *Proceedings in the National Academy of Sciences* 101 (16): 6045-9.

Buckland ST, Anderson DR, Burnham KP, and Laake JL. 1993. Distance Sampling: Estimating Abundance of Biological Populations. Chapman and Hall, London. Online.

Burney DA, Burney LP, Godfrey LR, Jungers WL, Goodman SM, Wright HT, and Jull AJT. 2004. A chronology for late prehistoric Madagascar. *Journal of Human Evolution* 47: 25-63.

Cornuet JM and Luikart G. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144: 2001-14.

Cowlishaw G and Dunbar RI. *Primate Conservation Biology*. Chicago: University of Chicago Press, 2000. Print.

Craul M, Chikhi L, Sousa V, Olivieri GL, Rabesandratana A, Zimmermann E, and Radespiel U. 2009. Influence of forest fragmentation on an endangered large-bodied lemur in northwestern Madagascar. *Biological Conservation* 142(12):2861-71.

Crowley BE. 2010. A refined chronology of prehistoric Madagascar and the demise of the megafauna. *Quaternary Science Reviews* 29: 2591-603.

Crowley BE, Godfrey LR, and Irwin MT. 2011. A glance to the past: Subfossils, stable isotopes, seed dispersal and lemur species loss in southern Madagascar. *American Journal of Primatology* 73: 25-37.

Dew JL and Wright PC. 1998. Frugivory and seed dispersal by four species of primates in Madagascar's eastern rain forest. *Biotropica* 30 (3): 425-37.

Di Marco M, Buchanan GM, Szantoi Z, Holmgren M, Grottolo Marasini G, Gross D, Tranquilli S, Boitani L, and Rondinini C. 2014. Drivers of extinction risk in African mammals: the interplay of distribution state, human pressure, conservation response and species biology. *Philosophical Transactions of the Royal Society B* 369 (1643): 20130198.

Dixo M, Metzger JP, Morgante JS, and Zamudio KR. 2009. Habitat fragmentation reduce genetic diversity and connectivity among toad populations in the Brazilian Atlantic Costal Forest. *Biological Conservation* 142:1560-9.

Earl DA and von Holdt BM. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics* 4: 359-61.

Ellison AM. 1996. An introduction to Bayesian inference for ecological research and environmental decision-making. *Ecological Applications* 6 (4): 1036-46.

Evanno G, Regnaut S, and Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 14: 2611-20.

Falush D, Stephens M, and Pritchard JK. 2003. Inference of population structure: Extensions to linked loci and correlated allele frequencies. *Genetics* 164: 1567-87.

Federman S, Dornburg A, Daly DC, Downie A, Perry GH, Yoder AD, Sargis EJ, Richard AF, Donoghue MJ, and Baden AL. 2016. Implications of lemuriform extinctions for the Malagasy flora. *Proceedings of the National Academy of Sciences* doi:10.1073/pnas.1523825113

Felsentein J. 1992.Estimating effective population size from samples of sequences: Inefficiency of pairwise and segregating sites as compared to phylogenetic estimates. *Genetics Research* 59: 139-47.

Frankham R. 2003. Genetics and conservation biology. *Comptes Rendus Biologies* 326: S22-9.

Frankham R. 2010. Challenges and opportunities of genetic approaches to biological conservation. *Biological Conservation* 143:1919–1927.

Fredsted T, Pertoldi C, Schierup MH, and Kappeler PM. 2005. Microsatellite analyses reveal fine-scale genetic structure in grey mouse lemurs (*Microcebus murinus*). *Molecular Ecology* 14: 2363-72.

Ganzhorn JU, Lowry PP, Schatz GE, and Sommer S. 2001. The biodiversity of Madagascar: one of the world's hottest hotspots on its way out. *Oryx* 35(4):346-8.

Geissmann T and Mutschler T. 2006. Diurnal distribution of loud calls in sympatric wild indris (*Indri indri*) and ruffed lemurs (*Varecia variegata*) : implications for call functions. *Primates* 47 : 393-396.

Gerber BD. 2010. Madagascar GIS: spatial information to aid research and conservation. http://filebox.vt.edu/users/bgerber/services.htm. Accessed 02 April 2016

Godfrey LR, Jungers WL, Schwartz GT, and Irwin MT. "Ghosts and Orphans: Madagascar's Vanishing Ecosystem" in *Elwyn Simons: A Search for Origins*, eds Fleagle JG and Gilbert CC. New York: Springer, 2008. pp 361–95.

Golden CD. 2009. Bushmeat hunting and use in the Makira Forest north-eastern Madagascar: a conservation and livelihood issue. Oryx 43: 386-92.

Gossens B, Setchell JM, James SS, Funk SM, Chikhi L, Abulani A, Ancrenaz M, Lackman-Ancrenaz I, and Bruford MW. 2006. Philopatry and reproductive success in Bornean orang-utans (*Pongo pygmaeus*). *Molecular Evolution* 15 (9): 2577-88.

Goudet J. 1995. FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity* 86 (6): 485-6.

Goudet J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). University of Lausanne, Lausanne. Available from http://www.unil.ch/dee/ page6767_en.html

Green GM and Sussman RW. 1990. Deforestation history of the eastern rain forests of Madagascar from satellite images. *Science* 248 (4952): 212-5.

Grinand C, Rakotomalala F, Gond V, Vaudry R, Bernoux M, and Vieilledent G. 2013. Estimating deforestation in tropical humid and dry forests in Madagascar from 2000 to 2010 using multi-date Landsat satellite images and the random forests classifier. *Remote Sensing of Environment* 139: 68–80

Haig AR, Matthes U, and Larson DW. 2000. Effects of natural habitat fragmentation on the species richness, diversity, and composition of cliff vegetation. *Canadian Journal of Botany* 78:786-97

Hale KA and Briskie JV. 2007. Decreased immunocompetence in a severely bottlenecked population of an endemic New Zealand bird. *Animal Conservation* 10: 2-10.

Harper GJ, Steininger MK, Tucker CJ, Juhn D, and Hawkins F. 2007. Fifty years of deforestation and forest fragmentation in Madagascar. *Environmental Conservation* 34: 1-9.

Herrera JP, Wright PC, Lauterbur E, Ratovonjanahary L, and Taylor LL. 2011. The effects of habitat disturbance on lemurs at Ranomafana National Park, Madagascar. *International Journal of Primatology* 32:1091-1108.

Hijmans, R.J., Guarino, L., Bussink, C., Mathur, P., Cruz, M., Barrentes, I. *et al*. 2004. DIVA-GIS. Vsn. 5.0. A geographic information system for the analysis of species distribution data. (manual available at: http://www.diva-gis.org).

Holmes SM, Baden AL, Brenneman RA, Engberg SE, Louis EE Jr, Johnson SE. 2013. Patch size and isolation influence genetic patterns in black-and-white ruffed lemur (*Varecia variegata*) populations. *Conservation Genetics* 14: 615-24.

Irwin MT, Johnson SE, and Wright PC. 2005. The state of lemur conservation in southeastern Madagascar: Population and habitat assessments for diurnal and cathemeral lemurs using surveys, satellite imagery and GIS. *Oryx* 39:204-18.

Jenkins RKB, Keane A, Rakotoarivelo AR, Rakotomboavonjy V, Randrianandrianina FH Razafimanahaka HJ, *et al*. 2011. Analysis of patterns of bushmeat consumption reveals extensive exploitation of protected species in eastern Madagascar. *PLoS ONE* 6: e27570. doi:10.1371/journal.pone.0027570.

Johns AD. 1988. Effects of "selective" timber extractions on rain forest structure and composition and some consequences for frugivores and folivores. *Biotropica* 20 (1): 31-7.

Jombart T. 2008. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 24: 1403-5. doi: 10.1093/bioinformatics/btn129

Jombart T, Devillard S, and Balloux F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics* 11: 94.

Kalinowski ST. 2005. HP-Rare: a computer program for performing rarefaction on measures of allelic diversity. *Molecular Ecology Notes* 5:187-9.

Kalinowski ST, Taper ML, and Marshall TC. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099-106. http://dx.doi.org/10.1111/j.1365-294x.2007.03089.x

Kremen C, Cameron A, Moilanen A, Phillips SJ, Thomas CD, Beentje H, Dransfield J, Fisher BL, Glaw F, Good TC, Harper GJ, Hijmans RJ, Lees DC, Louis EE Jr., Nussbaum RA, Raxworthy CJ, Razafimpahanana A, Schatz GE, Vences M, Vieites DR, Wright PC, Zjhra ML. 2008. Aligning conservation priorities across taxa in Madagascar with high-resolution planning tools. *Science* 320: 222-5.

Loehle C and Li BL. 1996. Habitat destruction and the extinction debt revisited. *Ecological Applications* 6 (3): 784-9.

Louis EE Jr., Ratsimbazafy JH, Razakamaharauo VF, Pierson DJ, Barber RC, and Brenneman RA. 2005. Conservation genetics of black and white ruffed lemurs, *Varecia variegata*, from Southeastern Madagascar. *Animal Conservation* 8:105-11

Luikart G, Allendorf FW, Cornuet JM, and Sherwin WB. 1998. Distortion of allele frequency distributions provides a test for recent population bottlenecks. *The Journal of Heredity* 89 (3): 238-47.

Martinez BT and Razafindratsima OH. 2014. Frugivory and seed dispersal patterns of the red-ruffed lemur, *Varecia rubra*, at a forest restoration site in Masoala National Park, Madagascar. *Folia Primatologica* 85: 228-43.

Meirmans PG and van Tienderen PH. 2004. GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792-4.

Mittermeier RA, Hawkins F, Rajaobelina A, and Langrand O. 2005. Wilderness conservation in a biodiversity hotspot. *International Journal of Wilderness* 11:42–5.

Mittermeier R, Louis EE Jr., Richardson M, Schwitzer C, Langrand O, Rylands AB, Hawkins F, Rajaobelina S, Ratsimbazafy J, Rasoloarison R, Roos C, Kappele PM, and Mackinnon J. 2010. Lemurs of Madagascar. 3rd Ed. Conervation International, Washington D.C.

Morin PA, Chambers KE, Boesch C, and Vigilant L. 2001. Quantitative polymerase chain reaction analysis of DNA from noninvasive samples for accurate microsatellite genotyping of wild chimpanzees (*Pan troglodytes verus*). *Molecular Ecology* 10: 1835-44.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB, and Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853-8.

Olivieri GL, Sousa V, Chikhi L, and Radespiel U. 2008. From genetic diversity and structure to conservation: Genetic signature of recent population declines in three mouse lemur species (*Microcebus* spp.). *Biological Conservation* 141: 1257-71.

Ostrowski FM, David J, Santoni S, McKhann H, Reboud X, Le Corre V, Camilleri C, Brunel D, Bouchez D, Faure B, and Bataillon T. 2006. Evidence for a large-scale population structure among accessions of Arabidopsis thaliana: possible causes and consequences for the distribution of linkage disequilibrium. *Molecular Ecology* 15 (6): 1507-17.

Pasbøll PJ, Bérubé M, and Allendorf FW. 2006. Identification of management units using population genetic data. *Trends in Ecology and Evolution* 22 (1): 11-6.

Perry GH, Melsted P, Marioni JC, Wang Y, Bainer R, Pickrell JK, Michelini K, Zehr S, Yoder AD, Stephen M, Pritchard J, and Gilad Y. 2013. Comparative RNA sequencing reveals substantial genetic variation in endangered primates. *Genome Research* 22: 602-10.

Piry S, Luikart G, and Cornuet JM. 1999. BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* 90:502–3.

Pritchard JK, Stephens M, and Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics* 155: 945-59.

Quéméré E, Louis EE Jr., Ribéron A, Chikhi L, and Crouau-Roy B. 2009. Non-invasive conservation genetics of the critically endangered golden-crowned sifaka (*Propithecus tattersalli*): high diversity and significant genetic differentiation over a small range. *Conservation Genetics* 11: 675– 84.

Quéméré E, Crouau-Roy B, Rabarivola C, Louis Jr EE, and Chikhi L. 2010. Landscape genetics of an endangered lemur (*Propithecus tattersalli*) within its entire fragmented range. *Molecular Ecology* 19:1606–1621.

RStudio Team. 2015. RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.

Radespiel U, Rakotondravony R, and Chikhi L. 2008. Natural and anthropogenic determinants of genetic structure in the largest remaining population of the endangered golden-brown mouse lemur, *Microsebus ravelobensis*. *American Journal of Primatology* 70: 860-70.

Rasoloarison RM, Goodman SM, and Ganzhorn JU. 2000. Taxonomic revision of mouse lemurs (*Microcebus*) in the western portions of Madagascar. *International Journal of Primatology* 21 (6): 936-1019.

Ratsimbazafy HJ. 2002. On the brink of extinction and the process of recovery: Responses of black-and-white ruffed lemurs (*Varecia variegata variegata*) to disturbance in Manombo Forest, Madagascar. PhD Dissertation, State University of New York, Syracuse, NY

Raymond M and Rousset F. 1995. Genepop (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-9.

Schwartz MK, Luikart G, and Waples R. 2006. Genetic monitoring as a promising tool for conservation and management. *Publications, Agencies and Staff of the U.S. Department of Commerce.* Paper 482.

Schwitzer C, Mittermeier RA, Davies N, Johnson S, Ratsimbazafy J, Razafindramanana J, Louis EE Jr., and Rajaobelia S. 2013. Lemurs of Madagascar: A strategy for their Conservation 2013-2016. IUCN. <www.primate-sg.org>. Downloaded on 05 December 2014.

Schaefer HM and Ruxton GD. *Plant-Animal Communication*. Oxford: Oxford University Press. 2011. Web.

Spielman D, Brook BW, Briscoe DA, and Frankman R. 2004. Does Inbreeding and Loss of Genetic Diversity Decrease Disease Resistance? *Conservation Genetics* 5(4):439-48.

Stylianou C, Pickles A, and Roberts SA. Using Bonferroni, BIC and AIC to assess evidence for alternative biological pathways: covariate selection for the multilevel Embryo-Uterus model. *BMC Medical Research Methodology* 13:73 .

Tavankar F, Majnounian B, and Bonyab AE. 2013. Felling and skidding damage to residual trees following selection cutting in Caspian forests of Iran. *Journal of Forest Science* 59 (5): 196-203.

Taylor BL and Dizon AE. 1999. First policy then science: why a management unit based solely on genetic criteria cannot work. *Molecular Ecology* 8: S11-6.

van Oosterhout C, van Heuven MK, and Brakefield PM. 2004. On the neutrality of molecular genetic markers: pedigree analysis of genetic variation in fragmented populations. *Molecular Ecology* 13:1025-34.

Vasey N and Tattersall I. 2002. Do ruffed lemurs for a hybrid zone? Distribution and discovery of Varecia, with systematic and conservation implications. *American Museum Novitates* 3376:1–26.

Waits LP, Luikart G, and Taberlet P. 2001. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. *Molecular Ecology* 10: 249-56.

Weir BS and Cockerham CC. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358– 70.

White FJ, Overdorff DJ, Balko EA, and Wright PC. 1995. Distribution of Ruffed Lemurs (*Varecia variegata*) in Ranomafana National Park, Madagascar. *Folia Primatologica* 64(3):124-31.

Whitlock MC and McCauley DE. 1999. Indirect measures of gene flow and migration: $F_{ST} \neq 1/(4Nm+1)$. *Heredity* 82: 117–25.

Wright PC. 1999. Lemur traits and Madagascar ecology: coping with an island environment. *Yearbook of Physical Anthropology* 42: 31-72.

Wright PC, Tecot SR, Erhart EM, Baden AL, King, SJ, and Grassi C. 2011. Frugivory in four sympatric lemurs: Implications for the future of Madagascar's forests. *American Journal of Primatology* 73: 585-602.

Wright PC, Erhart EM, Tecot S, Baden AL, Arrigo-Nelson SJ, Herrera J, Morelli TL, Blanco MB, Deppe A, Atsalis S, Johnson S, Ratelolahy F, Tan C, Zohdy S. 2012. Long-term lemur research at Centre Valbio, Ranomafana National Park, Madagascar. In: Kappeler PM, and Watts DP, editors. Long-term field studies of primates. Berlin Heidelberg: Springer p.67-100.

Wyner YM, Amato G, and Desalle R. 1999. Captive breeding, reintroduction, and the conservation genetics of black and white ruffed lemurs, *Varecia variegata variegata. Molecular Ecology* 8: S107-15.

APPENDIX I. Primer sequence, repeat motifs, and annealing temperatures of 9 *Varecia variegata* microsatellite markers from Louis *et al.*, 2005.

Marker	Sequence	Repeat Motif	Annealing Temp
51HDZ20	F: 5'-ATG ACT TGT AGC TTA AAT CCT TTG G-3'	$(CA)_{10}(TA)_5$	50°C
	R: 5'-TAC TTG GCT GAT TCG GGA G-3'		
51HDZ25	F: 5'-GTC AAA CGG GGA AAA TGC-3'	$(TGCACA)_4CACG(CA)_{11}$	54°C
	R: 5'-TCA AAT CGG TAG CTC TCG G-3'		
51HDZ204	F: 5'-AAT CAT GTT TTG TGG GAG GGG-3'	$(CA)_4A(CA)_{12}$	60°C
	R: 5'-GTA TAC CTC ACT GGC TCC CTG C-3'		
51HDZ247	F: 5'-AGG AAG GTA CAC TAA AAC AGA GAC T-3'	$(CA)_{14}$	50°C
	R: 5'-TGT ATC CTC CAT TTA TCT CCT TG-3'		
51HDZ560	F: 5'-CAC TTC TGC CTC CAA TCA CTC-3'	$(GT)_6(GC)_2AC(GT)_6(CTGT)_3$	52°C
	R: 5'-AAC ATC CCG TGG TCA CTA CAG-3'		
51HDZ598	F: 5'-ATT CAG AAG TGT TAC ATT TAC GGA GG-3'	$(CA)_8AGA(CA)_{15}$	50°C
	R: 5'-GAG TGG GTG GCA AGG TTC G-3'		
51HDZ691	F: 5'-CCA TGA CGT TAA TTC CTC TGC-3'	$(CA)_{17}$	50°C
	R: 5'-GCC ACC ATC ACC CAG TTG-3'		
51HDZ790	F: 5'-CCA CCC CAG TCC TGT CCT TA-3'	$(CA)_{10}$	50°C
	R: 5'-TTG TTG CCT CTC TGC CAA GTA G-3'		
51HDZ816	F: 5'-AGA GGC CAC TAC TGA CAA CG-3'	$(CA)_{19}$	52°C
	R: 5'-CCC CCA CAC ACA AAT ACT AAA C-3'		