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Aparna Chandrashekar  
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**Reconstructing the demographic history of the critically endangered black-and-white ruffed lemur (*Varecia variegata*) in Ranomafana National Park, Madagascar**

By

Aparna Chandrashekar

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

2018

Thesis Sponsor:

December 13, 2018

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December 13, 2018

Date

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## **ACKNOWLEDGEMENTS**

I would like to thank my advisor Dr. Andrea Baden for her unending support and guidance throughout my Master's program. She has been instrumental in helping me cultivate my skills as a budding conservation geneticist and in developing this project. In addition, I would like to thank Dr. Carlo Pacioni for his expertise and assistance for running these analyses. His knowledge regarding conservation management has truly made this project excel. I would like to thank my lab mates in the Primate Molecular Ecology Lab, who helped me keep sane through the ups and downs and to Amanda Mancini who was gracious enough to let me help on her project. Lastly, I'd like to express my gratitude towards my family and Louis Thompson. They have supported and encouraged me throughout my academic career.

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## ABSTRACT

Madagascar's moist forests have undergone extensive habitat modification within the last century, with deforestation rates progressively increasing in the last few years. Due to heavy fragmentation of their remaining range, the Critically Endangered black-and-white ruffed lemur (*Varecia variegata*) is facing catastrophic population declines, especially in the southern extent of its range, where genetic diversity is low and gene flow is almost absent. To understand the effects of large-scale landscape change and its influence on the genetic stability of endangered mammalian populations it is fundamental to determine the historical demography of a population. Here, I use microsatellite genotypes from *V. variegata* individuals in Ranomafana National Park to reconstruct their population history. Four sites were sampled within the park's boundaries and a total of 38 adult individuals were identified. Pairwise comparisons of the sampling sites indicated each locality as a distinct population (except for Vatorahanana and Mangevo); however, a Bayesian clustering method suggested evidence for one genetic cluster within the park. Extended Bayesian skyline analyses show that the ancestral effective population was stable during Madagascar's Late Pleistocene and Early Holocene's drastic climatic transformations. Mean ancestral effective population size was estimated to be ~4,000 before declining by 84% starting around 1,500 years ago. Though human arrival happened as early as 10,000 years ago, extensive settling may have begun around 2,300 years ago on the coasts of Madagascar. While archaeological data for the eastern humid rainforest region are sparse, evidence from charcoal abundance and linguistic data indicate that the interior regions of Madagascar were some of the last areas to be settled and this event may have taken up to seven centuries. The emergence of anthropogenic fire in this region, coupled with annual intense

cyclones, may have resulted in habitat modification too great for *V. variegata* to cope with, leading to population declines as seen with the Ranomafana population.

## **INTRODUCTION**

### **Natural History of Madagascar**

Madagascar is one of the most prominent biodiversity “hotspots” in the world due to its high levels of species endemism; unfortunately, the island is also under critical threat due to severe and extensive forest loss (Myers *et al.*, 2000). The separation from Africa 165 million years ago, and then from India 70 million years (Rakotosamimanana, 2003), has led to the unique biota found in Madagascar. However, the island has suffered from extreme habitat modification in the last millennia (Green and Sussman, 1990; Harper *et al.*, 2007; Vieilledent *et al.*, 2018), leading to the extinction of several species (Dewar, 2003; Burney *et al.*, 2004). The intense habitat loss and species extinctions are attributed to the extensive settling of humans on the island around 2,000 years ago (Burney *et al.*, 2004; Crowley 2010). Though the dominant narrative argues for heavy anthropogenic influence on environmental degradation, some suggest that past climatic fluctuations and vegetation changes prior to human arrival played a major role in shaping Madagascar’s biomes and fauna populations (Dewar and Wright 1993; Quémère *et al.*, 2012; Salmona *et al.*, 2017).

#### *Pre-Colonization (20,000-10,000 years ago)*

Madagascar’s climate history is complex owing to its long isolation from Africa millions of years ago. The earliest dated evidence for climate and vegetation change comes from Lake Tritrivakely in the highlands (Burney, 1987a; Burney *et al.*, 2004). The evidence from this site shows that much of Madagascar’s vegetation during the Pleistocene era, down to elevations of



around 1,000 meters, was ericoid bushland with composites and grasses (Burney, 1987a; Burney *et al.*, 2004). The island's humid forest zones were confined to small areas near the east coast, with isolated patches elsewhere (Burney, 1996; Burney *et al.*, 2004). Pollen, sedimentology, and paleolake-level evidence suggest that the climate at the Last Glacial Maximum was cool (mean temperature  $>4^{\circ}\text{C}$  colder than the present) and very dry (Burney, 1996; Burney *et al.*, 2004). Approximately 17,000 years before present saw the partial replacement of ericoid bush by savanna and woodland in the highlands, as well as deglaciation marked by warming conditions (Burney *et al.*, 2004). By 9,800 years BP Holocene type vegetation, most likely a mosaic of wooded grassland and woodland, had replaced the Pleistocene vegetation in the highlands (Burney *et al.*, 2004). Climatic desiccation, intense climatic dryness, occurred in the southwest around 4,000 years BP, which continued through the rest of the Holocene (Burney *et al.*, 2004). In addition to the climate changes occurring in the late Pleistocene and early Holocene era, pre-human Madagascar was subject to regular natural fire (Burney, 1987a, 1996). The prevalence of fire was limited to the central highlands and to the west, with scarcity in the east, north, and southwest regions due to these regions being either too wet or too dry to support the frequency of fires (Burney, 1996).

#### *Beginning of Human Arrival and Colonization (10,000-2,000 years ago)*

Multiple lines of evidence suggest that Madagascar went through waves of human migration from all corners of the Indian Ocean (Dewar and Wright, 1993; Serva *et al.*, 2012; Pierron *et al.*, 2014). However, there is no consensus as to when humans began settling on the island. The dominant narrative suggests that humans first settled in Madagascar around 2,000 years ago (Burney 1987b; Dewar and Wright, 1993; Perez *et al.*, 2003). Studies of the Malagasy language show a separation from its closest surviving linguistic relatives in the highlands of

Borneo about two millennia ago (Dewar and Wright, 1993). The occurrence of human-modified bones was found at the site of Taolambiby dating to  $2,324 \pm 43$  years BP (Perez *et al.*, 2003). Cut marks were found on the radius of the extinct sloth lemur (*Palaeopropithecus ingens*) suggesting that the flesh was removed with a sharp object (Perez *et al.*, 2003). In addition, pollen of the introduced *Cannabis/Humulus* found at Triterivakely in the central highlands was found at an interpolated age of 2,200 years BP (Burney 1987b). However, recent evidence for the timing of first human arrival has challenged this prevalent paradigm. Excavations at Lakaton'i Anja, have discovered small assemblages of microlithic tools dating to  $4,380 \pm 400$  years BP (Dewar *et al.*, 2013). A more recent study (Hansford *et al.*, 2018), provides a timing estimate that predates all other archaeological and genetic evidence by more than 6,000 years. Bones with anthropogenic marks (e.g., cuts and depression fractures) from a single elephant bird (*Aepyornis maximus*) individual were recovered at Christmas River (Hansford *et al.*, 2018). Researchers have dated the bones to 10,721-10,511 year B.P. (Hansford *et al.*, 2018), providing the earliest evidence for human modification on animals, as well as human arrival on Madagascar. These early arrivals could have had little impact on Madagascar's landscape, thus further evidence is needed to clarify the nature of these early settlements. However, the idea that Madagascar had remained untouched by humans until 2,000 years ago is now unsubstantiated (Dewar *et al.*, 2013; Douglass and Zinke, 2015; Hansford *et al.*, 2018).

#### *Settlement extension (2,000-500 years ago)*

It is not yet known from which part of the Indian Ocean the first settlers of Madagascar arrived, but it is suggested that they were most likely traders who lived on the coast because of their involvement in maritime commerce (Dewar, 2003). There is evidence indicating the growth of settlements on the west coast, with the first major port, Mahilaka, appearing on the northwest

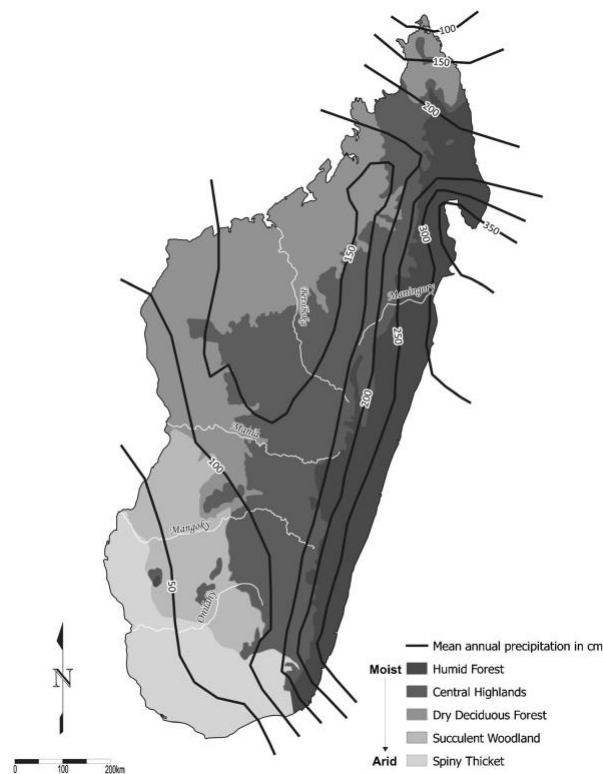
coast around 1150 A.D. (Dewar and Wright, 1993; Wright and Rakotoarisao, 2003). Burney and colleagues (2004) propose that Madagascar was first settled in the semi-arid southwest. Though, pre-existing fire ecology did occur naturally, dating of charcoal spikes after human arrival indicate that fire ecology changed nearly two millennia ago in the southwest (Burney *et al.*, 2004). Continuing this pattern, the dating suggests that the change in fire ecology spread up the west coast to wetter climates and within a century or two later at the higher lake Tritrivakely site (Burney, 1987b). Through charcoal and linguistic data, researchers postulate that humans gradually settled on the other coasts and then central highlands, with the humid forests of the low interior being some of the last areas settled (Wright and Rakotoarisao, 2003; Burney *et al.*, 2004; Serva *et al.*, 2012).

#### *Intensified Population Growth (500 years ago-present)*

Though, human impact on the environment was suggested to be limited for the first 1,100 years, in the last 500 human pressure intensified (Oliveri *et al.*, 2008). Estimates across Madagascar suggest that human population increased from 1.5 Million in 1800 (Campbell, 1991), to 4.5 Million in 1955, 11.5 Million in 1992, and 26.5 Million in 2018 (<http://www.worldometers.info-/world-population/madagascar-population/>). Pollen evidence shows that there was a drastic decline of coprophilous *Sporormiella* fungus spores, a proxy for megafaunal density (Burney *et al.*, 2004). Charcoal particle values increase by one to two orders of magnitude above background, indicating a higher frequency of fires affecting Madagascar's habitat and suggesting deforestation in the lowland rainforests starting around 1,000 A.D. (Burney *et al.*, 2004).

The complexities of Madagascar's biological and cultural geography make constructing a coherent narrative of the anthropogenic effects on the environment and fauna difficult (Douglass

and Zinke, 2015). The island has significant diversity in each of its five ecological zones: humid forest, central highlands, dry deciduous forest, succulent woodland, and spiny thicket (Figure 1; Douglass and Zinke, 2015). Thus, each region must be sufficiently sampled to outline the processes of human arrival and settlement (Douglass and Zinke, 2015). However, archaeological evidence has been limited to sites along the north, northeast, south, and southwest coasts, leaving large areas within Madagascar's dry western grasslands, deciduous forests, and this study's region, the eastern humid forests, sorely underrepresented in archaeological data (Douglass and Zinke, 2015). Though, other fields have offered persuasive arguments about the peopling of the interior of Madagascar (see Serva *et al.*, 2012), improved sampling of archeological sites will shed further light on the settling of the island. In lieu of these data, for the eastern region, we must currently rely on historical land cover and forest loss data, which is limited to the last 65 years (Green and Sussman, 1990; Harper *et al.*, 2007; Vieilledent *et al.*, 2018).



**Figure 1.** Madagascar's ecological zones as defined by an east-west precipitation gradient (map from Douglass and Zinke, 2015)

Forest cover change maps show that in Madagascar, forest fragmentation has been increasing since 1953 (Vieilledent *et al.*, 2018). The moist forests of the eastern region have significantly declined in forest cover from 8,578 Kha in 1953 to 4,410 Kha (Vieilledent *et al.*, 2018). The loss and fragmentation of the remaining tropical forests is a growing concern worldwide. Tropical forests are host to at least 50% of terrestrial species (Lovejoy, 1997) and contain 45% of above-ground carbon in vegetation (Watson *et al.*, 2000). However, tropical forests are being depleted unsustainably, at annual rates up to 4.7% (Achard *et al.*, 2002), leading to climate change and species extinctions (Cowlshaw and Dunbar, 2000; Harper *et al.*, 2007). Fragmentation has substantial effects on a forest's carbon storage capacity, whereby forest edges have 50% less carbon stocks than under a closed canopy (Brinck *et al.*, 2017). Deforestation poses a critical threat to species survival by destroying forest habitat and creating forest fragments too small to maintain viable populations (Cowlshaw and Dunbar, 2000; Harper *et al.*, 2007; Oliveri *et al.*, 2008; Vieilledent *et al.*, 2018). Within Madagascar, around 90% of species are forest dependent (Goodman and Benstead, 2005; Allnutt *et al.*, 2008). Deforestation between 1953 and 2000 has already led to the extinction of 9% of plant and invertebrate species (Allnutt *et al.*, 2008). The continuing deforestation (Vieilledent *et al.*, 2018) will only worsen these results, especially to the world's most endangered mammalian taxa, the lemuriform primates (Schwitzer *et al.*, 2013).

### ***Varecia variegata* Ecology and Conservation**

Black-and-white ruffed lemurs (*Varecia variegata*) are the largest extant members of the Lemnidae family (Vasey, 2003). They are medium sized (2.5 to 4.8 kg, Vasey, 2003; Baden *et al.*, 2008, 2014) primates found in the mid-to-low eastern rainforest corridor of Madagascar (Figure 1, Vasey, 2003). Sexes are monomorphic, showing no differences in body size or pelage

coloration (Vasey, 2003). Current taxonomy identifies three subspecies, *V.v. editorum*, *V. v. subcincta*, and *V. v. variegata*; this categorization is based on differences in pelage coloration and patterning (Vasey & Tattersall 2002; Andriaholinirina *et al.*, 2010; Mittermeier *et al.*, 2010). However, a recent species-wide investigation found support for only two distinct genetic clusters, a north and south cluster separated by the Mangoro River, calling into question the current taxonomy of this species (Baden *et al.*, 2014).

As arboreal quadrupeds, *Varecia* primarily use the highest forest strata available and seek out continuous arboreal pathways to travel through the forest (White *et al.*, 1995; Vasey, 2003). Compared to intact forest, degraded forest regions exhibit a decrease in canopy cover and connectivity (White *et al.*, 1995). Areas that are heavily degraded provide a significant locomotive challenge for this species by hindering their ability to move and forage in the canopy. In addition, *Varecia* is the most frugivorous living lemur, spending 74-90% of their feeding time on fruit (Balko, 1998; Vasey, 2003). However, two fruiting tree taxa that are preferentially exploited by *V. variegata*, *nato* (*Sidreoxylon*) and *ramy* (*Canarium sp.*), are selectively logged (White *et al.*, 1995), thereby depleting a critical resource for this species. Due to their locomotive ability and dietary requirements they are absent from heavily disturbed forest segments (White *et al.*, 1995; Vasey, 2003; Herrera *et al.*, 2011; Wright *et al.*, 2012), suggesting that forest structure is an important predictor of *V. variegata* abundance (Baden *et al.* in review).

*Varecia variegata* has suffered from extreme losses in population size, up to 80% declines, due to habitat loss in its remaining range (Vasey, 2003; Andriaholinirina *et al.*, 2010). The annual rate of deforestation from 1990 to 2014 has ranged from 0.4% to 1.6%, with mean rates doubling from 2005 (0.4%) to 2014 (1.1%; Vieilledent *et al.*, 2018). Habitat loss has reduced *V. variegata* populations to living in isolated forests fragments (Vasey, 2003; Irwin *et*

*al.*, 2005). Population declines due to hunting and severe fragmenting of the species has led to the *V. variegata* being classified as Critically Endangered (Andriaholinirina *et al.*, 2010; Schwitzer *et al.*, 2013) by the International Union for Conservation of Nature (IUCN). Population estimates of *V. variegata* range from 1,000 to 10,000 individuals (Ganzhorn *et al.*, 2001; Schwitzer *et al.*, 2013), though true estimates are likely closer to the lower end of this range. The patchwork distribution of this species throughout the eastern rainforest corridor make accurate population estimates difficult (Irwin *et al.*, 2005).

Within the southern portion of this species range is Ranomafana National Park (RNP). This park has been subjected to intensive and selective logging prior to its establishment (Wright *et al.*, 2012). However, approximately 50% of the remaining forest is categorized as primary vegetation (Wright *et al.*, 2012). As one of the largest remaining protected area in *V. variegata*'s range, it was paramount to evaluate the efficacy of maintaining gene flow and diversity within the park (Mancini and Baden unpubl.). Mancini and Baden (unpubl.) sampled from four localities within the southern parcel of the park and found that RNP was effective in maintaining gene flow within this area. Nevertheless, the population within this area had low levels of genetic diversity and significant evidence of a bottleneck signal (Mancini and Baden unpubl.). The signal was observed using summary statistic approaches, yet, because the it was found across three different mutation models (infinite alleles model, two phase model, and stepwise mutation model), it is considered a strong indicator (Gossens *et al.*, 2006; Mancini and Baden unpubl.). Coupled with the low levels of genetic diversity found within the population, there is strong evidence to suggest that the population within RNP underwent a past genetic bottleneck.

### **Demography and Conservation**

Imperative in conservation management is distinguishing between populations that naturally have low genetic variation from those that have reduced variation due to a severe reduction in population size, i.e., a bottleneck event (Pearse and Crandall, 2004). Effective population size ( $N_e$ ) is important to ascertain because it determines the loss of genetic variation rates, and whether fixation of deleterious alleles and inbreeding are likely to occur (Wright, 1969). It is crucial to detect reductions in population size in endangered species because immediate action may be necessary to avoid population extinction (Schwartz *et al.*, 1998). To understand the evolutionary forces that shape genetic diversity it is imperative to infer the demographic history of a species (Mitchell-Olds *et al.*, 2007). In some cases, historical hunting may provide some information on demographic history. However, in these instances reports only reflect number of killed animals, numbers which do not correlate with true population size (e.g., *Canis lupus*, Aspi *et al.*, 2006). Therefore, our knowledge of the past demographic history of endangered species is often incomplete.

In the last two decades, we have seen an increase in the development of molecular and computational methods used for inferring demographic history. Early methods relied heavily on the deviation of summary statistics from expected values under an equilibrium model (Luikart and Cornuet, 1998) as opposed to more recently developed methods using Bayesian approaches to date demographic events and estimate current and past effective population sizes (Beaumont, 1999; Storz and Beaumont, 2002; Beaumont, 2003). One of these more complex and realistic model-based approaches allows for the inference of several population size changes from microsatellite data (Heled and Drummond, 2008; Wu and Drummond, 2011). Mutations at microsatellite loci are more frequent in DNA, therefore making them ideal for detecting more recent patterns of gene flow and changes in population size (Slatkin, 1995). Using genetic data to



reconstruct a population's demographic history can complement historical accounts of species as well as shed light on major historic events such as bottlenecks, expansions, and migrations that have shaped a current population's genetic structure.

## **SPECIFIC AIMS**

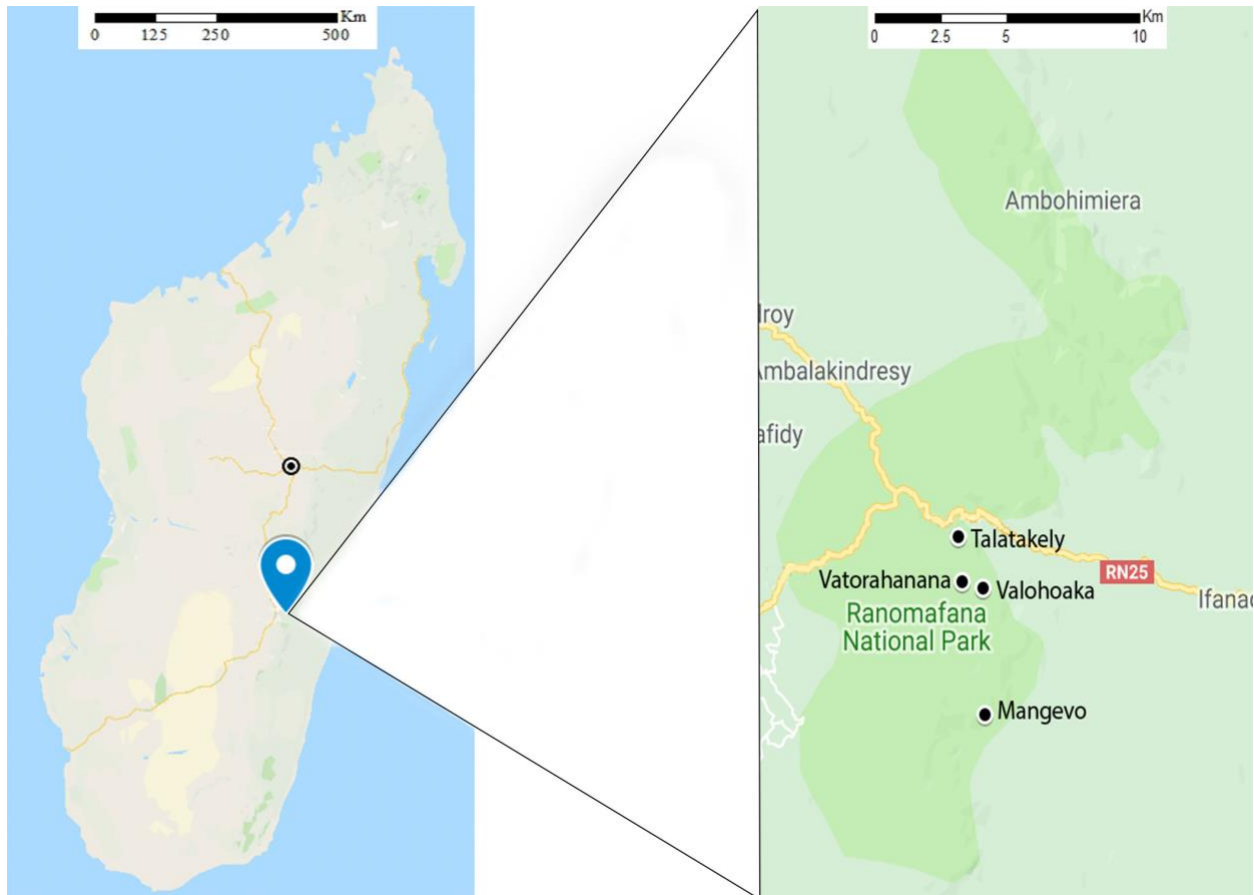
Ranomafana National Park has been effective in maintaining gene flow among sites within its boundaries, however, low levels of genetic diversity and a strong signal of a bottleneck have been detected (Mancini and Baden unpubl.). Therefore, the specific aims of this study were to infer the timing and quantify the extent of the population decline. To achieve this, I used microsatellite genotypes from a total of 38 *V. variegata* individuals from four localities in Ranomafana National Park (RNP; Mancini and Baden unpubl.). I then examined the RNP population for evidence of historical fluctuations in effective population size using extended Bayesian skyline plots (Heled and Drummond, 2008).

## **METHODS**

### **Study Site and Sample Collection**

**Study Site.** Ranomafana National Park (RNP; Figure 2), established in 1991, is located in southeastern Madagascar (21°16'S, 47°20'E, 41,000 ha, Wright *et al.*, 2012). The park is divided into three parcels –north, south, and west – by the Namorona River and a parallel paved road (Figure 2, Wright *et al.*, 2012). It is described as a montane rainforest and contains 13 sympatric lemur species (Wright *et al.*, 2012). Though, approximately 50% of the park is characterized as primary forest, from 1986-1989, anthropogenic disturbance (i.e., selective logging) was high

near the main road that bisects the park (Wright *et al.*, 2012). This disturbance ceased once the park was established (Wright *et al.*, 2012). In addition, natural habitat degradation occurs through intense annual cyclones that ravage the forest in this area (Ratsimbazafy, 2002; Wright *et al.*, 2012).



**Figure 2.** Ranomafana National Park with sampling localities.

**Sample Collection.** Fecal samples were collected noninvasively from adult *V. variegata* individuals from four localities within the southern parcel of RNP (Figure 2, Table 1) by Amanda Mancini and Centre Valbio Research Station (CVB) field technicians between June and August 2015.

**Table 1.** Sampling localities, geographic coordinates, and sample size (n) used in this study.

Site Name	Latitude	Longitude	n
Talatakely	S 21.2662 °	E 047.4255 °	4
Vatorahanana	S 21.2932 °	E 047.4282 °	12
Valohoaka	S 21.2969 °	E 047.4426 °	10
Mangevo	S 21.3722 °	E 047.4449 °	12

## DNA Extraction and Microsatellite Genotyping

**DNA Extraction and Microsatellite Genotyping.** Total genomic DNA was extracted using QIAmp DNA Stool Mini Kits (QIAGEN, Valencia, CA) following the manufacturer's protocol with an extended 65 hour incubation period (Mancini and Baden, unpubl.). Samples were amplified using a suite of ten microsatellite markers: 51HDZ20, 51HDZ25, 51HDZ160, 51HDZ204, 51HDZ247, 51HDZ560, 51HDZ598, 51HDZ691, 51HDZ790, and 51HDZ816 (Appendix I, Louis *et al.*, 2005). A total of 38 unique individuals were identified by Amanda Mancini and Andrea Baden (unpubl.) from the four sampling localities (Table 1).

## Population Genetic Analysis

**Summary statistics.** All ten loci were tested for the presence of null alleles using MICRO-CHECKER (van Oosterhout *et al.*, 2004) and for deviations from Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP v.4.2. Deviations were evaluated using a 10,000 iteration dememorization phase, followed by 100 batches of 10,000 iterations (Raymond & Rousset, 1995). Measures of genetic diversity, including number of alleles per locus (nA), mean number of alleles per locus (MNA), allelic richness (AR), Wright's  $F_{IS}$ , and observed ( $H_O$ ) and expected ( $H_S$ ) heterozygosities for each sampling location were calculated using GENODIVE (Meirmans & Van Tienderen, 2004). To account for uneven sampling between populations, allelic richness

(AR) was standardized to the smallest sample size in the dataset using HP-RARE 1.1 (Kalinowski, 2005).

**Population genetic structure.** Two methods were used to infer population structure, Wright's  $F_{ST}$  and a Bayesian clustering method.  $F_{ST}$  is a measure of genetic differentiation and uses pairwise comparisons to illustrate to what extent two populations are considered genetically distinct populations (Weir & Cockerham, 1984). Wright's  $F_{ST}$  was calculated using GENODIVE (Meirmans & Van Tienderen, 2004). A Bayesian clustering method was utilized in STRUCTURE v2.3.4 (Pritchard, Stephens, & Donnelly, 2000) to also infer genetic structure. This method infers the optimal number of genetic populations (K) through the Markov Chain Monte Carlo (MCMC) approach. Individuals were grouped solely on their multilocus genotypes as information regarding individual's geographic sampling was not provided. I evaluated the hypothesis K=1-7 (Evanno, Regnaut, & Goudet, 2005), using 100,000 iterations of MCMC following a burn-in of 50,000 iterations (Baden et al., 2014). I implemented 20 runs for each value of K, assuming admixture and correlated allele frequencies. The admixture model estimates the of the number of natural genetic clusters and detects historical population admixture (Falush, Stephens, & Pritchard, 2003; Ostrowski et al., 2006). The most likely number of genetic populations (K) was assessed by the highest value of mean  $L(K)$  using the program STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt, 2012).

### **Population Divergence and Demography Inference**

**Population Divergence.** To provide a calibration for the phylogenetic tree in the demographic analyses, and consequently, being able to accurately estimate the demographic changes in *V. variegata*, I carried out a phylogenetic analysis with published mtDNA sequences. For this analysis I aligned partial d-loop or control region sequences for *V. variegata* (n=32;

KJ700613: KJ700626, AF475873- AF475881, AF475890-AF475895 AF493668:AF493671), *Variegata rubra* (n=4, AF173504-AF173506, AF175880), and *Lemur catta* (n= 2, AF17586-AF175869) using MEGA7 (Kumar, Stecher, and Tamura 2015). *V. variegata* sequences were solely from RNP individuals sampled by Baden et al. (2014). All sequences were downloaded from GenBank. Divergence time was estimated using a Bayesian approach in Beast v.2.4.6 (Bouckaert *et al.*, 2014). The analysis was run using the BModelTest (Bouckaert and Drummond, 2017), which conducts a Bayesian model average across all possible evolutionary substitution models accounting for the uncertainty around the true model. I assumed a strict clock model, applied a gamma prior on the clock rate with shape 1 and scale 0.01, and  $1.0 \times 10^{-6}$  as starting value for this parameter. I constrained the internal node between *Lemur catta* and *Variegata ssp.* using the published estimated divergence time 23.42 million years (18.62-29.05 Myr, Hovarth *et al.*, 2008), with a lognormal prior with mean a standard deviation 0.12. The analysis was performed with a birth-death tree prior (Heled and Drummond, 2014) and a chain length of  $2.0 \times 10^9$  with outputs logged every  $4.0 \times 10^5$  steps. Tracer v1.6.0 was used to check chain convergence (Rambaut *et al.*, 2014). Trees were visualized in FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) and the age of the base of the *V. variegata* clade was used as calibration for the root in subsequent analyses.

**Population Demography.** To estimate changes in population size over time in a single population, the extended Bayesian skyline plot model (EBSP, Heled and Drummond, 2008) was fitted to the microsatellite data using BEAST v.1.8.3 (Drummond and Rambaut, 2007; Drummond *et al.*, 2012). The EBSP analysis is a coalescent-based method that exploits the relationship between the genealogy and the demographic history of a population (Pybus *et al.*, 2000; Ho and Shapiro, 2011). Through this analysis, I estimated changes in effective population

size through time based on the relationship between population size and the time between coalescent events (Pybus *et al.*, 2000; Ho and Shapiro 2011), with coalescent events being the convergence of two lineages back in time to a common ancestor (Drummond *et al.*, 2005). The analysis was performed using three out of twelve microsatellite substitution models as described by Saindudiin *et al.* (2004; Table 2) and implemented in BEAST by Wu and Drummond (2011). These models vary on three distinct features, if 1) mutation rate is independent of allele length (E= equal|independent and P= proportional); 2) probability that contraction is equal to expansion (U= unbiased, C= constant, L= linear); and if 3) mutation can change the length of an allele greater than 1 repeat (1= single-step and 2= two-step; Saindudiin *et al.*, 2004). For example, the PU2 model (Table 2) accounts for a microsatellite mutation rate that varies according to allele length, equal rates of contraction and expansion, and mutations of both single and multiple repeats (Saindudiin *et al.*, 2004, Allen *et al.*, 2012). The observed maximum length of any allele was 12 repeats which was the assumed as upper bound. The starting value of the clock rate was set to  $1.0 \times 10^{-4}$  (Ruiz-Garcia, 2005), but rates were estimated to account for variation across loci. The root of the tree was calibrated using a lognormal distribution and by setting the mean in real space to 88930.0 with a standard deviation of 0.42. A Poisson prior with mean  $\lambda = 0.6931$  was used for the number of changes in population history (Heled and Drummond, 2008). For each model, three independent BEAST runs were performed, each with a minimum chain length of  $1.0 \times 10^9$  with outputs logged every  $1.0 \times 10^5$  steps. Tracer v1.6.0 was used to check chain convergence (Rambaut *et al.*, 2014) and to verify that the combined run's effective sampling size (ESS) values were greater than 150 after a burn-in of 10%. The software package ggplot2 (Wickman, 2016) was used in RStudio (RStudio Team, 2015) to graph the resulting plot.

**Table 2.** 12 models of microsatellite evolution as described by Sainudiin et al (2004). **Bold** denotes models used in this study.

<b>Mutation Model</b>	<b>Description</b>
<b>PU2</b>	Proportional-rate, unbiased, two-step
EC2	Equal-rate, constant-bias, two-step
EL2	Equal-rate, linear-bias, two-step
PC2	Proportional, constant-bias, two-step
PU1	Proportional-rate, unbiased, one-step
EU2	Equal-rate, unbiased, two-step
PC1	Proportional, constant-bias, one-step
<b>EU1</b>	Equal-rate, unbiased, one-step
EC1	Equal-rate, constant-bias, one-step
<b>PL2</b>	Proportional-rate, linear-bias, two-step
PL1	Proportional-rate, linear-bias, one-step
EL1	Equal-rate, linear-bias, one-step

## RESULTS

### Summary Statistics

Nine out of ten loci were polymorphic, while locus 51HDZ204 was dimorphic (Table 1). The number of alleles per locus ranged from 2 to 6 alleles (Table 1), with a mean of 4 alleles per locus. There was no evidence of linkage disequilibrium between marker pairs and no loci deviated significantly from the Hardy-Weinberg equilibrium (Table 1). Allelic richness ranged from 1.90 to 3.10 across the sampling localities of *V. variegata* and mean allelic richness was  $2.68 \pm 0.55$  (Table 4). The mean observed heterozygosity across sampling sites was  $0.592 \pm 0.131$ , while mean expected heterozygosity was  $0.530 \pm 0.146$ . Overall  $F_{IS}$  was -0.134 and values ranged from -0.001 at Vatorahanana to -0.247 at Talatakely.

**Table 3.** Characters of 10 microsatellite markers amplified in 38 *V. variegata* samples, including the number of alleles per locus ( $n_A$ ), observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity, and deviations from Hardy-Weinberg Equilibrium (HWE). Significant p values ( $p < 0.05$ ) are shown in **bold**.

Marker	Size Range	Annealing Temp	$n_A$	$H_o$	$H_e$	HWE	GenBank
<b>51HDZ20</b>	220-236	50	6	0.530	0.693	0.0856	AF468499
<b>51HDZ25</b>	169-173	54	3	0.358	0.328	1.0000	AF468500
<b>51HDZ160</b>	234-242	54	4	0.618	0.589	0.9625	AF468502
<b>51HDZ204</b>	134-140	60	2	0.608	0.504	0.4734	AF468503
<b>51HDZ247</b>	248-258	50	6	0.915	0.772	0.6800	AF468504
<b>51HDZ560</b>	257-261	52	3	0.463	0.595	0.8486	AF468508
<b>51HDZ598</b>	195-207	51	3	0.592	0.547	0.8377	AF468509
<b>51HDZ691</b>	232-250	50	5	0.523	0.749	0.9142	AF468512
<b>51HDZ790</b>	209-213	50	3	0.463	0.488	0.3800	AF468513
<b>51HDZ816</b>	279-289	54	5	0.848	0.786	0.9510	AF468514

**Table 4.** Allelic diversity within each of the 4 sampling localities, including mean number of alleles per locus (MNA), allelic richness (AR), observed ( $H_o$ ) and expected ( $H_e$ ) heterozygosity, inbreeding coefficient ( $F_{IS}$ ), and p-values of Hardy-Weinberg Equilibrium (HWE) test calculated using 10,000 iterations.

Site	n	MNA	AR(SE)	$H_o$	$H_e$	$F_{IS}$	HWE
<b>Talatakely</b>	4	1.900	1.900(0.57)	0.400	0.321	-0.247	1.0000
<b>Vatorahanana</b>	12	3.600	3.100(0.88)	0.641	0.640	-0.001	0.4097
<b>Valohoaka</b>	10	3.400	2.700(0.75)	0.632	0.542	-0.165	0.9112
<b>Mangevo</b>	12	3.700	3.030(0.66)	0.695	0.618	-0.124	0.8432
<b>Overall</b>	38	3.150	2.680(0.55)	0.592	0.530	-0.134	-

## Population Genetic Structure

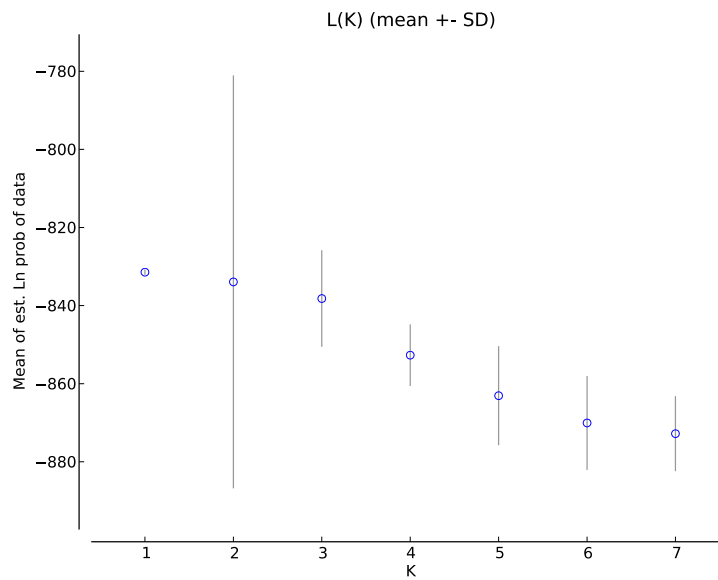
Mean  $F_{ST}$  over all sampling localities was  $0.082 \pm 0.044$  (Table 5). The pairwise comparison of Vatorahanana-Mangevo did not have significant  $F_{ST}$  values, suggesting that there no differentiation between the two sites. However, all other sites yielded significant pairwise comparisons, indicating these sites within RNP are potentially genetically distinct populations.

**Table 5.** Pairwise  $F_{ST}$  values (above diagonal) and significance of  $F_{ST}$  values (below diagonal) among sampling localities of *V. variegata*. Significant values indicated with \* ( $p < 0.0083$  after Bonferroni corrections).

	Talatakely	Vatorahanana	Valohoaka	Mangevo
<b>Talatakely</b>	--	0.147	0.330	0.135
<b>Vatorahanana</b>	*	--	0.086	0.027
<b>Valohoaka</b>	*	*	--	0.084
<b>Mangevo</b>	*	NS	*	--



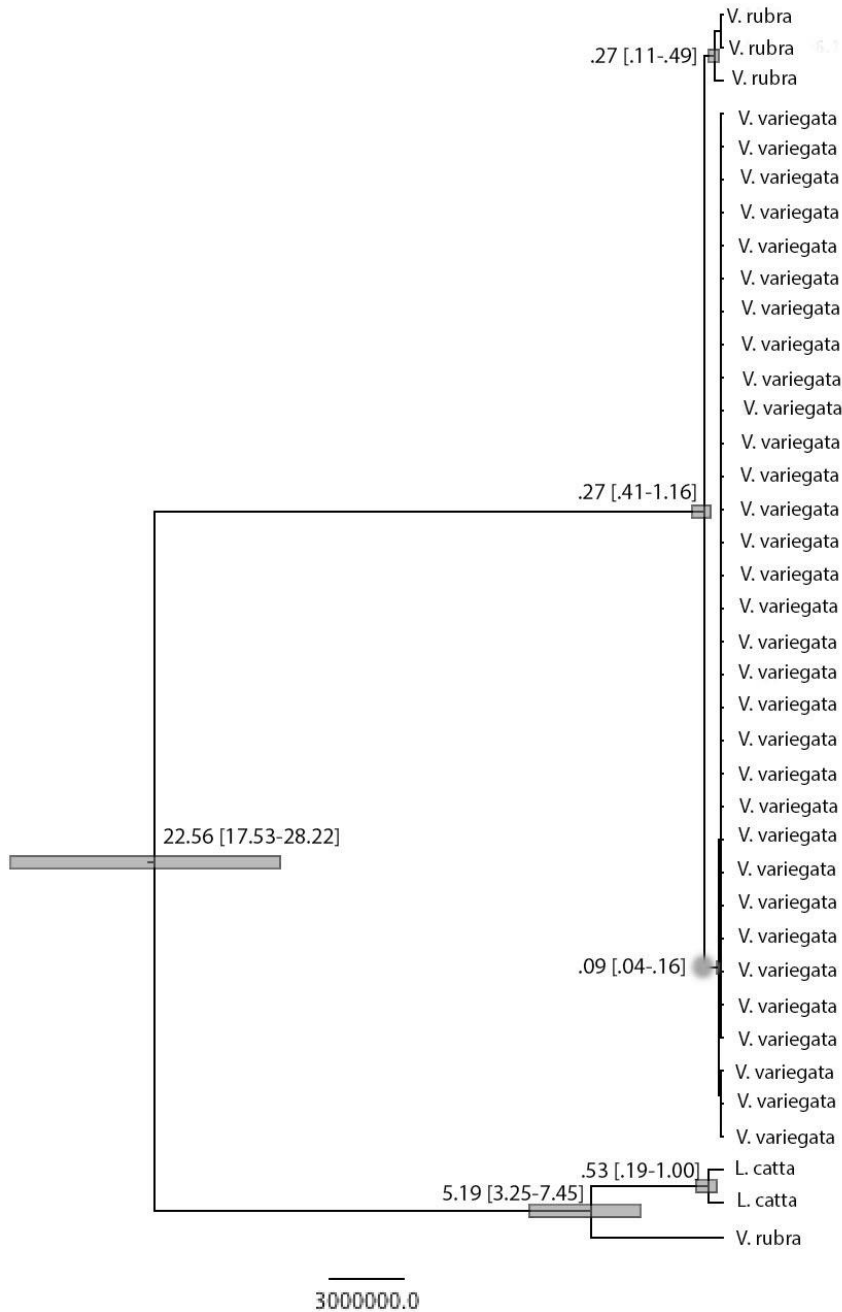
STRUCTURE analysis (Figure 3) identified one genetic cluster within RNP. The mean  $L(K)$  indicated that the likelihood is close between  $K=1$  and  $K=2$ , however, the standard deviation of  $K=2$  is large indicating little stability in the results. Hence suggesting that  $K=1$  is the most likely number of genetic clusters.



**Figure 3.** STRUCTURE analysis. Mean  $L(K)$  indicating  $K=1$  as the number of clusters within RNP.

## Divergence Time

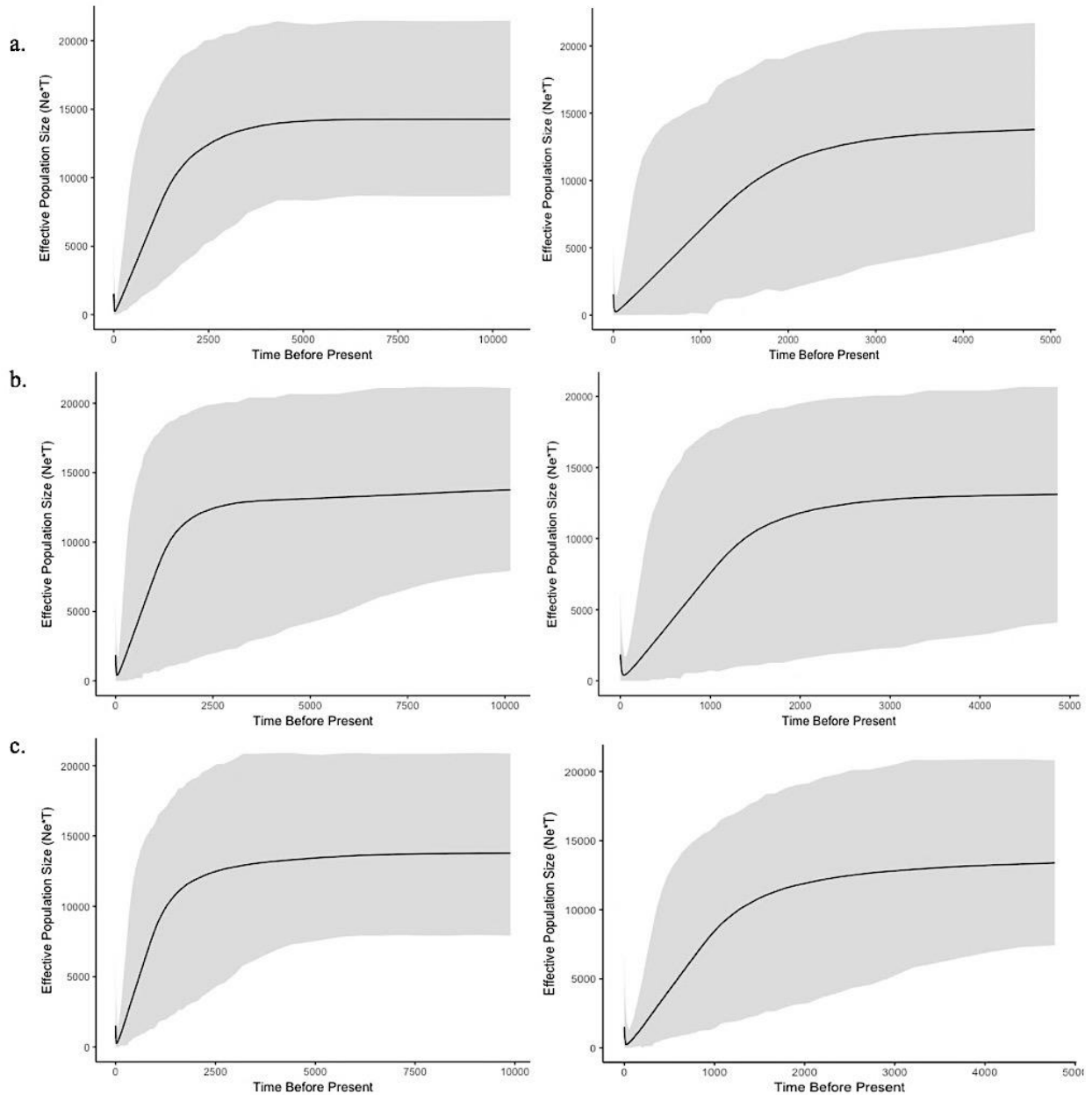
The phylogenetic reconstruction of the RNP *V. variegata* population estimated a divergence time of 88,930 Kyr (95% HPD 35,367-164,860, Figure 4).



**Figure 4.** Ultrametric tree showing Bayesian divergence estimates in millions of years. Shaded gray boxes indicate 95% HPD and dot indicates RNP *V. variegata* node.

## Population Demography Inference

Coalescent extended Bayesian skyline plots (EBSP) were used to estimate the magnitude and timing of effective population size changes in the RNP *V. variegata* population. All mutation models found that the *V. variegata* population at RNP experienced a population collapse in its recent past (Figure 5). The runs rejected a constant population size with the posterior probabilities of the inferred number of population size changes over time being the highest at 2 for all models. The PU2 model suggests a gradual decline starting around 2,000 years ago (2,175-2,600 years BP), with a more drastic decline starting around 1,500 years ago (1,478-1,787 years BP). The EU1 and PL2 model suggest a decline starting around 1,500 years ago (1,290-1,818 years BP). By assuming that the average generation time is 3 years (Baden, pers. comm.), the mean ancestral effective population size ( $N_e$ ) was estimated to be around 4,728 to 4,888 (95% HPD 2,640-7,141). Estimations of current  $N_e$  varied between models, with the PU2 model estimating a population size around 684 (95% HPD 2-1,992), the EU1 model estimating a population size around 820 (95% HPD 8-2,313), and the PL2 model estimating a population size of around 746 (95% HPD 6-2,363). However, all models projected a decrease in  $N_e$  of about 84% (83-85%).



**Figure 5.** Extended Bayesian Skyline Plot for the 10 microsatellite loci (a. PU2, b. EU1, c. PL2). The gray shading corresponds to the 95% HPD around the mean effective population size ( $N_e$ ) times generation time ( $T$ ). Cropped to 10,000 and 5,000 years prior.

## DISCUSSION

### Demographic History of *V. variegata* in Ranomafana National Park

The extended Bayesian skyline plot (EBSP) analysis confirms that *V. variegata* population size decreased in Ranomafana National Park (RNP) between approximately 1,500

years ago and today. Data suggest that RNP once had large effective population sizes (~4,000) prior to the bottleneck (~1,290-1,818 years ago), which then decreased dramatically by 84%. The population decline was gradual and most likely occurred in the last 1,500 years.

The EBSP analysis suggests that prior to the crash, the population was stable, with minor fluctuations in effective population size throughout the late Pleistocene and early Holocene, prior to human arrival. Radiocarbon dating of the extinct giant lemurs, elephant birds, and hippos has shown that the “extinction window,” the duration from which the earliest human evidence to last occurrence of extinct megafauna, overlaps with humans (Burney *et al.*, 2004). Extinct lemurs *Hadropithecus stenognathus*, *Pachylemur insignis*, *Mesopropithecus pithecoides*, and *Daubentonia robusta* were still present near the end of the First Millennium AD and *Archaeolemur* sp., *Megaladapis edwardsi*, and *Palaeopropithecus ingens* survived until 830±60 yr BP, 630±50 yr BP, and 510±80 yr BP, respectively (Burney *et al.*, 2004). These dates, and the growing corpus of dates regarding the extinction of several Malagasy species, have made clear that the major climate and vegetation changes of the late Pleistocene and pre-human Holocene were survived by most of the megafauna (Burney *et al.*, 2004). In addition, while there is evidence for range shrinkage in the late Holocene for some lemurs (Godfrey *et al.*, 1999; Lawler, 2011; Baden *et al.*, 2014), there have been no pre-human disappearances detected in the history of these animals, including the *V. variegata* population at RNP.

There are five hypotheses used to explain the megafauna extinctions seen in Madagascar: The *Great Drought*, which hypothesized that extinctions occurred due to the spread of semiarid conditions (Mahé and Sourdat, 1972); *Blitzkrieg*, which proposed that first contact overkill of large fauna (Martin, 1984); *Biological Invasion*, which theorized that introduced animals disrupted natural vegetation and caused competition with native species (Dewar, 1984);

*Hypervirulent Disease*, which suggested that humans introduced unknown pathogens which were lethal to a wide array of mammals, birds, and reptiles (MacPhee and Marx, 1997); and the *Great Fire* hypothesis, theorized by Humbert (1927), postulates that the landscape was transformed rapidly by the introduction of fire by humans. The resulting forest loss caused the extinction of Malagasy's megafauna (Humbert, 1927). Humbert (1927) posed that this pattern was simultaneous throughout Madagascar. Recent charcoal spikes dating disputes this pattern and suggests that human fire occurred at different times in Madagascar (Burney *et al.*, 2004). Finally, the *Synergy* hypothesis postulates that a full array of human impacts played a role in species declines and extinctions (Burney, 1999). However, additional factors, such as climate change, may have amplified the anthropogenic effects (Burney, 1999). This process was very slow and varied in extent between regions (Burney, 1999).

There is not much information of when early human societies settled and affected the habitats of the interior mid-to-low rainforests of Madagascar. But following Burney *et al.* (2004), the interior of Madagascar was one of the last regions to be settled and may have taken up to seven centuries or more to inhabit. Though there is evidence of frequent fire altering the habitat of Madagascar prior to human arrival, the eastern rainforest was too wet to support frequent fires (Burney, 1996; Burney *et al.*, 2004). The arrival of humans in Madagascar drastically altered the fire ecology by increasing the burning and by spreading fire to environments that were too dry or wet to support natural fires (Burney *et al.*, 2004). Results from the present study suggest that the Ranomafana *V. variegata* population began declining about 800 years after the arrival of humans; one possible cause could have been the appearance of fire in this region. However, these results do not support a simultaneous and rapid rate of species decline as hypothesized by the *Great Fire*, but instead support the *Synergy* hypothesis as the rates were gradual and varied

by region. The effective population change inferred by the analysis suggest a steady rate of decline, indicating that anthropogenic fire is not the sole cause of *V. variegata* declines in RNP. In addition, RNP is affected by intense cyclones (Wright *et al.*, 2012) that naturally degrade the forest. This coupled with human introduced fire may have prevented the population from recovering, lending more support to the *Synergy* hypothesis, in that lemur population declines and extinctions were multifaceted.

### **Conservation and Management**

Biodiversity is lost at an astonishing rate each year due to the rise of threats such as climate change, invasive species, emerging diseases, and deforestation (Pimm *et al.*, 2014). Estimated extinctions rates are 100-1,000 times higher than those observed in the past (Pimm *et al.*, 2014). Madagascar is under tremendous conservation pressure as it contains endemic plants and vertebrates amounting to at least 2% of total species world-wide (Myers *et al.*, 2000). Paramount to maintaining Madagascar's habitat diversity are the frugivorous lemurs, such as *V. variegata*. *Varecia variegata* is both an indicator and umbrella species of Madagascar (Vasey, 2003). It has strict habitat requirements, such that it is not found in heavily disturbed forest fragments (White *et al.*, 1995), and its large home range spans a myriad of other plant and animal taxa (Vasey, 2003). In addition, a close relative to *Varecia*, *Pachylemur*, has only recently become extinct (Vasey, 2003). Thus, there is a dire need to understand their extinction risk and key factor in detangling this risk is knowing how many individuals are in a population.

Population census size ( $N_C$ ) and effective population size ( $N_e$ ) are important to ascertain because they can help predict the extinction risk of populations. However, these parameters are often difficult estimate, especially  $N_C$ , which is more informative in conservation management. Effective population size is described as the number of individuals that would be required in an

ideal Wright-Fisher population to yield the same level of diversity as what is observed in a sampled population (Fisher, 1930; Wright, 1931; Luikart *et al.*, 2010). While census size is defined as the actual number of individuals in a population (Luikart *et al.*, 2010). A population's true census size is often difficult to quantify and any method short of counting each individual is subject to misinterpretation of the value (Luikart *et al.*, 2010). *Varecia variegata* is particularly hard to observe, due to their preference for spending time in the high canopy (Vasey, 2003), as well as difficulty differentiating among individuals. Therefore, counting methods can be subject to considerable error. Effective population size estimates have limitations as well. In coalescent methods, as seen with this study, the amount of uncertainty in the posterior distribution can make it difficult to draw definitive results.

There is potential for inferring  $N_C$  from  $N_e$  (or vice versa) by using the  $N_e/N_C$  ratio. Census size is usually larger than effective population size, with  $N_e$  averaging between 10-50% of the census size for most species (Hare *et al.*, 2011). By inferring the  $N_e/N_C$  ratio, ecologists can determine the ecological factors that reduce  $N_e$  below the  $N_C$  (Kalinowski and Waples, 2002; Luikart *et al.*, 2010). However,  $N_e/N_C$  ratio is likely to change over time in species with variable life histories (Luikart *et al.*, 2010). Long-term studies done on salmonids (Palstra and Ruzzante, 2008) and red flour beetles (Pray *et al.*, 1996) documented the variation of this ratio over time due to effects of immigration on  $N_e$  and fluctuating reproductive success. For example, high  $N_C$  in red beetles led to a lower  $N_e/N_C$  ratio, while high  $N_C$  in salmonids had the opposite effect (Luikart *et al.*, 2010). When excluding life history variables, the average  $N_e/N_C$  ratio across a range of species is 0.34, in solely mammals it is 0.46, but estimates including life history variables resulted in an average ratio of 0.11 (Frankham, 1995).



Though management efforts focus on census size, effective population size has valuable applications of its own. By inferring  $N_e$ , we can assess a population's ability to adapt and persist after stochastic events (Hare *et al.*, 2011), such as natural cyclone events (Vasey, 2003; Wright *et al.*, 2012) or high mortality from new pathogens. Stochastic events such as those described contribute to genetic drift, which is the random loss of alleles from a population due to individual mortality or lack of reproduction. The effect of genetic drift on a population acts strongly on populations with low  $N_e$  values. The loss of alleles from a population decreases its overall genetic diversity, which has deleterious effects such as increasing the chance of allele fixation and inbreeding, as well as lessening the effect of natural selection (Hare *et al.*, 2011; Heller *et al.*, 2011). Thus, some studies suggest that an  $N_e$  of ~1,000-5,000 is necessary to ensure genetic security and the long-term survival of a species (Lynch and Lande, 1998; Allen *et al.* 2012). The results of this study show that the *V. variegata* population at Ranomafana National Park is below the minimum (~684-820) needed ensure its long-term survival. Reforestation programs are currently being implemented by Centre ValBio Research station along the eastern boundary of the park near Ranomafana village. As one of the most effective seed dispersers in Malagasy rainforests, *V. variegata* are the only lemur species capable of dispersing large seeded trees species such as *ramy* (*Canarium sp.*; Martinez and Razafindratsima, 2014; Federman *et al.*, 2016). Their increased ability in dispersing a variety of tree species will not only enable successful gene flow between sites in the park but also be help regenerate the forest and forest corridors.

### **Future Directions**

To ensure the robustness of the demographic analysis, this study will be broadened to include additional *V. variegata* genotypes from three new sites (Sahavoemba, Ambodiaviavy,

and Sakaroa) within RNP and five additional sites (Malazamasina, Madiorano, Tatamaly, Ambodivanana, and Mandiandry) surrounding the park. The current sampling of each locality is limited resulting in discordant results between the  $F_{ST}$  and *STRUCTURE* analyses. However, the conclusion that the four localities used in this study is part of the same genetic cluster is supported by Baden et al. (2014), who identified a genetic cluster over a wider geographic range south of the Mangoro river. The addition of the eight new sites will provide a more encompassing sampling of the *V. variegata* population at RNP and throughout the Ranomafana – Andringitra Corridor. In addition, to better understand the human-lemur dynamics at RNP, a demographic analysis of five other diurnal lemurs found in the park will be done: *Haplemur griseus*, *Eulemur rubriventer*, *Eulemur rufifrons*, and *Propithecus edwardsi*. By comparing the demographic history of lemurs across body size, dietary requirements, locomotor patterns and life history variables, we can see if there are similar demographic trends occurring within the same environment. Unlike other regions of Madagascar, there is little paleontological data regarding the timing of human settlement in this area. This comparison could shed light on whether the emergence of human induced fire caused the lemur population declines as seen in the *V. variegata* at RNP.

## CONCLUSION

Madagascar has a complex cultural and biogeographic history, and the relationship between human colonization and lemur population declines, as well as extinction, is multifaceted. My results suggest that the black-and-white *V. variegata* population in Ranomafana National Park has been declining for the last 1,500 years. During the peopling of Madagascar, fire was introduced to regions that were not previously affected by natural fire. This

change in fire ecology may have modified *V. variegata*'s habitat and led to heavily degraded forest patches which are unsuitable for this species. The results presented here demonstrate that demographic analyses can be used to shed light on the historical population dynamics of extant lemur species (Olivieri *et al.*, 2008; Lawler, 2011; Quémère *et al.*, 2012; Salmona *et al.*, 2017). These analyses are particularly useful when combined with data from paleoecology, archaeology, and historical records. It is vital to note that lemur population sizes can historically fluctuate due to climatic variation (Quémère *et al.*, 2012; Salmona *et al.*, 2017), without the presence of human disturbance. Therefore, it is important to detangle lemur population dynamics so that we can separate out anthropogenic effects from natural ones and strengthen our understanding of how these mechanisms govern the dynamics of the world's most endangered mammals.

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**APPENDIX I.** Marker, primer sequence, repeat motif, annealing temp, and GenBank accession number of the 10 microsatellite markers used in this study.

<b>Marker</b>	<b>Sequence</b>	<b>Repeat Motif</b>	<b>Base Pair Size</b>	<b>Annealing Temp</b>	<b>GenBank Accession no.</b>
51HDZ20	F: 5'-ATG ACT TGT AGC TTA AAT CTT TTG G-3' R: 5'-TAC TTG GCT GAT TCG GGA G-3'	(CA)10(TA)5	221-241	50	AF468499
51HDZ25	F: 5'-GTC AAA CGG GGA AAA TGC-3' R: 5'-TCA AAT CGG TAG CTC TCG G-3'	(TGCACA)4CACG(CA)11	169-175	54	AF468500
51HDZ160	F: 5'-GCT CTG TTA TCT CTG TCT CAC TTC C-3' R: 5'-GAT TTT GAT TAG TCT TTT TTA CAT GG-3'	(CA)14	237-245	54	AF468502
51HDZ204	F: 5'-AAT CAT GTT TTG TGG GAG GGG-3' R: 5'-GTA TAC CTC ACT GGC TCC CTG C-3'	(CA)4A(CA)12	129-139	60	AF468503
51HDZ247	F: 5'-AGG AAG GTA CAC TAA AAC AGA GAC T-3' R: 5'-TGT ATC CTC CAT TTA TCT CCT TG-3'	(CA)14	249-265	50	AF468504
51HDZ560	F: 5'-CAC TTC TGC CTC CAA TCA CTC-3' R: 5'-AAC ATC CCG TGG TCA CTA CAG-3'	(GT)6(GC)2AC(GT)6(CTGT)3	253-259	52	AF468508
51HDZ598	F: 5'-ATT CAG AAG TGT TAC ATT TAC GGA GG-3' R: 5'-GAG TGG GTG GCA AGG TTC G-3'	(CA)8AGA(CA)15	201-217	51	AF468509
51HDZ691	F: 5'-CCA TGA CGT TAA TTC CTC TGC-3' R: 5'-GCC ACC ATC ACC CAG TTG-3'	(CA)17	233-251	50	AF468512
51HDZ790	F: 5'-CCA CCC CAG TCC TGT CCT TA-3' R: 5'-TTG TTG CCT CTC TGC CAA GTA G-3'	(CA)10	211-215	50	AF468513
51HDZ816	F: 5'-AGA GGC CAC TAC TGA CAA CG-3' R: 5'-CCC CCA CAC ACA AAT ACT AAA C-3'	(CA)19	280-292	54	AF468514