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Combatting the Illegal Pet Trade: Using Molecular Techniques to Determine the Provenience of Illegal Ring-Tailed Lemur (Lemur catta) Pets

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Combatting the Illegal Pet Trade: Using Molecular Techniques to Determine the Provenience of Illegal Ring-Tailed Lemur (*Lemur catta*) Pets

by

Jessica Knierim

Submitted in partial fulfillment of the requirements for the degree of Master of Arts in Animal Behavior and Conservation, Hunter College The City University of New York

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Thesis Sponsor:

December 18, 2017

Date

Dr. Andrea Baden

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Signature of Second Reader
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COMBATTING THE ILLEGAL PET TRADE IN *L. CATTA*

Abstract

The ring-tailed lemur (*Lemur catta*) was once widespread across southern Madagascar. However, anthropogenic activities such as habitat loss, hunting for bushmeat, and live capture for the illegal pet trade have caused *L. catta* populations to plummet in the past decade. Here I compare genotypes of illegal wild-caught pet and confiscated ex-pet *L. catta* to those from wild populations to determine their source localities. Fecal samples were collected from 20 wild-caught pet *L. catta* and 5 wild individuals from Isalo National Park. DNA was extracted and amplified at seven polymorphic loci following Parga et al. (2012, 2015). To determine the geographic origin of captive and confiscated lemurs, their genotypes were matched to a geographically-referenced allele frequency database generated from a reference library of 30 adults sampled from three wild *L. catta* populations in south-central Madagascar (Anja, Sakaviro, Tsinjoriake). Evidence for two genetic clusters was provided by both a Bayesian cluster analysis and a multivariate clustering method (Discriminate Analysis of Principle Components), suggesting genetic clustering based on geographic location. Mean number of alleles per locus of the wild populations ranged from 4.29-6.00. Observed and expected heterozygosity ranged from 0.690-0.857 and 0.727-0.739, respectively. These populations maintained moderate levels of genetic diversity but lower levels of allelic richness as compared to other populations of *L. catta*. Additionally, most captive individuals could not be assigned to a source population, likely because sampling does not represent the species’ entire geographic range. This is a pilot study and additional sampling of both captive and wild populations of *L. catta* will be needed to accurately pinpoint provenience of *L. catta* in the pet trade. Ultimately, results of this study can be used to help determine geographic “hot spots” of wildlife trafficking.
for which targeted conservation initiatives – including heightened security and increased conservation outreach – can be developed.

**INTRODUCTION**

Madagascar’s endemic lemurs represent more than 20% of the world’s primate species and 30% of family-level diversity, and yet, they are the world’s most endangered group of mammals (Schwitzer et al., 2014). The island’s vast levels of endemic biodiversity are unparalleled by any other country and set it apart as a biodiversity hotspot (Ganzhorn et al., 2001; Myers et al., 2000; Schwitzer et al., 2014). Despite the need to preserve the country’s endemic and biodiverse species, lemurs are facing extinction due to human driven disturbances including habitat loss and fragmentation and hunting for the bushmeat (Borgerson, McKean, Sutherland, & Godfrey, 2016; Golden, 2009) and illegal pet trades (Estrada et al., 2017; Reuter, Gilles, et al., 2016).

The Endangered (Andriaholinirina et al., 2014) ring-tailed lemur (*Lemur catta*), a flagship species (Mittermeier et al., 1992), represents one of over 100 lemur species and is perhaps Madagascar’s most widely recognized and well-studied species of lemur (Sauther et al., 2015). Historically, this species has been widespread and abundant throughout the southern regions of Madagascar (Goodman, Rakotoarisoa, & Wilme, 2006; Gould, 2007; Gould, Sussman, & Sauther, 2003; Sauther et al., 2015; Sauther, Sussman, & Gould, 1999; Sussman et al., 2006), but loss of habitat and overexploitation via poaching for the illegal bushmeat and pet trade over the past decade have caused extirpations and severe population reductions throughout their geographic range (Gould & Sauther, 2016; LaFleur & Gould, 2009). Despite the species’ historic ability to cope in degraded habitats, the stark loss of nearly half of their habitat in just the past 40 years may prove too much for this traditionally abundant species to overcome.
(Brinkmann et al., 2014; LaFleur, Clarke, Ratzimbazafy, et al., 2017). This severe habitat loss, coupled with their disappearance from extant forests due to hunting and live capture for the illegal pet trade have caused this species to be included in the list of “The World’s 25 Most Endangered Primates” (LaFleur, Clarke, Ratzimbazafy, et al., 2017).

*L. catta* are just one of the many primate species threatened by live capture for the pet trade. The global exotic pet trade affects thousands of primates every year, with some estimates suggesting ~40,000 live captured primates are traded annually on international markets (Karesh et al., 2005). The demand for primate pets contributes to declining populations of many endangered species, including the Critically Endangered Central American Spider Monkey (*Ateles geoffroyi*; Smith, 2005). Live capture for the pet trade is the greatest threat facing the Critically Endangered Javan slow loris (*Nycticebus javanicus*) and is one of the main threats to the entire *Nycticebus* genus (Nekaris et al., 2010). Additionally, adult Critically Endangered Bornean orangutans (*Pongo pygmaeus*) are killed in order to capture infants for the pet trade (Husson et al., 2017; Rijksen & Meijaard, 1999). While the wildlife trade is a major threat to many primate species, the number of live primates exported annually has been steadily increasing since 1995, with 3,500 more live primates being exported every year (Nijman et al., 2011).

Thousands of lemurs are affected by Madagascar’s illegal pet trade every year and *L. catta* is the most common lemur species kept in captivity in the country (Reuter 2015; Reuter, Gilles, Wills, & Sewall, 2016). As of yet, there is no data on which wild populations *L. catta* are being taken from in order to fuel the demands of the pet trade. In this thesis, I employ forensic genetic techniques to identify the source populations from which wild-caught current and ex-pet *L. catta* were captured and inform conservation actions.
Ecology and Conservation Status of *Lemur Catta*

*L. catta* is an exceptionally flexible species, in terms of both its habitat and diet (Gould, 2007). It occupies a diverse range of habitats across southern, southwestern, and south-central Madagascar, including gallery, littoral, and dry deciduous forests, brush and scrub, spiny bush, high-altitude ericoid bush, rocky-outcrop mixed vegetation, and mangroves (Cameron & Gould, 2013; Goodman et al., 2006; Gould, 2007; Sauther et al., 1999). Some of these habitats also experience the island’s most extreme climate conditions, from the hottest and driest climates to the coldest (Cameron & Gould, 2013). *Lemur catta* live in female philopatric multi-male/multi-female groups averaging 11 to 17 individuals but these group sizes vary widely across sites (Cawthon Lang, 2005; Jolly, 2003; Sauther et al., 1999; Sussman, 1991). Males disperse from their natal group once they reach sexual maturity (3-4 years) and will again disperse on average every 3.5 years throughout their lifetime (Sauther et al., 1999; Sussman, 1992).

*L. catta*, a medium-sized strepsirrhine (average 2.2 kg) (Gould et al., 2003; Sussman, 1991), is considered an “opportunistic omnivore” and has a remarkably flexible diet (Gould, 2007; LaFleur & Gould, 2009; Sauther et al., 1999). Its diet can consist of both ripe and unripe fruit, young and mature leaves, leaf and flower stems, flowers, soil, dead wood, cactus, insect larvae, spiders, spider webs, caterpillars, cicadas, locusts, and occasionally small birds and chameleons (Gould, 2007; Jolly, 1966; Kelley, 2011; Oda, 1996; Rasamimanana & Rafidinarivo, 1993; Sauther, 1992, 1998; Sauther et al., 1999; Simmen et al., 2006; Simmen, Hladik, & Ramasiarisoa, 2003; Sussman, 1972; Yamashita, 2002). In addition to their diet, their social behavior and activity budgets also vary with changing environmental conditions (Sauther et al., 1999). For example, *L. catta* live in denser populations with smaller home ranges in gallery
forests which typically have an abundance of resources versus populations that live in drier forests (O’Conner, 1987; Sussman, 1991).

As a result of L. catta’s behavioral plasticity, they are able to survive in highly fragmented habitats. Unfortunately, this ability has become requisite for their survival due to the severe forest loss throughout the species’ geographic range. In just the past 40 years, L. catta have lost over 45% of their habitat (Brinkmann, Noromiarilanto, Ratovonamana, & Buerkert, 2014; LaFleur et al., 2017) and are projected to lose 68% by 2080 from climate change alone (Brown & Yoder, 2015). Despite L. catta’s flexibility and ability to survive in degraded landscapes (Gould & Gabriel, 2015), they are dependent on key resources and reach a threshold beyond which they are unable to survive in deforested areas (LaFleur, Clarke, Ratzimbazafy, et al., 2017; Sauther et al., 1999). Because L. catta is the most terrestrial of all extant lemurs, they are usually able to disperse across non-forested areas (Goodman et al., 2006). However, fragmentation throughout their geographic range is too severe in many areas to allow for male dispersal (Gould & Sauther, 2016). Because of this, L. catta populations are declining in accordance with their shrinking forest habitats (LaFleur, Clarke, Ratzimbazafy, et al., 2017; LaFleur, Clarke, Reuter, et al., 2017).

Today, many L. catta populations live in small, isolated forest remnants which impedes their ability to disperse and results in reduced gene flow, decreased genetic variability and increased inbreeding (Craul et al., 2009; Holmes et al., 2013; Olivieri et al., 2008; Parga et al., 2012; Radespiel, Rakotondravony, & Chikhi, 2008). Lemur catta’s restriction to isolated fragments of forest throughout their historic range has been a major driver of population declines which has caused the species to be reclassified from ‘Near Threatened’ to ‘Endangered’ (Andriaholinirina et al., 2014; Bodin et al., 2006; Cameron & Gould, 2013; Sussman et al.,
The ongoing fragmentation of forests in Madagascar has already driven local extinctions and is leaving other populations with low densities while also threatening the genetic diversity of the remaining populations (Bodin et al., 2006; Ganzhorn et al., 2001; Gould & Gabriel, 2015; Sussman et al., 2003).

Following a coup d'état in 2009, Madagascar has experienced political unrest and financial instability, which has only exacerbated the threats facing *L. catta* and the country’s other endemic primates (Schwitzer et al., 2014). Since 2009, law enforcement has relaxed and there has been a marked increase in illegal logging, slash and burn agriculture, mining, and bushmeat hunting (Barrett et al., 2010; Cameron & Gould, 2013; Innes, 2010; Jenkins et al., 2011; Schwitzer et al., 2014). Although it is traditionally considered *fady* (taboo) to hunt and consume *L. catta*, the instability in the country has led to changing cultural norms and people have begun poaching these lemurs for food (LaFleur, 2013; Sauther et al., 2013). There has also been an uptick in commercial hunting of lemurs, a practice which was not previously seen in the country (Reuter, Randell, et al., 2016; Schwitzer et al., 2014). Because bushmeat hunting and live-capture for the pet trade are often inextricably linked, the latter has also increased (LaFleur, Clarke, Reuter, et al., 2017; Reuter, Gilles, et al., 2016; Sauther et al., 2013). As a result of being subjected to living in small isolated fragments, *L. catta* are more severely affected by hunting and live capture, which has already resulted in local extinctions in regions where they were formerly abundant and very low densities in the remaining populations (Gould & Sauther, 2016).

**The Problem: Lemurs in the Pet Trade**

Given the Endangered status of *L. catta*, the live capture of these animals for the pet trade poses a serious threat to the species’ survival. *Lemur catta* were recently added to the list of the ‘World’s 25 Most Endangered Primates,’ which cites the illegal pet trade as one of the leading
causes of the species rapid population decline (LaFleur, Clarke, Ratzimbazafy, et al., 2017). Humans have a long history of overexploiting wildlife to the point of extinction and it continues to pose a serious threat to globally threatened animals (Baillie, Hilton-Taylor, & Stuart, 2004). As the human population continues to grow, so does the wildlife trade industry, including the bushmeat and wildlife trade (Corlett, 2007). Research tends to focus on the extent and implications of the bushmeat trade rather than the live capture of wildlife, but live animals are often caught as byproducts of hunting in order to be sold in markets or kept as pets (Corlett, 2007; Duarte-Quiroga & Estrada, 2003; Fa et al., 1995; Reuter, Gilles, et al., 2016). The widespread and increasing presence of the bushmeat trade in Madagascar could positively correlate to the live capture of lemurs. Until recently, traditional taboos had prevented many people from hunting *L. catta* and other mammals, but rapidly changing social norms after the political coup and the well-established trade routes of the radiated tortoise (*Astrochelys radiata*) pet trade have resulted in people actively poaching *L. catta* (Sauther et al., 2013). Adult *L. catta* are targeted for the growing demand of luxury bushmeat and the infant and juveniles are taken to cities to be sold as pets (Sauther et al., 2013).

It is difficult to assess the numbers of primates in the wildlife trade, especially in domestic trades, but some estimates suggest that 40,000 live primates are traded globally each year (Karesh et al., 2005) and, with domestic trades, this number could be in the hundreds of thousands (Nijman et al., 2011). The extent of the international wildlife trade is better understood than domestic trades due to the Convention of the International Trade in Endangered Species of Wild Fauna and Flora (CITES), an international agreement which was designed to protect endangered species from overexploitation by monitoring, regulating, and outlawing the international trade of endangered species. All primate species are protected by CITES and are
listed in either of the two highest levels of protection, Appendix I or Appendix II. *Lemur catta* is classified under Appendix I, granting it the highest level of protection in the international trade because it is threatened with extinction. The trade of the *L. catta* is only permitted in exceptional cases. While there have been some discrepancies in the numbers of species traded internationally by CITES (Blundell & Mascia, 2005), the scope of the international trade of primates and other endangered species, is much better monitored than domestic trades (Nijman et al., 2011).

It can be difficult to monitor domestic trades of wildlife because laws preventing wildlife trade are often not enforced. It is, however, becoming increasingly clear that domestic demands in countries throughout the tropics are a large driver in the primate trade (Ceballos-Mago & Chivers, 2010; Ceballos-Mago, González, & Chivers, 2010; Duarte-Quiroga & Estrada, 2003; Nijman, Martinez, & Shepherd, 2009; Shepherd, 2010). In Madagascar, the wildlife trade had previously been much less formal and organized than the markets in Asia and Africa, but recent studies have found that specialized sellers are selling large quantities of wildlife products that rival the markets in other sub-Saharan African countries (Reuter, Gilles, et al., 2016; Reuter, Randell, et al., 2016). Much of the research on the illegal wildlife trade in Madagascar focuses on bushmeat (Golden et al., 2014; Randrianandrianina, Racey, & Jenkins, 2010; Razafimanahaka et al., 2012), but it is becoming increasingly apparent that live capture is widespread. Amphibians and reptiles in the country are being taken from the wild to supply the international pet and medicinal markets at unsustainable rates, which is driving some species to extinction (Andreone, Mercurio, & Mattioli, 2006; Reuter et al., 2016). It is harder to get accurate estimates of mammals in the pet trade than birds and reptiles because the majority of birds and reptiles are sold in open markets while mammals are mostly sold in smaller quantities and by request, making it harder for scientists and law enforcement to find them easily (Bush, Baker, &
Macdonald, 2014). It was, however, found that between 2010 and mid-2013 an estimated 28,000 lemurs were illegally kept in captivity (Reuter et al., 2016). Surveys indicated that the most common lemur observed in captivity were *L. catta*, accounting for 28% of observed sightings, while no other lemur accounted for even 10% of captive lemur sightings (Reuter, 2015). *Lemur catta* are the most terrestrial of all strepsirhines species (Cameron & Gould, 2013; Jolly, 1966; Sussman, 1972), which may make them easier to capture than the more arboreal species and may partially account for their high rates in the pet trade.

The practice of live capturing lemurs to keep them as pets is illegal both internationally and domestically (Ordonnance No. 60-128, 1962) and can domestically result in confiscation and a fine. Despite this, a quarter of Malagasy people have seen a lemur being kept as a pet and nearly 30,000 lemurs were kept illegally over the course of three years (Reuter & Schaefer, 2017). These lemurs are kept by both individuals as pets and by hotels to make money from tourists (Reuter & Schaefer, 2017). The ownership of lemurs may have adverse effects on both the captive animal’s well-being and on the fitness of the wild populations or sub-populations (Ceballos-Mago et al., 2010; Reuter & Schaefer, 2016). Although Madagascar does not, many other countries regulate the ownership of primates and mandate minimum requirements for the captive conditions (Reuter & Schaefer, 2016). These baseline regulations usually require the primate is provided with food akin to what they would eat in the wild; the animal is given adequate space to exhibit natural behaviors, such as climbing and jumping; the primate is given species appropriate enrichment; and they are housed socially, unless the species is naturally solitary (Reuter & Schaefer, 2016). In Madagascar, these minimum standards for animal-welfare are often not met for captive lemurs. Reuter and Schaefer (2016) found that the majority of pet lemurs were restrained by a rope or chain or were kept in a cage. These cages did not provide
adequate space for the lemurs to exhibit their natural behaviors, such as jumping and climbing. In hotels, however, nearly all lemurs appeared to be free roaming and habituated in order to attract tourists. Captive lemurs were also fed human food instead of their natural diet and the majority of lemurs were reported to be in “poor health.” Assessments of pet lemurs being in “poor health” included lemurs that were underweight, having lacerations from restraints, and/or exhibiting stereotypical behavior (repetitive pacing, indicating poor mental health). Many captive lemurs were also aggressive, which often led to them being further restrained (Reuter & Schaefer, 2016).

In addition to the welfare issues of keeping lemurs as pets, the live capture of these animals may also have a negative effect on the long-term viability of wild populations. The effects of live capture and hunting on wild populations has not yet been studied in *L. catta*, but we can make inferences based on research done on other primates. In a study of the effects of hunting and the pet trade on yellow-breasted capuchin monkey (*Sapajus xanthosternos*), da Silva et al. (2016) found that the live capture of infant females had a greater negative impact on population viability than did the capture of infant males. This is likely due to the reproductive habits of the species because the loss of a female has a direct effect on the number of offspring. In comparison, the loss of a male does not affect the number offspring because he would be replaced by another reproductive male who copulates with multiple females (da Silva et al., 2016). Unlike the *S. xanthosternos* who have an interbirth interval of two years, which exacerbates the problem (Canale, 2010; da Silva et al., 2016; Fedigan, 1993; Fragaszy, Visalberghi, & Fedigan, 2004), 75-80% of *L. catta* give birth every year, which may help offset the population viability deficits that are associated with live capture (Gould et al., 2003; Jolly et al., 2002; Koyama et al., 2001). These high rates of the reproduction help *L. catta* recover from
years with especially high infant mortality (Cawthon Lang, 2005; Sauther et al., 1999), but because the surviving infants are targeted for the pet trade, the practice is likely still unsustainable. Additionally, the recent severe fragmentation of *L. catta* habitat has led to small, isolated populations (Gould & Sauther, 2016), which will likely exacerbate ecological effects of live capture on these small populations. Only three sites (Berenty Reserve, Bezà Mahafaly Reserve, and Anja Private Reserve) are known to have *L. catta* populations over 200 and many other sited have been extirpated of the species or have fewer than 30 individuals left, such as Kirindy-Mitea National Park, the Makay Massif, and the Mikea forest (Gould & Sauther, 2016; LaFleur, Clarke, Reuter, et al., 2017).

**Wildlife Forensic Genetics**

In order to combat the illegal pet lemur trade, we must first understand where wild individuals are being captured. Using molecular wildlife forensic methods, one can determine the source population of an individual pet of unknown origin by comparing its genetic signature to conspecifics in the wild. More specifically, by employing multilocus genotypes and statistical assignment methods, an individual can be assigned or matched to a population or geographic region while being excluded from others (Manel, Berthier, & Luikart, 2002). Determining geographic origin of illegal pets or illegal wildlife products (e.g., elephant ivory) can lead to the identification of geographic poaching ‘hotspots’, insight into wildlife trade networks, and potentially aid in the reintroduction of live captured animals back into the wild (Ogden & Linacre, 2015).

These wildlife forensic techniques have been applied to multiple species that are severely affected by the wildlife trade, including African elephants, leopards in India, and slow lorises in Vietnam (Blair et al., 2017; Mondol et al., 2015; Wasser et al., 2008). It was previously believed
that shipments of ivory confiscated in Asia was sourced broadly from across Africa. However, Wasser et al. (2008) found that most of the ivory from the two of the largest poaching rings sourced their ivory from a poaching hotspot in central Africa. Mondol et al.’s (2015) study on determining provenience of leopard skins invalidated the commonly held assumption that pelts sized in small quantities were from local leopards. This research indicated that leopard poaching is widespread throughout the Indian subcontinent and there are poaching hotspots in central India (Mondol et al., 2015). Additionally, preliminary findings on pygmy slow lorises (Nycticebus pygmaeus) in Vietnam have corroborated the trend that wildlife is trafficked from the south of the country to the north (Blair et al., 2017). The findings of these three studies will provide invaluable information for developing targeted conservation plans.

To accurately identify the geographic origin of an individual using assignment methods, the source population (or birthplace) needs to be genetically distinct from other potential source populations (i.e., reference populations) (Ogden & Linacre, 2015). If the origin is unknown, reference populations must represent the species’ entire geographic range in order to ensure all possible populations are considered for accurate assignment. Because the L. catta has a wide and diverse range across southern Madagascar, we need a broad sampling to effectively assign captive pets to their geographic origin. As of yet, there has not been a comprehensive study on the genetics of the entire population of L. catta. To date, two studies have investigated the genetics of wild populations in southwestern Madagascar (Parga et al., 2012) and in south-central Madagascar (Anja Community Reserve, Tsaranoro Valley Forest, and Sakaviro) (Clarke, 2015; Clarke et al., 2015).

Parga et al. (2012) primarily investigated the presence of a population genetic bottleneck of two populations of L. catta in southwestern Madagascar: Beza Mahafaly Special Reserve
(BMSR) and Tsimanampetsotsa National Park (TNP). While they found evidence of a recent population genetic bottleneck of these lemurs due to climate events and human disturbances, they also reported observed and expected heterozygosity. These statistics are useful in determining the genetic diversity and health of the populations. *L. catta* in both BSMR (600 ha) and TNP (48,000 ha) showed high levels of heterozygosity (BMSR $H_O=0.80$, MNA=8.62; TNP $H_O=0.80$, MNA=9.13) (Parga et al., 2012) that were consistent with or higher than other studies using the same microsatellite markers (Jekielek & Strobeck, 1999; Pastorini et al., 2005; Zaonarivelo et al., 2007).

Comparatively, three populations in south-central Madagascar had similar levels of heterozygosity but lower levels of the mean number of alleles across all loci. Clarke’s (2015, et al 2015) study sites were smaller and had small population sizes: Anja (34 ha), Sakaviro (14 ha), and Tsaranoro (46 ha). Despite the sites being severely fragmented and having small populations of *L. catta*, they still maintained moderate genetic diversity (Anja $H_O=0.88$, MNA=5.17; Sakaviro $H_O=0.79$, MNA=4.83; Tsaranoro $H_O=0.72$, MNA=5.83) when compared to the larger populations in the southwest. Clarke et al. (2015) also found that there was little genetic difference between the populations, but the differences increased as a product of increasing geographic distance. While these populations are not currently revealing a loss in genetic diversity, it is likely because they are exhibiting a lag response due to the recent intense fragmentation. Should this be the case, genetic diversity will markedly decrease as the effects of fragmentation catch up to the *L. catta*’s uncanny ability to be immune to environmental stressors (Clarke et al., 2015).

If we are to effectively determine the origins of *L. catta* being kept as pets, we will need to include these populations as potential source populations as well as other wild *L. catta*
populations across their range. Population genetic data will need to be collected from other extant populations throughout southwestern Madagascar as well as southern Madagascar.

**SPECIFIC AIMS**

This research serves as a pilot study to assess the plausibility of determining the provenience of individual wild-caught rescued and current pet *L. catta* based on a sampling of four wild populations (i.e., source populations). The specific aims of this study are to 1) assess the genetic diversity and structure of the four source populations, and 2) assign individual wild-caught rescued and current pets to their wild population of origin, thereby identifying provenience of capture for these animals. To do this, I performed microsatellite genotyping for a total of n=55 wild and captive *L. catta*. I genotyped samples from 5 wild individuals from Isalo National Park in southwestern Madagascar, and 20 captive individuals – those that are currently or were rescued from being kept as pets – at seven polymorphic loci. I will also include the genotypes of the three previously genotyped populations in south-central Madagascar: Anja (n=10), Sakaviro (n=10), and Tsaranoro (n=10) (Clarke, 2015; Clarke et al., 2015). All individuals were assessed at the same seven polymorphic loci. This study will serve as a foundation for future research into the origin of *L. catta* pets when more potential source populations are included.

**METHODS**

**Study Sites and Sample Collection**

**Study Sites.** Noninvasive fecal samples were opportunistically collected from wild *L. catta* individuals in Isalo National Park and from captive *L. catta* around southern Madagascar by Tara A. Clarke (TAC) and Marni LaFleur (ML). Isalo National Park (22°29.26' S, 45°22.73'
E) is located in southwestern Madagascar and has several dense but isolated populations of *L. catta*, but their population size estimates are unknown (Gould & Sauther, 2016; LaFleur, Clarke, Reuter, et al., 2017; Sussman et al., 2003). The 81,500 ha park’s landscape is characterized by large rock massifs of sandstone and intrusive igneous rock formations (Sussman et al., 2003). The majority of the park consists of exposed rock or grass savannah (Hawkins, 1999). There is tree savannah interspersed between the grass savannah and in the valleys of the rock formations are dense, closed-canopy forests where rainwater collects (Hawkins, 1999; Sussman et al., 2003).

Fecal samples were also collected from captive *L. catta* at the Lemur Rescue Center (LRC; 23°07′19″S 43°37′17″E) and from individuals being kept as pets. The LRC is located within the Reniala Botanical Reserve and currently houses 26 *L. catta* that were rescued from the illegal pet and bush meat trade (LaFleur et al., 2015). The center has been taking in *L. catta* since 2011, when Malagasy officials began confiscating illegally owned *L. catta* pets (Gould & Andrianomena, 2015; LaFleur et al., 2015). Samples were also collected from ring-tailed lemurs being kept as personal pets in Toliara (23°21′22″S 43°41′28″E) and at a hotel in Tsinjoriake (23°32′08″S 43°44′43″E) in southwestern Madagascar. These individuals were discovered by citizen reports to the Pet Lemur Survey Project.

For a reference of potential source populations, I used the genotypes of 30 adult *L. catta* from three wild populations: Anja, Sakaviro, Tsaranoro (Clarke, 2015; Clarke et al., 2015) (Table 1). These populations are in the central highland region of Madagascar and are all comprised of mixed xerophytic, deciduous vegetation, and large granite outcrops (Cameron & Gould, 2013; Clarke, 2015; Clarke et al., 2015; Gabriel, 2013). This region of Madagascar has been almost completely deforested over the last 50 years and has just a few small forest fragments scattered throughout (Gould & Gabriel, 2015). The Anja Reserve (21°51′12″S
46°50'40"E; 34 ha) has a population of ~220 *L. catta* (Cameron & Gould, 2013); Sakaviro (21°47'03"S 46°52'02"E; 14 ha) has a population of ~27 *L. catta* (Gould & Andrianomena, 2015); and Tsaranoro Valley (22°05'11"S 46°46'14"E; 46 ha) has a population of ~80 *L. catta* (Gould & Andrianomena, 2015). The Anja and Sakaviro fragments are ~12 km apart and are both isolated from other forest fragments due to highways and anthropogenic savannah, making dispersal unlikely (Clarke, 2015; Clarke et al., 2015; Gould & Andrianomena, 2015; Gould & Gabriel, 2015). The Tsaranoro fragment is 27 km southwest of Anja and is <5 km away from two other forest fragments in the Tsaranoro valley (Gould & Andrianomena, 2015). These three fragments may comprise a single breeding population of *L. catta* (Clarke, 2015).
Figure I. Map of sampling localities. Wild populations are marked blue (Isalo National Park, Sakaviro, Anja Community Reserve, and Tsaranoro Valley Forrest) and captive individuals are marked green (Lemur Rescue Center, Hotel in Tsinjoriake, and personal pets in Toliara). The historical geographic distribution of *L. catta* in yellow is based on Goodman et al. (2006).

Table I. Sampling localities, geographic coordinates, and sample sizes of wild populations and the sampling size of the captive *L. catta* used in this study.

<table>
<thead>
<tr>
<th>Site Name</th>
<th>Latitude</th>
<th>Longitude</th>
<th>n</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anja</td>
<td>21°51'12&quot; S</td>
<td>46°50'40&quot; E</td>
<td>10</td>
<td>Clarke et al., 2015</td>
</tr>
<tr>
<td>Isalo National Park</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canyon of Maki</td>
<td>22°29'16&quot; S</td>
<td>45°22'44&quot; E</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Campsite</td>
<td>22°32'31&quot; S</td>
<td>45°22'44&quot; E</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Sakaviro</td>
<td>21°47'03&quot; S</td>
<td>46°52'02&quot; E</td>
<td>10</td>
<td>Clarke et al., 2015</td>
</tr>
<tr>
<td>Tsaranoro</td>
<td>22°05'11&quot; S</td>
<td>46°46'14&quot; E</td>
<td>10</td>
<td>Clarke et al., 2015</td>
</tr>
<tr>
<td>Captive Individuals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lemur Rescue Center</td>
<td>23°07'19&quot; S</td>
<td>43°37'17&quot; E</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Hotel in Tsinjoriake</td>
<td>23°32'08&quot; S</td>
<td>43°44'43&quot; E</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Illegal pet in Toliara</td>
<td>23°21'22&quot; S</td>
<td>43°41'28&quot; E</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>
**Sample Collection.** Fecal samples were collected by TAC and ML from Isalo in July 2016 and from captive *L. catta* in July and August 2016. At Isalo, samples were randomly collected from individuals (n=3) that came through a tourist campsite (22°32′31″S 45°22′44″E) and from individuals (n=2) in the Canyon of Maki (22°29′16″S 45°22′44″E). Samples were collected from ex-pet lemurs at the LRC and were opportunistically collected from pet lemurs the field researchers encountered in their travels. All samples were immediately stored in RNA*later* reagent (Ambion, Austin, Tex., USA) solution to prevent DNA degradation. Samples were transported to the Primate Molecular Ecology Lab (PMEL; Hunter College of the City University of New York, New York, NY) where they were stored at -80°C.

**DNA Extraction and Microsatellite Genotyping**

**DNA extraction.** Genomic DNA was extracted from samples genotyped in this study using QIAamp DNA Stool Mini Kits (QIAGEN, Valencia, CA). Extraction followed the manufacturer’s protocol with one modification: starting material was increased to 300mg to account for the weight of the RNA*later* solution.

**Microsatellite genotyping.** Samples were amplified at seven highly polymorphic microsatellite markers that have been shown to reliably amplify fecal DNA: L-2 (Merenlender, 1993), Efr09 (Jekielek & Strobeck, 1999), Lc5, Lc6, Lc7 (Pastorini et al., 2005), 69HDZ267, 69HDZ299 (Zaonarivelo et al., 2007) (Appendix). Extraction products were amplified via PCR in a 13 µL reaction volume using HotStarTaq DNA polymerase Master Mix, BSA, primer pairs, and 3 µL gDNA using annealing temperatures specified in the literature (Appendix). PCR products were run at the Yale DNA Analysis Facility on Science Hill on an ABI 3730xl Genetic Analyzer (Applied Biosystems) and an internal size standard, Rox-500, was added. Allele calls
were made using GeneMarker Software v.2.6.7 (SoftGenetics) and were checked for consistency across plates by including positive controls. Genotype assignment was based on multiple independent reactions. Heterozygous genotypes were confirmed with at least two independent reactions and homozygous individuals were confirmed with five independent reactions (Morin et al., 2001; Taberlet et al., 1996).

**Population Genetic Analysis**

**Genetic diversity.** All loci were tested for the presence of null alleles using the program MICRO-CHECKER (van Oosterhout et al., 2004). Loci were tested for deviations from Hardy-Weinberg equilibrium using the program GENEPOP v.4.2 and 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), and observed (H\textsubscript{O}) and expected (H\textsubscript{S}) heterozygosities for each sampling location were calculated using GENODIVE (Meirmans & Van Tienderen, 2004). Allelic richness (AR) was standardized to the smallest sample size in the dataset to account for uneven sampling between populations using HP-RARE 1.1 (Kalinowski, 2005). Wright’s F\textsubscript{IS} was then calculated to measure deviations from Hardy–Weinberg equilibrium with 10,000 permutations (Weir & Cockerham, 1984).

It should be noted that for all analyses of the Anja, Sakaviro and Tsaranoro populations (Clarke, 2015; Clarke et al., 2015), only the seven microsatellite markers that I used for my genotyping of the captive individuals and the Isalo population were used in the analysis (Appendix).
Population genetic structure. To assess the genetic distances between the sampling localities, I calculated Wright’s $F_{ST}$ (Weir & Cockerham, 1984) using $G_{ENODIVE}$ (Meirmans & Van Tienderen, 2004). $F_{ST}$ is a measure of genetic differentiation and uses pairwise comparisons to illustrate whether and to what extent two populations are considered genetically distinct populations.

To infer population genetic structure from my sample of 55 *L. catta*, I first used a model-based Bayesian clustering method in *STRUCTURE* v2.3.4 (Pritchard, Stephens, & Donnelly, 2000). This method uses the Markov Chain Monte Carlo (MCMC) approach to infer the optimal number of genetic populations ($K$) and groups individuals into those groups based on their multilocus genotypes. No a priori information regarding the individuals’ geographic sampling locations was provided so clusters were formed solely on genetic information. I evaluated the hypothesis $K=1-7$, three more than the number of wild populations (Evanno, Regnaut, & Goudet, 2005), using 100,000 iterations of MCMC following a burn-in of 50,000 iterations (Baden et al., 2014; Holmes et al., 2013). I implemented 20 runs for each value of $K$, assuming admixture and correlated allele frequencies. The admixture model lets us estimate the of the number of natural genetic clusters and detect of historical population admixture (Falush, Stephens, & Pritchard, 2003; Ostrowski et al., 2006). The most likely number of genetic populations ($K$) was assessed using the highest value of $\Delta K$ (Evanno et al., 2005) using the program *STRUCTURE HARVESTER* v0.6.94 (Earl & vonHoldt, 2012). *STRUCTURE HARVESTER* calculates the second order rate of change of the likelihood distribution ($\Delta K$), which indicates the most pronounced subdivision within the data and the optimal number of genetic clusters.

To corroborate my *STRUCTURE* analysis, I used a Discriminant Analysis of Principle Components (DAPC) (Jombart, Devillard, & Balloux, 2010) in R Studio, using the adegenet
package (Jombart, 2008; RStudio Team, 2015). This multivariate method identifies clusters of genetically related individuals that maximize between-group variability and minimize within-group variability by using a set of retained principle components (determined by the user to optimize variance explained). The optimal number of clusters is determined by the number of clusters with the smallest Bayesian Information Criterion (BIC) value. Because the DAPC is not a model-based analysis and cannot be driven by an underlying population genetic model, it is more flexible than Bayesian clustering methods.

To further substantiate both the S\textsc{structure} and DAPC results, I performed a principal coordinates analysis (PCoA) with a standard genetic distance matrix (Nei, 1978) using GENALEX v.6.5 (Peakall & Smouse, 2012).

Lastly, I executed an exclusion test (Cornuet et al., 1999) in GeneClass2 (Piry et al., 2004). This type of assignment test calculates the likelihood that an individual in question belongs to each candidate population by comparing its genotypes to 10,000 simulated genotypes of each reference population. The likelihood of the individual belonging to each reference population can be used to rule out one or more of the source populations as the population of origin. Unlike the S\textsc{structure} analysis, this assignment test does not assume the true population of origin was sampled from. I used the partial Baysean method (Rannala & Mountain, 1997) and performed Monte-Carlo resampling simulations following Paetkau et al. (2003).

\section*{RESULTS}

\textbf{Genetic Diversity}

All seven loci (described in Table II) were polymorphic with 10-16 alleles. Individuals were pooled across sampling localities and there was no evidence of linkage disequilibrium
across markers. Two loci were found to significantly deviate from Hardy-Weinberg equilibrium (L-2 and Lc7; Table II), although they did not deviate at any one site specifically and were therefore used in further analysis. Allelic richness varied from 3.12-3.24 across the sampling localities of wild *L. catta* and was higher among the captive individuals (3.84 ± 0.23). The mean allelic richness was 3.30 ± 0.487 when pooled across sampling sites (Table III). The mean observed heterozygosity across sampling sites was 0.794 ± 0.074, while the mean expected heterozygosity was 0.762 ± 0.057. Overall *F*$_{IS}$ was -0.042 (95% confidence interval of -0.127 to 0.050). *F*$_{IS}$ values ranged from -0.183 at Sakaviro to 0.054 at the captive group, with all populations significantly deviating from 0 except for Anja. Mean *F*$_{ST}$ over all sampling localities was 0.108 ± 0.037. Nearly all pairwise comparisons between sampling localities had significant *F*$_{ST}$ values except Isalo-Anja (Table IV). This suggests that nearly all sampling locations were genetically distinct.
Table II. Characteristics of 7 microsatellite markers amplified in 55 *L. catta* 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.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Size Range</th>
<th>Annealing Temp</th>
<th>n_A</th>
<th>H_o</th>
<th>H_e</th>
<th>HWE</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-2</td>
<td>179-203</td>
<td>48</td>
<td>11</td>
<td>0.816</td>
<td>0.785</td>
<td><strong>0.0160</strong></td>
<td>----</td>
</tr>
<tr>
<td>Efr09</td>
<td>92-126</td>
<td>54</td>
<td>13</td>
<td>0.688</td>
<td>0.799</td>
<td>0.1072</td>
<td>AF104091</td>
</tr>
<tr>
<td>Lc5</td>
<td>127-151</td>
<td>60</td>
<td>10</td>
<td>0.629</td>
<td>0.717</td>
<td>0.5160</td>
<td>AY366441</td>
</tr>
<tr>
<td>Lc6</td>
<td>248-270</td>
<td>60</td>
<td>10</td>
<td>0.811</td>
<td>0.761</td>
<td>0.2706</td>
<td>AY366442</td>
</tr>
<tr>
<td>Lc7</td>
<td>172-198</td>
<td>60</td>
<td>12</td>
<td>0.745</td>
<td>0.688</td>
<td><strong>0.0396</strong></td>
<td>AY366443</td>
</tr>
<tr>
<td>69HDZ267</td>
<td>156-178</td>
<td>55</td>
<td>16</td>
<td>0.907</td>
<td>0.758</td>
<td>0.8883</td>
<td>EF093488</td>
</tr>
<tr>
<td>69HDZ299</td>
<td>238-262</td>
<td>58</td>
<td>12</td>
<td>0.963</td>
<td>0.828</td>
<td>0.7803</td>
<td>EF093489</td>
</tr>
</tbody>
</table>

Table III. Allelic diversity within each of 4 sampling localities and of the captive *L. catta*, including mean number of alleles per locus (MNA), allelic richness (AR), observed (H_O) and expected (H_S) heterozygosity, inbreeding coefficient (F_IS), and significant deviations from Hardy-Weinberg Equilibrium (HWE) calculated using 10,000 iterations. Significant values (p < 0.05) are shown in bold.

<table>
<thead>
<tr>
<th>Site</th>
<th>N</th>
<th>MNA</th>
<th>AR (SE)</th>
<th>H_O</th>
<th>H_S</th>
<th>F_IS</th>
<th>HWE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captive</td>
<td>20</td>
<td>9.57</td>
<td>3.84 (0.231)</td>
<td>0.816</td>
<td>0.863</td>
<td>0.054</td>
<td>0.4108</td>
</tr>
<tr>
<td>Isalo</td>
<td>5</td>
<td>4.29</td>
<td>3.17 (0.731)</td>
<td>0.857</td>
<td>0.736</td>
<td>-0.165</td>
<td>0.8076</td>
</tr>
<tr>
<td>Anja</td>
<td>10</td>
<td>5.00</td>
<td>3.13 (0.440)</td>
<td>0.747</td>
<td>0.739</td>
<td>-0.011</td>
<td>0.0940</td>
</tr>
<tr>
<td>Sakaviro</td>
<td>10</td>
<td>4.86</td>
<td>3.12 (0.375)</td>
<td>0.860</td>
<td>0.727</td>
<td>-0.183</td>
<td><strong>0.0032</strong></td>
</tr>
<tr>
<td>Tsaranoro</td>
<td>10</td>
<td>6.00</td>
<td>3.24 (0.659)</td>
<td>0.690</td>
<td>0.738</td>
<td>0.064</td>
<td>0.6422</td>
</tr>
<tr>
<td>Overall</td>
<td>55</td>
<td>5.94</td>
<td>3.30 (0.487)</td>
<td>0.794</td>
<td>0.762</td>
<td>-0.042</td>
<td>-</td>
</tr>
</tbody>
</table>

Table IV. Pairwise FST values (above diagonal) and indication of significance FST values (below diagonal) among sampling localities of *L. catta*. Significant values indicated with * (P < 0.005 after Bonferroni corrections).

<table>
<thead>
<tr>
<th></th>
<th>Captive</th>
<th>Isalo</th>
<th>Anja</th>
<th>Sakaviro</th>
<th>Tsaranoro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Captive</td>
<td>-</td>
<td>0.076</td>
<td>0.067</td>
<td>0.093</td>
<td>0.089</td>
</tr>
<tr>
<td>Isalo</td>
<td>*</td>
<td>-</td>
<td>0.114</td>
<td>0.174</td>
<td>0.169</td>
</tr>
<tr>
<td>Anja</td>
<td>*</td>
<td>NS</td>
<td>-</td>
<td>0.087</td>
<td>0.087</td>
</tr>
<tr>
<td>Sakaviro</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>0.126</td>
</tr>
<tr>
<td>Tsaranoro</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>-</td>
</tr>
</tbody>
</table>
Population Genetic Structure and Assignments

A STRUCTURE analysis (Fig. I) identified two genetic clusters, as indicated by the highest value of ΔK. Sampling localities appeared to primarily cluster geographically, with the western most sampling location, Isalo, primarily clustering into cluster 2 and the more eastern populations (Anja, Sakaviro, and Tsaranoro) primarily clustering into cluster 1. The majority of captive individuals (18 of 20), clustered together with the Isalo population. Although Isalo had a small sample size (n=5), this could potentially suggest the captive *L. catta* are from more westerly populations, including those in or around Isalo.

![Figure II. STRUCTURE analysis. Each bar represents the of proportional membership (Q) of an individual lemur belonging to two clusters.](image)

Results from the Discriminant Analysis of Principle Components (DAPC) also suggest that two genetic clusters are present in the data. Like the STRUCTURE analysis, the captive individuals mainly formed one cluster and the Anja, Sakaviro, and Tsaranoro populations formed another. However, there was only weak evidence for classifying the data in two versus one clusters, as indicated by the difference in the Bayesian Information Criterion (ΔBIC) between the $K = 1$ and $K = 2$ was $ΔBIC = 1.5$. 

A principal component analysis (PCoA; Fig. III) supported the results of the STRUCTURE and DAPC analyses, in that there was loose clustering of the Isalo population along axis 2. The majority of the captive *L. catta* also clustered along axis 2 with the western Isalo population. However, clusters were not well defined. The first axis accounted for 11.51% of the total molecular variance while another 8.22% of the variance was explained by Axis 2, which separated Isalo from the more eastern populations in the central highlands.
Population assignment tests of the four wild populations in GeneClass resulted in individuals being accurately assigned 97.14% of the time to their respective population. However, these individuals were excluded from all other populations in only 20.5% of these assignments. One individual from Sakaviro was incorrectly assigned to Anja (99.9% Anja, 98.6% Sakaviro). When captive individuals were assigned to the four wild populations, 3 individuals (15%) could be assigned to a population while being excluded from all other populations. One individual was excluded from Isalo but not from the other three populations. However, 16 captive individuals (80%) of the 20 were excluded from all populations and could not be assigned to any of the sites in this study.
DISCUSSION

Genetic Differentiation and Diversity of Wild Populations

**Genetic Differentiation.** My analyses suggest that the three populations of ring-tailed lemurs in the central highlands (Anja, Sakaviro, and Tsaranoro) represent one genetic cluster and the lemurs in Isalo National Park may be a genetically distinct population. The results of both my STRUCTURE analysis and Discriminant Analysis of Principal Components (DAPC) when comparing all four wild populations and the captive individuals clustered the Anja, Sakaviro, and Tsaranoro populations together. The STRUCTURE analysis, however, grouped 26.67% of the central highland lemurs (3 Anja, 2 Sakaviro, and 3 Tsaranoro) with the Isalo and captive cluster and the DAPC grouped 10% of these lemurs (1 Anja and 2 Sakaviro) into the captive and Isalo cluster. Only one individual from Sakaviro was placed in the other group in both the STRUCTURE analysis and DAPC, while the other individuals were only grouped in the other cluster in one of the two analyses. The STRUCTURE analysis clustered all the Isalo individuals together along with the majority of the captive individuals, while the DAPC only clustered 40% with the captive individuals. Although the clustering was not identical across both analyses, they both showed general trends of genetic clustering based on geographic location and that the captive individuals were clustering with the more southwestern population, Isalo, rather than the three populations in the central highlands. The principal component analysis also supported these data as it demonstrated loose clustering based on geographic location, with the captive ring-tailed lemurs grouping more closely with the Isalo population, rather than the other three populations. The small number of samples (n=5) from Isalo could be interfering with the results, which may become clearer as more individuals are genotyped from that population.
Pairwise $F_{ST}$ comparisons also support the evidence that genetic differences in populations increases as a product of geographic distance because three of the four highest $F_{ST}$ values were between Isalo and the other three wild populations with only the Sakaviro-Tsaranoro comparison being more closely related than the Isalo-Anja comparison (the only nonsignificant result). Yet, Sakaviro and Tsaranoro are the farthest apart (34 km) among the highland populations, supporting the evidence that the populations are more significantly different the farther away they are due to the severe habitat fragmentation in Madagascar. These results are similar to those found in *Microcebus ravelobensis* ($F_{ST} = 0.01$-$1.15$) which showed evidence of isolation-by-distance, although the populations were still exhibiting levels of genetic differentiation during a time when their forest fragments may have been connected (Olivieri et al., 2008). This indicates that fragmentation did not occur too long ago and that they are still exhibiting historic, rather than current, structuring due to a time lag.

**Genetic Diversity.** Levels of observed heterozygosity were similar to those found in the Beza Mahafaly Special Reserve (BMSR) and Tsimanampetsotsa National Park (TNP), though MNA was lower (BMSR $H_o$=0.79, MNA=8.29; TNP $H_o$=0.79, MNA=8.43) (Parga et al., 2012). Parga et al. (2012) used eight microsatellite markers, but for this analysis and comparison I used seven microsatellite markers. To compare our populations, I only used the seven microsatellite markers that were identical to those utilized in my analysis. Although Isalo National Park is the largest fragment in this analysis (81,500 ha), it showed the lowest levels of allelic diversity (MNA=4.29) yet some of the highest levels of heterozygosity ($H_O$=0.86). The genetic diversity in Isalo were similar to the smallest fragment, Sakaviro (MNA=4.86, $H_O$=0.86). At the time of this analysis, only five individuals were genotyped from this populations, which could skew the
results. More individuals will need to be genotyped from Isalo before we can conclude that Isalo is exhibiting significant losses of allelic diversity.

Additionally, levels of genetic variability within *L. catta* in south-central and southwestern Madagascar were similar to those found in other strepsirrhines, including *Propithecus tattersalli* (MNA=6.3, $H_o=0.69$) and *Microcebus* spp. (MNA=4.38–6.50, $H_o=0.47–0.69$) (Olivieri et al., 2008; Quéméré et al., 2010).

**Assignment of Captive Lemurs**

The cumulative results of my assignment tests indicate that the vast majority of the pet lemurs sampled did not originate from any of the four wild populations used in this study. When the origin population of sampled individuals are not known, it is recommended that both Bayesian assignment tests (STRUCTURE; Pritchard et al., 2000) and Bayesian exclusion tests (Cornuet et al., 1999) are used to avoid assignment errors (Manel et al., 2002; Manel, Gaggiotti, & Waples, 2005). Using the results of both tests, just one individual can be assigned to Tsaranoro. This individual had not been confiscated by Malagasy authorities and taken to the Lemur Rescue Center (LRC), but was instead being kept at a hotel Tsinjoriable on the southwestern coast of Madagascar, ~350km southwest of Tsaranoro. This suggests that the hotel did not capture this lemur from the populations in the nearby forests, but instead may have bought the lemur from a wildlife trader. Another individual from the LRC was narrowed down to coming from Anja, Sakaviro, or Tsaranoro, but the exact population is unknown. The other two individuals that were assigned to a population in the exclusion test were not assigned to those populations in the STRUCTURE analysis, so they cannot be definitively assigned to a population.
The results of all three assignment tests indicate that the majority of the captive individuals came from populations that were not included in this analysis. Thus, 80% of the captive individuals were excluded from all four populations. In both the STRUCTURE and DAPC analyses, most of the captive individuals formed one cluster, while the Anja, Sakaviro, and Tsaranoro populations formed another. While Isalo clustered with the captive individuals in the STRUCTURE analysis and was split between the two clusters in the DAPC, the principal coordinates analysis indicated it was loosely separated from the other populations due to its geographic distance. More individuals are currently being genotyped from this population, which will help strengthen these results.

The wild populations in this study are geographically isolated, but they are exhibiting a time lag in their loss of genetic diversity due to the sites being fragmented relatively recently. As time goes on, these populations will likely become more genetically distinct and assigning individuals to a source population will become more well-defined. As this happens, effective population sizes will decrease, gene flow will be reduced, genetic variability will decrease, and inbreeding will increase (Craul et al., 2009; Holmes et al., 2013; Olivieri et al., 2008; Parga et al., 2012; Radespiel et al., 2008).

Future Study and Conservation Implications

While this pilot study did show evidence of genetic clustering of L. catta, the results made it clear that sampling needs to be greatly broadened in order to reflect the full range of L. catta geographic range. Additional genotyping is underway, including samples from Berenty Private Reserve in southern Madagascar, populations near Tolaria, southwestern Madagascar, and additional samples from Isalo National Park. Additional reference genotypes will be
included from Beza Mahafaly Special Reserve (BMSR) and Tsimanampetsotsa National Park (TNP) (Parga et al., 2012). The inclusion of these sites will more closely represent the entire population of *L. catta* and I expect that genetic clustering based on geographic distance will become more evident and there may be more than two genetic clusters. These data will allow for more accurate assignment of pet lemurs to their wild population of origin. Because most of the captive samples collected for this study were located near the southwestern coast of Madagascar and *L. catta* are known to be sold in the southwestern city, Toliara (Sauther et al., 2013), it is likely that many of these pet lemurs came from sites in this region of the country. However, recent studies have found that several populations in this area near the western coast have recently been extirpated due to mining, hunting, and live capture (e.g. Ranobe, Reniala Private Reserve, Ifaty forest) while other populations are nearly locally extinct (e.g. Tsinjoriake) (Gould & Sauther, 2016; LaFleur, Clarke, Reuter, et al., 2017). These local extinctions may be the reason that the hotel in this region did not get their lemur from a local population, but instead from Tsaranoro, which is ~350km away but has larger *L. catta* populations remaining. When sites with extant populations from this region are included (populations near Toliara, BSMR, and TNP), I expect that provenience for many of the pet *L. catta* will be from this region. If it is found that majority of pet lemurs were sourced from more distant populations, it will allow us to better understand trade routes.

When sampling is broadened in the future, this project will represent the first comprehensive analysis of the genetic health of ring-tailed lemurs throughout their entire range and will provide critical guidance for conservation management. There have been surprisingly few studies on the genetic diversity of ring-tailed lemurs and given their recent population declines, it is imperative to obtain information that can help preserve the species long-term.
viability in the wild. Future genetic analyses will allow for the identification of populations that maintain healthy levels of genetic diversity and gene flow which will inform potential release sites for the possible future reintroduction of wild caught pet ring-tailed lemurs.

This study, when completed, will represent one of the first reference genetic databases with the intent of identifying poaching hotspots in non-human primates, and will be the first time these techniques are used to track the pet trade exclusively, rather than wildlife products. A similar study is currently being conducted to identify trade networks and provenience of pygmy slow lorises (*Nycticebus pygmaeus*) in Vietnam (Blair et al., 2017). This type of genetic database has also been successfully developed for tracing confiscated elephant ivory to its origin in Africa (Wasser et al., 2015) and to identify the origin of confiscated leopard skins in India (Manel et al., 2002; Mondol et al., 2015). This relatively new application of these forensic techniques has directly impacted the conservation of these species. For example, prior to Wasser et al.’s (2008) study on ivory poaching, it was widely believed that ivory dealers were sourcing products from across Africa as stock became available. However, the study revealed that two of the largest ivory poaching syndicates both sourced all of their ivory from similar locations in and around Zambia (Wasser et al., 2008). This information was only revealed after performing genetic assignment techniques because ivory was moved to many different intermediary countries before being shipped to its destination in Asia as a means of reducing risk by distancing the syndicates from the source. Previous techniques of tracing ivory only began at the shipping origin location which could be very far from where the animals were poached (Wasser et al., 2008).

Only recently have studies in Madagascar shown that the country’s wildlife trade is becoming formalized and that wildlife products are being transported large distances (Reuter, Randell, et al., 2016). It was previously believed that wild caught lemurs and bushmeat were
sourced locally, but this project could potentially reveal more formalized trade routes. The one confidently assigned individual in this study was sourced ~350 km away from the hotel where it was kept, which could be early evidence of trade routes. As broader data is added to this research, trade routes could become more evident, or it may reveal that the majority of pet lemurs are sourced locally. Knowing this information will allow for more targeted and effective conservation measures to be developed. If most trades are happening locally, targeted education and community outreach may be an effective conservation strategy. However, if this study finds that the trade of pet *L. catta* is organized and trade routes are discovered, education efforts may not be sufficient to combat the trade. Instead, stricter law enforcement may be necessary.

Although Madagascar’s laws prohibit the buying, selling, and owning of lemurs they are not strictly enforced; thus, this study could provide crucial insight into trade networks and mitigate much of the investigative efforts that would otherwise be needed to identify and crack down on trade networks. Potential poaching hotspots and trade routes identified by this study could help law enforcement more efficiently and effectively stop the trade of live lemurs at the source.

This novel approach to understanding the primate pet trade will give us critical insight into the growing conservation issue that is the wildlife trade. By identifying poaching hotspots where lemurs are being captured, conservationists will be able to implement targeted actions that will be more effective than broad outreach campaigns. By combining forensic methods with conservation genetics, it is possible to directly apply scientific research into investigative processes. As such, this emerging and growing field is gaining enthusiasm from government agencies, nonprofit organizations and the scientific research communities alike as a promising tool for combatting the growing illegal wildlife trade industry (Alacs et al., 2010; Ogden, Dawnay, & McEwing, 2009). If applied to other species, these methods could help the estimated
hundreds of thousands of primates affected by the wildlife trade every year (Nijman et al., 2011) as well as any species that is threatened by the wildlife trade.
References


**APPENDIX.** Primer size range, sequence, repeat motifs, annealing temperatures, and GenBank accession number of the seven microsatellite markers used.

<table>
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<th>Repeat Motif</th>
<th>Annealing Temp (°C)</th>
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References: (1) Merenlender, 1993; (2) Jekielek and Strobeck; (3) Pastorini et al., 2005; (4) Zaonarivelo et al., 2007