Molecular phylogenetics and conservation genetics of sportive lemurs (*Lepilemur* spp.) in northwestern Madagascar

Von der Naturwissenschaftlichen Fakultät
der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades eines
Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Dipl.-Biol. Mathias Craul

geboren am 22.11.1973 in Hannover

2009

Referenten: Prof. Dr. Elke Zimmermann

Korreferentin: PD Dr. Heike Hadrys

Tag der Promotion: 27. Juli 2009

To my family

Table of contents

Chapter 1

Abstract

Molecular phylogenetics and conservation genetics of sportive lemurs (*Lepilemur* spp.) in northwestern Madagascar

The aim of this thesis was to investigate for the first time the phylogeographic pattern and processes in a large-bodied lemur genus, *Lepilemur*, in northwestern Madagascar. Both ancient (genetic drift and speciation) and recent (habitat fragmentation due to anthropogenic disturbances) processes of genetic differentiation should be disentangled in order to develop effective conservation strategies. Sportive lemurs (*Lepilemur* spp.) are medium-sized nocturnal primates that occur in different types of primary and secondary forests throughout Madagascar. Several recent taxonomic revisions resulted in an extraordinary increase of recognized species with poorly known distributions. Northern and northwestern Madagascar, a part of the island that is subject to high levels of human interventions, has hardly been studied in this respect. The first aim was to clarify the biogeography and phylogenetic relationships of sportive lemurs in northwestern Madagascar. We tested predictions derived from two biogeographic models by exploring the genetic and morphological divergence among populations of *Lepilemur*. By using the phylogenetic analyses of mtDNA sequence data, molecular diagnostic sites and phenotypic morphometric traits, we uncovered two previously undetected species. Moreover, the distribution patterns of the seven species of *Lepilemur* in the study region contradict the two existing biogeographic models. Brief species descriptions are provided and a new biogeographic model is proposed (the 'large river model'). The second aim was to develop microsatellite markers for conservation genetics studies of the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*). We isolated 21 microsatellites. Seventeen loci amplified and were found to be polymorphic. The third aim was to investigate the effects of forest fragmentation on presence, abundance and genetic diversity in *L. edwardsi*. The loss and fragmentation of forest habitats are well known consequences of human activities. One result of this study is the disappearance of *Lepilemur* from many fragments due to hunting. In addition, mtDNA and microsatellite markers detected a negative influence of forest fragmentation on genetic diversity, and revealed signals of a past demographic bottleneck. Given the results, urgent conservation actions are needed and should concentrate on an effective protection in order to ensure the long-term survival of *L. edwardsi* and the other sportive lemur species of the region.

Keywords: *Lepilemur*, conservation, phylogenetics

Chapter 2
Zusammenfassung

Molekulare Phylogenie und Naturschutzgenetik von Wieselmakis (*Lepilemur* spp.) im Nordwesten Madagaskars

Das Ziel dieser Studie war es, die phylogeographischen Muster und Prozesse einer größeren Lemurengattung, *Lepilemur*, im Nordwesten Madagaskars zu untersuchen. Dabei sollten sowohl historische (genetische Drift und Speziation) als auch rezente (Habitatfragmentierung beruhend auf anthropogenen Störungen) Prozesse genetischer Differenzierung herausgearbeitet werden, um effektive Naturschutz-Strategien zu entwickeln. Wieselmakis (*Lepilemur* spp.) sind mittelgroße, nachtaktive Primaten, die in Madagaskars Primär- und Sekundärwäldern vorkommen. Aufgrund einer Reihe von taxonomischen Revisionen ist die Anzahl beschriebener Wieselmakiarten außergewöhnlich stark angestiegen. Die Verbreitung dieser neu beschriebenen Arten ist jedoch kaum bekannt. Der Norden und Nordwesten Madagaskars mit seinen starken anthropogenen Einflüssen wurde in diesem Zusammenhang bisher kaum untersucht. Das erste Ziel dieser Studie war es daher, die Biogeographie und Phylogenie der Wieselmakis im Nordwesten Madagaskars aufzuklären. Wir testeten die Voraussagen zweier biogeographischer Modelle, indem wir die genetische und morphologische Divergenz zwischen verschiedenen Populationen von Wieselmakis untersuchten. Mit Hilfe phylogenetischer Analysen von molekularen und morphologischen Daten entdeckten wir zwei bislang unbekannte Arten. Die Verbreitungsmuster der insgesamt sieben Wieselmakiarten der Region widersprachen den beiden biogeographischen Modellen. Daraufhin postulierten wir ein neues biogeographisches Modell, das „large river model“. Das zweite Ziel war die Entwicklung von Mikrosatelliten für naturschutzgenetische Studien des Milne-Edwards’ Wieselmakis (*Lepilemur edwardsi*). Wir isolierten 21 Mikrosatelliten, von denen 17 amplifizierten und polymorph waren. Das dritte Ziel war die Untersuchung der Effekte von Waldfragmentierung auf das Vorkommen, die Abundanz und die genetische Diversität von *L. edwardsi*. Der Verlust und die Fragmentierung von Waldhabitaten sind wohl bekannte Konsequenzen anthropogener Aktivitäten. Ein Ergebnis dieser Studie ist das Verschwinden der Wieselmakis von vielen Fragmenten aufgrund von Bejagung. Zusätzlich entdeckten wir mit Hilfe von mitochondrialer DNS und Mikrosatelliten einen negativen Einfluss von Waldfragmentierung auf die genetische Diversität und Signale eines demographischen Flaschenhalses. Aufgrund dieser Ergebnisse sind dringend Naturschutzaktivitäten nötig, um einen effektiven Schutz und dadurch das Überleben von *L. edwardsi* und der anderen Wieselmakiarten der Region auf lange Sicht zu gewährleisten.

Schlagwörter: *Lepilemur*, Naturschutz, Phylogenie

Chapter 3
General introduction

3.1 Origins of the Malagasy fauna

Madagascar, India and Africa were once part of the supercontinent Gondwana. This land mass began to fragment approximately 170 million years ago, and by 83 million years, all major components that we recognize today, were separated by tracts of water (Masters et al., 2006). Today, Madagascar is located in the Indian Ocean southeast of Africa, separated by the Mozambique Channel (Fig 1-1). With a total of almost 590 000 km^2, Madagascar is the world's fourth largest island. It stretches 1 600 km north south across 14° latitude (~12°S to 26°S) and measures nearly 600 km across at its widest point.

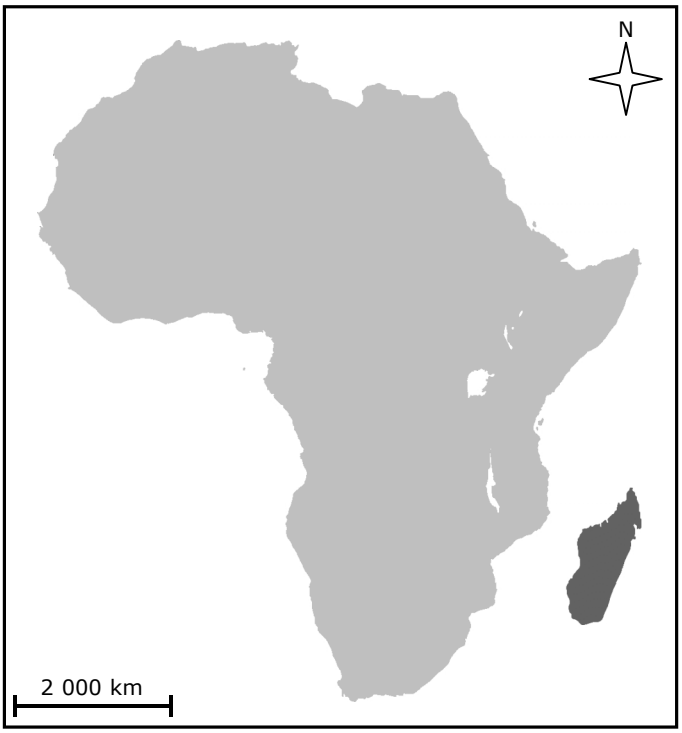

Figure 1-1: Map of Africa (light) and Madagascar (dark).

Madagascar has an asymmetric topography. Along latitude 22°S, the land surface rises rapidly to 2 000 m within 100 km from the Indian Ocean and then gradually drops down over 400 km to the Mozambique Channel in the west. A steep drainage system flanks the east coast, whereas the wide west coast plains support a series of large river deltas.

Madagascar has a wide range of climatic zones, from semi-desert in the southwest to tropical forest in the east and north. The interior vegetation cover of Madagascar is also

different from its coasts. Large areas of the central highlands of Madagascar (about 1 200 m) support savannah grasslands, with heath and peatlands at higher altitudes that, in some places, nearly reach 2 800 m.

Madagascar is one of the world's hottest biodiversity hot spots and contains 3.2 % and 2.8 % of the total global endemic plants and vertebrates, respectively (Ganzhorn et al., 2001; de Wit, 2003). The biogeographical origins of the extant terrestrial and freshwater vertebrate fauna of Madagascar are one of the greatest unsolved questions of natural history (Wallace, 1892; Vences et al., 2001). About 96 % of the 4 220 Malagasy tree species and large shrubs are restricted to Madagascar (e.g. six out of eight species of Baobab trees are found only in Madagascar (Baum et al., 1998)), which is an extremely high level of endemism. Many of the world's chameleons live only in Madagascar, and all Malagasy frog genera are endemic, except for one, shared with Southeast Asia. Lemurs, Madagascar's primates, are 100 % endemic. Scientists have long discussed the circumstances that have created this general pattern and the origins of the ancestral lineages. There are two major hypotheses, Gondwana vicariance followed by neoendemism (Simpson, 1940, 1951; Masters et al., 2006), and Cenozoic dispersal followed by paleoendemism (Millot, 1953; Yoder & Nowak, 2006). The former does not seem to be very likely, since cretaceous terrestrial fossils on Madagascar have not yet revealed linkages to the extant Malagasy fauna and flora, and the few existing molecular-clock data rather support a Cenozoic origin of the major groups at a time when Madagascar was already isolated (Yoder et al., 1996a; Vences et al., 2001, Raxworthy et al., 2002). Analyzing the evolutionary history of organisms by using cladistic methods brought up vicariance biogeography, an approach that makes biogeographic predictions on the basis of significant tectonic or environmental events that split up existing ecosystems. In the case of Madagascar, separation after the fragmentation of Gondwana provides the primary framework for such vicariance hypotheses. The lack of a continuous terrestrial fossil complicates rigorous testing, but newly recovered fossils of endemic frogs show that this may change (Asher & Krause, 1998).

Simple vicariance models alone cannot explain the origin of all animal groups that originated in the Cenozoic. On Madagascar, problematic cases include lemurs and chameleons and also some freshwater fish. The sister group of the indigenous freshwater fish (cichlids) of Madagascar are found in the East African great lakes (Vences et al., 2001), and

molecular estimates of divergence between major cichlid lineages are younger than the separation of Madagascar from East Africa. Similarly, the global chameleon fossil record goes back to 20 Ma, yet cladograms based on molecular and morphological evidence from 52 chameleon species suggest a Malagasy origin for chameleons with multiple radiations to Africa and other Indian Ocean islands (Raxworthy et al., 2002). If this interpretation of the chameleon radiation is correct, then Madagascar provides an independent evolutionary source at least for some taxonomic groups. In contrast, the lemuriform radiation suggests that adapiforms travelled in the opposite direction. Thus, 'two-way traffic' may have been as important as vicariance in shaping the biogeography of Madagascar. Another disputed point is the explanation of the extraordinary radiations on Madagascar. In all, there are more than 9 700 extant plant (16.4 per 100 km^2) and 770 vertebrate (1.3 per 100 km^2) species endemic to Madagascar, respectively (Myers et al., 2000; Tyson, 2000).

3.2 Biodiversity and phylogeny of lemurs

The lemuroid radiation on Madagascar is the most diverse and extensive of all extant primate radiations. Lemuroid body sizes span the entire range observed among extant primates, from the smallest (approx. 30 g) to the largest (approx. 197,500 g) (Godfrey et al., 1995; Rowe, 1996), and diets, locomotor specializations and social organization vary accordingly (Fleagle, 1999).

The history of this radiation has been difficult to investigate due to the complete absence of any primate fossil record on Madagascar prior to 26 000 years ago (Simons et al., 1995). Thus, the only way to reveal the historical background is through reconstructions of phylogenetic relationships among the living and recently extinct taxa. Much effort has been driven towards the end of generating a reliable phylogeny for the Lemuroidea, based on morphology, physiology, behaviour, and molecular genetics (DelPero et al., 2001). Almost all possible relationships have been proposed, and most nodes have been contested.

During the last decade, nucleotide sequencing has become increasingly popular, and the amount of genetic data stored in public databases has grown enormously (e.g. Adkins & Honeycutt, 1994; Yoder, 1994; Porter et al., 1995; Yoder, 1996; Yoder et al., 1996a, b; Porter et al., 1997; Yoder, 1997; Arnason et al., 1998; Goodman et al., 1998; Stanger-Hall & Cunningham, 1998; Yoder & Irwin, 1999; Wyner et al., 2000; DelPero et al., 2001; Pastorini

et al., 2001, 2003; Yang & Yoder, 2003; Poux & Douzery, 2004; Roos et al., 2004; Yoder & Yang, 2004; Olivieri et al., 2007). Some species (e.g. *Lemur catta*, *Microcebus murinus*) have been particularly well studied because of their use as outgroups or as examples of ancestral primates in studies of molecular evolution (DelPero et al., 2006); others (e.g. indriids and megaladapids) have been relatively neglected.

The living lemurs comprise of over 90 species grouped into 5 families (Mittermeier et al., 2008). Including the recently extinct subfossil lemurs, the number of species increased even more, classified into 22 genera and 7 families (DelPero et al. 2001). The closest relatives of lemurs are the galagos (bushbabies) of Africa and the lorises of Africa and Asia.

The relationship between the different Lemuroidea families was long disputed and is still controversial. In the last few decades, molecular methods have been widely applied with the aim to clarify these relations. However, a diverse array of tree topologies was generated. Most recent studies based on mtDNA and/or nuclear sequences and SINE (short interspersed nuclear elements) integrations yielded eight different trees (Yoder et al., 1996a; Yoder et al., 1996b; Yoder & Irwin, 1999; DelPero et al., 2001; Pastorini et al., 2003; Yang & Yoder, 2003; Poux & Douzery, 2004; Roos et al., 2004). To find a consensus, DelPero et al. (2006) reconstructed a composite molecular data set of about 6 400 bp and analysed it with different methods. However, even this large data set revealed different trees. The only certainty seems to be that the family Daubentoniidae is the basal in-group taxon of the Lemuroidea.

Within families and genera, molecular phylogenetics has been applied mostly to determine species identities and species boundaries (e.g. for *Microcebus*: Rasoloarison et al., 2000; Louis et al., 2006b; Olivieri et al., 2007; Radespiel et al., 2008). As a consequence the number of nominal *Microcebus* species has constantly been revised and increased over time.

The genus *Lepilemur* has long been neglected in terms of molecular phylogenetics. Initially, Schwarz (1931) and Hill (1953) recognized only two species of *Lepilemur*: *L. mustelinus* from the eastern rainforests and *L. ruficaudatus* from the western and southern dry forests of Madagascar. *L. ruficaudatus* was further divided into two subspecies, *L. ruficaudatus ruficaudatus* and *L. ruficaudatus leucopus*. Since then, the taxonomy of the sportive lemurs has been repeatedly revised (Petter & Petter-Rousseaux, 1960; Rumpler & Albignac, 1975; Petter et al., 1977; Tattersall, 1982; Jenkins, 1987; Mittermeier et al., 2008; Ravoarimanana et al., 1999, 2004; Thalmann & Geissmann, 2000; Groves, 2001; Rumpler

et al., 2001; Thalmann & Ganzhorn, 2003). Similar to the genus *Microcebus*, the genus *Lepilemur* has undergone a dramatic expansion to 24 recognized species through the taxonomic revisions of Andriaholinirina et al. (2006; described three new species), Louis et al. (2006a; described 11 new species), Rabarivola et al. (2006; described one new species), and Lei et al. (2008; described one new species). Additionally, Zinner et al. (2007) rose important concerns about the sportive lemurs recently described in northwestern Madagascar, expressing the need for comparative analyses including the holotypes (specifically *L. dorsalis* and *L. grandidieri*) and respective data sets, along with an in-depth morphological analysis.

To complete our knowledge a connective approach is needed since many areas have not been sampled at all. The distribution of a species cannot easily be deduced from the data available. A region especially neglected is the North-West of Madagascar, where previous samples originated only from the Ankarafantsika National Park (*Lepilemur edwardsi*). The phylogenetic relationship between the numerous newly described species also remains unclear, since the published phylogenetic trees are inconsistent with one another. For conservation purposes and a better understanding of how the species have evolved it is important to know the distribution limits of these species and the phylogenetic relationship between them. So far, only simplistic phylogeographic scenarios such as a north/south or an east/west division have been suggested. However, there are other theories on how adaptive radiation in the many endemic species of Madagascar may have taken place. Two major models have been proposed to explain this radiation. The best known (Martin, 1972a, 1995) suggests that major rivers and mountains jointly acted as effective barriers to gene flow and thereby facilitated allopatric speciation processes. The second, a more recent model (Wilmé et al., 2006), uses an analysis of Quaternary climatic shifts in the context of watersheds to explain the process of speciation on the island. Quaternary paleoclimatic variation has played an important role in the distribution and speciation of organisms (Hewitt, 2000; Straka, 1996; Burney, 1997). During periods of glaciations, when the climate was cooler and drier, natural habitats at lower elevations experienced more-pronounced arid conditions than did zones at higher elevations (Haffer, 1969). Riverine habitats acted as buffers for the maintenance of more mesic local conditions and potential corridors for retreat toward higher altitudinal zones. The influence of these climatic shifts was not equal across watersheds, and those with sources at relatively low elevations would have experienced more-notable ecological shifts, associated

with aridification, and greater levels of habitat isolation than those occurring at higher elevations. Nevertheless, no attempt has so far been undertaken to test these theories on a large-bodied nocturnal lemur genus on a finer spatial scale. One aim of my study is therefore to perform for the first time a fine-scaled sampling on a large-bodied nocturnal lemur, sportive lemurs, in northwestern Madagascar in order to i) define the distribution limits of all sportive lemur species in that region, ii) determine the phylogenetic relationships between these species, and iii) test to what extent the two biogeographic hypotheses mentioned above predict species diversity in sportive lemurs.

3.3 Conservation of lemurs and the use of genetics for conservation purposes

Madagascar's diverse fauna and flora is currently highly endangered. Only about 2 000 years ago humans settled on Madagascar. As a direct result of human activities, many areas on Madagascar are now deforested or highly fragmented. Archaeological findings as well as pollen and charcoal profiles indicate that until 100 years ago human densities remain relatively low, with the exception of a few bigger but rather short-lived cities (e.g. Radimilahy, 1997). Nevertheless, even these early settlers may have had a considerable impact on the environment. Some studies aimed to quantify people's role on Madagascar's Holocene extinction (e.g. Burney et al., 2004). However, so far only a few studies on extant species have been conducted in order to investigate if there are signatures of population decrease that correspond to these archaeological findings. Alternatively, the impact of early settlers on extant species may have remained negligible, compared to what happened in the last century when human population growth and land conversion has become particularly rapid (e.g. Sussman et al., 1994; Olivieri et al., 2008).

Today, the major threats to lemurs include deforestation (land clearing for grazing, firewood and charcoal production, construction, agriculture, selective logging, fires, mining activities), hunting for food and capturing for the pet trade. Small distribution ranges coupled with deforestation result in low total individual numbers. The remaining populations are threatened by habitat fragmentation.

The enormous destruction of natural habitats in Madagascar by humans forces us to initiate programmes for the conservation of its endangered species, such as the lemurs.

Some 2 000 years ago, the lemurs of Madagascar (> 90 species among 15 genera currently alive in Madagascar, which represents about 15 % of the whole diversity among primates (Martin, 2000; Mittermeier et al., 2008; Tattersall, 2007)) inhabited a wide variety of wooded terrains, from forests to open woodlands and marshlands (Godfrey et al., 1997). A spectacular variety of life histories derived from a single ancestral primate that colonized Madagascar around 60 Ma (Yoder & Yang, 2004). But human activities, such as overhunting and habitat modifications, led at least 17 species, belonging to nine different genera, to eventual extinction (Simons, 1997; Godfrey & Jungers, 2003). Several entire families, the Archaeolemuridae, Palaeopropithecidae, and Megaladapidae, disappeared.

Many taxa in Madagascar are affected by the recent landscape changes. Some conservation projects (e.g. Durbin et al., 2003) have chosen certain species to represent a particular ecosystem in order to achieve protection. Usually, these flagship species are known for their vulnerability, attractiveness or distinctiveness to gain best support and acknowledgement from the public and the scientific community. The protection action established for such key species will also protect its environment and influence conservation of entire ecosystems.

At present, our knowledge of the population biology of many taxa is still rather limited. In the past, conservation biology has been influenced mainly by ecology, but the necessity of genetic approaches has been widely recognized during the last 15 years (Frankel & Soule, 1981; Schoenewald-Cox et al., 1983; Fiedler & Jain, 1992; Loeschke et al., 1994; Avise & Hamrick, 1996). The structure of natural populations, e.g. the amount of genetic variability, the degree of genetic diversity among local populations and, of course, correlations between local environmental conditions and genetic variability should be known in order to optimize in situ conservation programmes.

Conservation genetics is a relatively newly recognized subdiscipline of conservation biology and aims to evaluate and to minimize the risk of decline or extinction due to genetic factors. It thereby provides conservationists and environmental managers with new insights into the extent of genetic diversity present in a population.

A general concern for the conservation of an endangered species in its natural habitat is the maintenance of genetic variation within populations, particularly when the remaining populations become fragmented and reduced in size. The loss of genetic variation can lead to

short-term reduction of fitness (Allendorf & Leary, 1986; Primack, 1993; Lacy, 1997; Frankham & Ralls, 1998). Changes in population size and density may also change behavioural mechanisms, with negative consequences to gene flow (Greenwood, 1980; Johnson & Gaines, 1990; Bohonak, 1999). Detectable changes in allele frequencies can occur between subsequent generations, and can be an indicator for demographic changes (Luikart et al., 1999).

Conservation biologists are concerned that small populations may enter an 'extinction vortex' (Burney et al., 2004). Small populations are susceptible to extinction as a result of demographic, environmental and genetic factors. The idea is that the interaction of these factors may result in the number of individuals becoming smaller and smaller by negative feedback loops until they are driven to extinction. Extinction can be the result of complex interactions. The population may be first forced to a small size by habitat fragmentation. Random genetic drift has a large effect on the genetic diversity of small populations. It is unlikely that the sex ratio will remain equal in a small population. Consequently, the effective population size can approach zero. The effects of inbreeding will begin to alter the average fitness. All factors interact to reduce the census and effective population size, as well as the genetic diversity. They put small populations at great risk. After a loss of genetic diversity, the individuals present in the population may not be able to resist diseases or environmental changes anymore. At this point, the population may be driven to extinction.

Genetic analyses of natural populations have allowed biologists to ask a wide variety of questions, which previously could only be answered by extensive observations. A number of genetic markers have proven to be useful, among which are microsatellite loci. Microsatellites consist of 2-6 bp long repeat units that are repeated many times and provide the basis for an extensive polymorphism. Recently, microsatellites have been increasingly used in genetics (e.g. Beaumont, 1999; Radespiel et al., 2008; Turner et al., 2004). There are some advantages of microsatellites compared to other markers. Microsatellite loci are found in large numbers, are highly polymorphic and are relatively evenly spaced throughout the genome (Edwards et al., 1991). Of the loci examined by Edwards et al. (1991), about 50 % are polymorphic. Further, most loci are neutral which makes them compatible with the assumptions of most theoretical population genetic models. Finally, microsatellites have been found to be

polymorphic even in populations that have low levels of allozyme and mitochondrial variation (Estoup et al., 1995a, 1995b, 1996; Paetkau & Strobeck, 1994).

Microsatellites are increasingly used in the field of conservation genetics (e.g. Michaux et al., 2005; Wu et al., 2007; McDevitt et al., 2009). They allow the calculation of genetic diversity, genetic differentiation, genetic structure and inbreeding. In addition, they can provide estimations of genetic bottleneck effect. Since microsatellites are very taxon-specific, they need to be newly developed for each taxonomic group. In lemurs microsatellites were developed for a number of genera (e.g. *Microcebus*, *Propithecus*, *Hapalemur*, *Eulemur*, and *Phaner*). For the large-bodied lemur genus *Lepilemur* microsatellites are not yet available.

In this study, I developed 17 microsatellites for *L. edwardsi* in order to investigate the genetic diversity and demographic changes of this endangered lemur species.

The data collected for this thesis will contribute to a better understanding of the consequences of habitat loss and fragmentation on the genetic diversity of sportive lemurs in northwestern Madagascar. The accumulation of this kind of data is essential to decide which kind of conservation measures are priorities in the near future.

3.4 Introduction of the model – Sportive lemurs (*Lepilemur* spp.)

Sportive lemurs are medium-sized nocturnal primates that are the only extant genus of the family Lepilemuridae (Harcourt & Thornback, 1990; Mittermeier et al., 2008). In general, the biology and conservation status of these species are only poorly known (Rasoloharijaona et al., 2001; Ravaoarimanana et al., 2001). All *Lepilemur* are listed in the category 'vulnerable' or 'endangered' in the IUCN Red Data Book (www.redlist.org), because of the rapid loss of forest habitats and the high threats imposed by poaching. Sportive lemurs are 'slow clingers and leapers' (Richard & Dewar, 1991), preferentially using vertical supports for travelling. Various authors observed that they rest in tree holes or sometimes in dense open vegetation during daytime (e.g. Petter et al., 1977; Harcourt & Thornback, 1990; Rasoloharijaona et al., 2003; Mittermeier et al., 2008).

All sportive lemurs are folivorous. There is some information available on their feeding ecology (e.g. Hladik et al., 1971; Ganzhorn, 1993; Thalmann, 2001), seasonal activity, and locomotor ecology (e.g. Warren, 1994, 1997; Schmid & Ganzhorn, 1996; Nash, 1998; Drack et al., 1999). The socioecology and communication of most species, however, is only poorly

understood (Mueller & Thalmann, 2000, but see: Rasoloharijaona et al., 2006). Previously, sportive lemurs were reported to live mainly solitarily (e.g. Petter et al., 1977; Jolly, 1988; Kappeler, 1998). Recent field observations emphasized that the Milne Edwards' sportive lemur (*Lepilemur edwardsi*) generally uses holes of living or dead trees as daily sleeping sites, and that it was seen often in groups of 2-3 animals (Petter et al., 1977; Warren, 1994; Rasoloharijaona et al., 2000; Thalmann, 2001). It was therefore hypothesized that the Milne Edwards' sportive lemur forms a dispersed monogamous social organization (Mueller & Thalmann, 2000; Rasoloharijaona et al., 2000), related to the quality and distribution of sleeping sites (Rasoloharijaona et al., 2003; Rabesandratana, 2006). Overall, pair partners do not differ in body length or body mass (Rasoloharijaona et al., 2003). Pairs use a very limited number of sleeping holes in their home range, which do not differ in quality between sexes. Pairs use these sites exclusively and defend them jointly against neighbours and strangers by loud call displays (Rasoloharijaona et al., 2006; Mendez-Cardenas et al., 2008). The main mating season extends from May to June, as indicated by the presence of males with high testes volumes and estrous females (Randrianambinina et al., 2007). In the mating season, early postmating season, and postparturition season, sexes do not differ in body mass, but in August and November females have a significantly higher body mass than males, reflecting pregnancy. Gravidity in females lasts for about four to five months (Randrianambinina et al., 2007). Females give birth to a single offspring, which is left in its first days of life in the hole of a sleeping tree; later on, it is transported orally by its mother and parked in the dense vegetation during foraging (Petter et al., 1977; Rasoloharijaona & Zimmermann, personal observations).

Climate conditions in the dry deciduous forest of northwest Madagascar are harsh, with large daily temperature fluctuations of up to 20°C throughout the year (Radespiel et al., 1998; Rasoloharijaona et al., 2001). Suitable tree holes may provide some degree of thermal insulation and may buffer large temperature differences in such a highly seasonal environment (Charles-Dominique, 1971; Hladik, 1980; Radespiel et al., 1998). Known predators of sportive lemurs are boas, nocturnal and diurnal raptors, and the largest Malagasy carnivore, the fossa (*Cryptoprocta ferox*; Goodman et al., 1993).

3.5 Aims of the study

Based on the current knowledge on sportive lemurs, this study investigates three main fields of interest: Sportive lemur biogeography, phylogeny, and conservation genetics. The thesis will be presented in three chapters that are in various stages of publication. The region studied is the North-West of Madagascar. Extensive genetic sampling, development of genetic markers, and genetic analyses of nuclear microsatellite loci and mtDNA genes were utilised in this study to answer the following questions:

Sportive lemur biogeography (Chapter II)

- What is the distribution of *L. edwardsi*, *L. sahamalazensis*, and *L. dorsalis* in northwestern Madagascar?
- What limits the distribution of different sportive lemur species?
- Do sportive lemurs follow the Martin (1995) or the Wilmé et al. (2006) model in northwestern Madagascar?

Sportive lemur phylogeny and phylogeography (Chapter II, III and IV)

- What are the morphometric characteristics of the different species?
- What is the level of interspecific genetic differentiation?
- Which are the phylogenetic relationships between the different sportive lemur species?
- Can we detect genetic signatures of Pleistocene climate and phylogeographic changes?

Sportive lemur conservation genetics (Chapter II, III and IV)

- What is the level of intraspecific genetic differentiation?
- Is the level of intraspecific genetic differentiation comparable between species?
- Has forest fragmentation affected the genetic diversity of populations?
- Has hunting affected the abundance of sportive lemurs?
- How genetically differentiated are populations of *L. edwardsi*?
- Can signatures of population size changes be detected in present populations of *L. edwardsi*?
- Can the changes in population size of *L. edwardsi* be dated and related to human activities?
- How well protected are the different species in northwestern Madagascar?
- Can the genetic data be related to field observations on habitat condition in order to formulate adequate conservation recommendations?

3.6 References

Adkins RM and Honeycutt RL (1994) Evolution of the primate cytochrome c oxidase subunit II gene. *Journal of Molecular Evolution*, **38**, 215–231.

Allendorf F and Leary R (1986) Heterozygosity and fitness in natural populations of animals. In: *Conservation Biology: the Science of Scarcity and Diversity* (ed. Soule M), pp. 57-76. Sinauer Associates Sunderland MA.

Andriaholinirina N, Fausser J-L, Roos C, Zinner D, Thalmann U, Rabarivola C, Ravoarimanana I, Ganzhorn J U, Meier B, Hilgartner R, Walter L, Zaramody A, Langer C, Hahn T, Zimmermann E, Radespiel U, Craul M, Tomiuk J, Tattersall I and Rumpler Y (2006) Molecular phylogeny and taxonomic revision of the sportive lemurs (*Lepilemur*, Primates). *BMC Evolutionary Biology*, **6**, 17.

Arnason U, Gullberg A and Janke A (1998) Molecular timing of primate divergences as estimated by two nonprimate calibration points. *Journal of Molecular Evolution*, **47**, 718–727.

Asher RJ and Krause D (1998) The first pre-Holocene (Cretaceous) record of Anura from Madagascar. *Journal of Vertebrate Palaeontology*, **18**, 696–699.

Avise J and Hamrick J (1996) *Conservation Genetics - Case Histories from Nature.* New York: Chapman & Hall.

Baum DA, Small RL and Wendel JF (1998) Biogeography and floral evolution of baobabs (adansonia bombacaceae) as inferred from multiple data sets. *Systematic Biology*, **47**, 181–207.

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. *Genetics*, **153**, 2013–2029.

Bohonak AJ (1999) Dispersal gene flow and population structure. *Quaterly Review of Biology*, **74**, 21–45.

Burney DA (1997) Theories and facts regarding Holocene environmental change before and after human colonization. In: *Natural change and human impact in Madagascar* (eds. Goodman SM and Patterson BD), pp. 75-89. Smithsonian Institution Press Washington DC.

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

Charles-Dominique P (1971) Eco-ethologie des prosimiens du Gabon. *Biologica Gabonica*, **7**, 121–228.

de Wit MJ (2003) MADAGASCAR: Heads It's a Continent Tails It's an Island. *Annual Review of Earth and Planetary Sciences*, **31**, 213–248.

Delpero M, Masters JC, Cervella P, Crovella S, Ardito G and Rumpler Y (2001) Phylogenetic relationships among the Malagasy lemuriforms (Primates: Strepsirrhini) as indicated by mitochondrial sequence data from the 12S rRNA gene. *Zoological Journal of the Linnean Society*, **133**, 83–103.

DelPero M, Pozzi L and Masters JC (2006) A composite molecular phylogeny of living lemuroid primates. *Folia Primatologica*, **77**, 434–445.

Durbin J, Rakotoniaina L and Randriamahefasoa J (2003) Project Alaotra: using endangered species as flagships for community-based wetland conservation. In: *The Natural History of Madagascar* (eds. Goodman SD and Benstead JP), pp. 1551-1555. Chicago: The University of Chicago Press.

Edwards A, Civitello A, Hammond H and Caskey C (1991) DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics*, **49**, 746–756.

Estoup A, Tailliez C, Cornuet JM and Solignac M (1995a) Size homoplasy and mutational processes of interrupted microsatellites in two bee species *Apis mellifera* and *Bombus terrestris* (Apidae). *Molecular Biology and Evolution*, **12**, 1074–1084.

Estoup A, Garnery L, Solignac M and Cornuet JM (1995b) Microsatellite variation in honey bee (*Apis mellifera* L.) populations: hierarchical genetic structure and test of the infinite allele and stepwise mutation models. *Genetics*, **140**, 679–695.

Estoup A, Solignac M, Cornuet JM, Goudet J and Scholl A (1996) Genetic differentiation of continental and island populations of *Bombus terrestris* (Hymenoptera: Apidae) in Europe. *Molecular Ecology*, **5**, 19–31.

Fiedler P and Jain S (1992) *Conservation Biology - The Theory and Practice of Nature Conservation Preservation and Management* New York: Chapman & Hall.

Fleagle JG (1999) *Primate Adaptation and Evolution* Elsevier Science Oxford UK.

Frankel O and Soule M (1981) *Conservation and evolution* Cambridge University Press.

Frankham R and Ralls K (1998) Inbreeding leads to extinction. *Nature*, **392**, 441–442.

Ganzhorn JU (1993) Flexibility and constraints of *Lepilemur* ecology. In: *Lemur social systems and their ecological basis* (eds. Kappeler PM and Ganzhorn JU), pp. 153-165. New York: Plenum Press.

Ganzhorn JU, Lowry II PP, Schatz GE and Sommer S (2001) The biodiversity of Madagascar: one of the world's hottest hotspots on its way out. *Oryx*, **35**, 346-348.

Godfrey LR and Jungers WL (2003) Subfossil lemurs. In: *The Natural History of Madagascar* (eds. Goodman SM and Benstead JP), pp. 1248-1258. Chicago: The University of Chicago Press.

Godfrey LR, Sutherland MR, Paine RR, Williams FL, Boy DS and Vuillaume-Randriamanantena M (1995) Limb joint surface areas and their ratios in Malagasy lemurs and other mammals. *American Journal of Physical Anthropology*, **97**, 11–36.

Godfrey L, Jungers W, Reed K, Simons E and Chatrath P (1997) Subfossil lemurs: Inferences about past and present primate communities. In: *Natural Change and Human Impact in Madagascar* (eds. Goodman SM and Patterson BD), pp. 218-256. Smithsonian Institution Press Washington DC.

Goodman SM, O'Connor S and Langrand O (1993) A review of predation on lemurs: implications for the evolution of social behavior in small nocturnal primates. In: *Lemur social systems and their ecological basis* (eds. Kappeler P and Ganzhorn JU), pp.51-66. New York: Plenum Press.

Goodman SM, Porter CA, Czelusniak J, Page SL, Schneider H, Shoshani J, Gunnell G and Groves CP (1998) Toward a phylogenetic classification of Primates based on DNA evidence complemented by fossil evidence. *Molecular Phylogenetics and Evolution*, **9**, 585–598.

Greenwood P (1980) Mating systems philopatry and dispersal in birds and mammals. *Animal Behaviour*, **28**, 1140–1162.

Groves CP (2001) *Primate taxonomy*. Smithsonian Institution Press Washington DC.

Haffer J (1969) Speciation in Amazonian Forest Birds. *Science*, **165**, 131–137.

Harcourt C and Thornback J (1990) *Lemurs of Madagascar and the Comors*. IUCN Gland Switzerland.

Hill W (1953) *Primates Comparative Anatomy and Taxonomy Volume I – Strepsirhini*. Edinburgh: University Press.

Hladik A (1980) The dry forest of the west coast of Madagascar: climate phenology and food available for prosimians. In: *Nocturnal Malagasy primates Ecology physiology and behavior* (eds. Charles-Dominique P, Cooper H, Hladik A, Hladik C, Pages E, Pariente G, Petter-Rousseaux A, Schilling A and Petter J), pp.3-40. New York: Plenum Press.

Hladik C, Charles-Dominique P, Valdebouze P, Delort-Laval J and Flanzy J (1971) La caecotrophie chez un Primate phyllophage du genre *Lepilemur* et les correlations avec les particularites de son appareil digestif. *Comptes-rendus de l'Académie des Sciences de Paris*, **272**, 3191–3194.

Johnson M and Gaines M (1990) Evolution of Dispersal: Theoretical Models and Empirical Tests Using Birds and Mammals. *Annual Review of Ecology and Systematics*, **21**, 449–480.

Jolly A (1988) Lemur social behaviour and primate intelligence. In: *Machiavellian intelligence* (eds. Byrne R and Whiten A). Oxford: Clarendon Press.

Kappeler P (1998) Nests tree holes and the evolution of primate life histories. *American Journal of Primatology*, **46**, 7–33.

Lacy R (1997) Importance of genetic variation to the viability of mammalian populations. *Journal of Mammalogy*, **78**, 320–335.

Lei R, Engberg S, Andriantompohavana R, McGuire S, Mittermeier RA, Zaonarivelo J, Brenneman R and Louis Jr EE (2008) Nocturnal Lemur Diversity at Masoala National Park. *Special Publications of the Museum of Texas Tech University*, **53**, 1–48.

Louis Jr EE, Engberg S, Lei R, Geng H, Sommer J, Randriamampionona R, Randriamanana J, Zaonarivelo J, Andriantompohavana R, Randria G, Prosper, Ramaromilanto B, Rakotoarisoa G, Rooney A and Brenneman R (2006a) Molecular and Morphological Analyses of the Sportive Lemurs (Family Megaladapidae: Genus *Lepilemur*) Reveals 11 Previously Unrecognized Species. *Special Publications Museum of Texas Tech University*, **49**, 1–47.

Louis Jr EE, Coles M, Andriantompohavana R, Sommer J, Engberg S, Zaonarivelo J, Mayor M and Brenneman R (2006b) A Revision of the Mouse Lemurs (*Microcebus*) of Eastern Madagascar. *International Journal of Primatology*, **27**, 347–389.

Luikart G, Cornuet J-M and Allendorf FW (1999) Temporal Changes in Allele Frequencies Provide Estimates of Population Bottleneck Size. *Conservation Biology*, **13**, 523–530.

Martin RD (1972) Adaptive radiation and behaviour of the Malagasy lemurs. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences*, **264**, 295–352.

Martin RD (1995) Prosimians: from obscurity to extinction? In: *Creatures of the dark: the nocturnal prosimians* (eds. Alterman L, Doyle GA and Izard MK), pp. 535-563. New York: Plenum Press.

Martin RD (2000) Origins Diversity and Relationships of Lemurs. *International Journal of Primatology*, **21**, 1021–1049.

Masters JC, de Wit MJ and Asher RJ (2006) Reconciling the Origins of Africa India and Madagascar with Vertebrate Dispersal Scenarios. *Folia Primatologica*, **77**, 399–418.

McDevitt AD, Mariani S, Hebblewhite M, Decesare NJ, Morgantini L, Seip D, Weckworth BV and Musiani M (2009) Survival in the Rockies of an endangered hybrid swarm from diverged caribou (*Rangifer tarandus*) lineages. *Molecular Ecology*, **18**, 665–679.

Mendez-Cardenas M, Randrianambinina B, Rabesandratana A, Rasoloharijaona S and Zimmermann E (2008) Geographic variation in loud calls of sportive lemurs (*Lepilemur* ssp) and their implications for conservation. *American Journal of Primatology*, **70**, 828–838.

Michaux JR, Hardy OJ, Justy F, Fournier P, Kranz A, Cabria M, Davison A, Rosoux R and Libois R (2005) Conservation genetics and population history of the threatened European mink *Mustela lutreola* with an emphasis on the west European population. *Molecular Ecology*, **14**, 2373–2388.

Millot J (1953) Le continent de Gondwana et les méthodes de raisonnement de la biogéographie classique. *Annales des Sciences Naturelles – Zoologie*, **15**, 185–219.

Mittermeier RA, Konstant WR, Hawkins F, Louis Jr EE, Langrand O, Ratsimbazafy J, Rasoloarion R, Ganzhorn JU, Rajaobelina S, Tattersall I and Meyers DM (2008) *Lemurs of Madagascar* 3nd ed Washington DC: Conservation International.

Mueller A and Thalmann U (2000) Origin and evolution of primate social organisation: a reconstruction. *Biological Reviews*, **75**, 405–435.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA and Kent J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853–858.

Olivieri G, Zimmermann E, Randrianambinina B, Rasoloharijaona S, Rakotondravony D, Guschanski K and Radespiel U (2007) The ever-increasing diversity in mouse lemurs: three new species in north and northwestern Madagascar. *Molecular Phylogenetics and Evolution*, **43**, 309–327.

Olivieri G, Sousa V, Chikhi L and Radespiel U (2008) Genetic signature of recent population declines in the endangered golden-brown mouse lemur (*Microcebus ravelobensis*). *Biological Conservation*, **141**, 1257–1271.

Paetkau D and Strobeck C (1994) Microsatellite analysis of genetic variation in black bear populations. *Molecular Ecology*, **3**, 489–495.

Pastorini J, Martin RD, Ehresmann P, Zimmermann E and Forstner MRJ (2001) Molecular phylogeny of the lemur family Cheirogaleidae (Primates) based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, **19**, 45–56.

Pastorini J, Thalmann U and Martin RD (2003) A molecular approach to comparative phylogeography of extant Malagasy lemurs. *Proceedings of the National Academy of Sciences U S A*, **100**, 5879–5884.

Petter JJ and Petter-Rousseaux A (1960) Remarque sur la systématique du genre *Lepilemur*. *Mammalia*, **24**, 76–86.

Petter JJ, Albignac R and Rumpler Y (1977) Mammifères lémuriens (Primates prosimiens). In: *Faune de Madagascar* (), pp. 1-513. Paris: CNRS/ORSTOM.

Porter CA, Sampaio I, Schneider H, Schneider MP, Czelusniak J and Goodman M (1995) Evidence on primate phylogeny from epsilon-globin gene sequences and flanking regions. *Journal of Molecular Evolution*, **40**, 30–55.

Porter CA, Page SL, Czelusniak J, Schneider H, Schneider MP, Sampaio I and Goodman M (1997) Phylogeny and Evolution of Selected Primates as Determined by Sequences of the epsilon-Globin Locus and 5' Flanking Regions. *International Journal of Primatology*, **18**, 261–295.

Poux C and Douzery EJP (2004) Primate phylogeny evolutionary rate variations and divergence times: a contribution from the nuclear gene IRBP. *American Journal of Physical Anthropology*, **124**, 1–16.

Primack R (1993) *Essentials of Conservation Biology* Sinauer Associates Sunderland MA.

Rabarivola C, Zaramody A, Fausser J-L, Andriaholinirina N and Roos C (2006) Cytogenetic and molecular characteristics of a new species of sportive lemur from Northern Madagascar. *Lemur News*, **11**, 45–49.

Rabesandratana A and Zimmermann E (2006) Regional biogeograpgy and conservation status of the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*) in the Ankarafantsika National Park northwestern Madagascar. *5 Göttinger Freilandtage: Primate Diversity: Past Present and Future*.

Radespiel U, Cepok S, Zietemann V and Zimmermann E (1998) Sex-specific usage patterns of sleeping sites in grey mouse lemurs (*Microcebus murinus*) in northwestern Madagascar. *American Journal of Primatology*, **46**, 77–84.

Radespiel U, Olivieri G, Rasolofoson DW, Rakotondratsimba G, Rakotonirainy O, Rasoloharijaona S, Randrianambinina B, Ratsimbazafy JH, Ratelolahy F, Randriamboavonjy T, Rasolofoharivelo T, Craul M, Rakotozafy L and Randrianarison RM (2008) Exceptional diversity of mouse lemurs (*Microcebus* spp) in the Makira region with the description of one new species. *American Journal of Primatology*, **70**, 1033–1046.

Radimilahy M (1997) Mahilaka an eleventh- to fourteenth-century Islamic port. In: *The Natural History of Madagascar* (eds. Goodman SM and Benstead JP), pp. 342-363. Chicago: The University of Chicago Press.

Randrianambinina B, Mbotizafy S, Rasoloharijaona S, Ravoahangimalala R and Zimmermann E (2007) Seasonality in Reproduction of *Lepilemur edwardsi*. *International Journal of Primatology*, **28**, 783–790.

Rasoloarison RM, Goodman SM and Ganzhorn JU (2000) Taxonomic Revision of Mouse Lemurs (*Microcebus*) in the Western Portions of Madagascar. *International Journal of Primatology*, **21**, 963–1019.

Rasoloharijaona S (2001) *Contribution à l'etude comparative de la communication vocale et de la vie sociale de deux formes de* Lepilemur *(Geoffroy 1858) (Lepilemuridae Rumpler et al 1972) provenant de la forêt seche de l'Ouest et de la forêt humide de l'Est de Madagascar*. PhD thesis Université d'Antananarivo, Antananarivo, Madagascar.

Rasoloharijaona S, Rakotosamimanana B and Zimmermann E (2000) Infanticide by a Male Milne-Edwards' Sportive Lemur (*Lepilemur edwardsi*) in Ampijoroa NW-Madagascar. *International Journal of Primatology*, **21**, 41–45.

Rasoloharijaona S, Rakotosamimanana B, Randrianambinina B and Zimmermann E (2003) Pair-specific usage of sleeping sites and their implications for social organization in a nocturnal Malagasy primate the Milne Edwards' sportive lemur (*Lepilemur edwardsi*). *American Journal of Physical Anthropology*, **122**, 251–258.

Rasoloharijaona S, Randrianambinina B, Braune P and Zimmermann E (2006) Loud calling spacing and cohesiveness in a nocturnal primate the Milne Edwards' sportive lemur (*Lepilemur edwardsi*). *American Journal of Physical Anthropology*, **129**, 591–600.

Ravaoarimanana B (2001) *Apport de la biologie moléculaire à la taxonomie et à l'étude de la spéciation des* Lepilemur septentrionalis *Complémentarité des techniques morphologiques cytogénétiques et moléculaires*. PhD thesis Université Louis Pasteur, Strasbourg, France.

Raxworthy CJ, Forstner MRJ and Nussbaum RA (2002) Chameleon radiation by oceanic dispersal. *Nature*, **415**, 784–787.

Richard A and Dewar R (1991) Lemur Ecology. *Annual Review of Ecology Evolution and Systematics*, **22**, 145–175.

Roos C, Schmitz J and Zischler H (2004) Primate jumping genes elucidate strepsirrhine phylogeny. *Proceedings of the National Academy of Sciences U S A*, **101**, 10650–10654.

Rowe N (1996) *A Pictorial Guide to the Living Primates*. East Hampton Pogonias Press.

Rumpler Y and Albignac R (1975) Intraspecific chromosome variability in a lemur from the North of Madagascar: *Lepilemur septentrionalis* species nova. *American Journal of Physical Anthropology*, **42**, 425–430.

Rumpler Y, Ravaoarimanana B, Hauwy M and Warter S (2001) Cytogenetic arguments in favour of a taxonomic revision of *Lepilemur septentrionalis*. *Folia Primatologica*, **72**, 308–315.

Schmid J and Ganzhorn J (1996) Resting metabolic rates of *Lepilemur ruficaudatus*. *American Journal of Primatology*, **38**, 169 –174.

Schoenewald-Cox C, Chambers S, MacBryde B and Thomas L (1983) *Genetics and Conservation - A Reference for Managing Wild Animals and Plant Populations*. London: Benjam/Cummings.

Schwarz E (1931) A revision of the genera and species of Madagascar Lemuridae. *Proceedings of the Zoological Society of London*, 399–428.

Simons E (1997) Lemurs: Old and New. In: *Natural Change and Human Impact in Madagascar* (eds.Goodman SM and Patterson BD),pp. 218-256. Smithsonian Institution Press Washington DC.

Simons E, Burney D, Chatrath P, Godfrey L, Jungers W and Rakotosamimanana B (1995) AMS 14 dates on extinct lemurs from caves in the Ankarana Massif of northern Madagascar. *Quaternary Research*, **43**, 249–254.

Simpson GG (1940) Mammals and land bridges. *Journal of the Washington Acadamy of Science*, **30**, 137–163.

Simpson GG (1951) Probabilities of dispersal in geologic time. *Bulletin of the American Museum of Natural History*, **99**, 163–176.

Stanger-Hall K and Cunningham CW (1998) Support for a monophyletic lemuriformes: overcoming incongruence between data partitions. *Molecular Biology and Evolution*, **15**, 1572–1577.

Straka H (1996) Histoire de la végétation de Madagascar oriental dans les derniers 100 millénaires. In: *Biogéographie de Madagascar* (ed. Lourenco WR), pp. 37-47. Editions de l'ORSTROM Paris.

Sussman R, Green G and Sussman L (1994) Satellite imagery human ecology anthropology and deforestation in Madagascar. *Human Ecology*, **22**, 333–354.

Tattersall I (1982) *The primates of Madagascar* New York: Columbia University Press.

Tattersall I (2007) Madagascar's lemurs: cryptic diversity or taxonomic inflation? *Evolutionary Anthropology*, **16**, 12–23.

Thalmann U (2001) Food resource characteristics in two nocturnal lemurs with different social behavior: *Avahi occidentalis* and *Lepilemur edwardsi*. *International Journal of Primatology*, **22**, 287–324.

Thalmann U and Geissmann T (2000) Distribution and Geographic Variation in the Western Woolly Lemur (*Avahi occidentalis*) with Description of a New Species (*A. unicolor*). *International Journal of Primatology*, **21**, 915–941.

Thalmann U and Ganzhorn J (2003) *Lepilemur*, Sportive Lemur. In: *The Natural History of Madagascar* (eds. Goodmann SM and Benstead JP), pp. 1336-1340. Chicago: The University of Chicago Press.

Turner T, Dowling T, Broughton R and Gold J (2004) Variable microsatellite markers amplify across divergent lineages of cyprinid fishes (subfamily Leusicinae). *Conservation Genetics*, **5**, 279–281.

Tyson P (2000) *The Eighth Continent: Life Death and Discovery in the Lost World of Madagascar* New York: Harper Collins.

Vences M, Freyhof J, Sonnenberg R, Kosuch J and Veith M (2001) Reconciling fossils and molecules: Cenozoic divergence of cichlid fishes and the biogeography of Madagascar. *Journal of Biogeography*, **28**, 1091–1099.

Wallace A (1892) *Island Life* 2nd ed London: Macmillan.

Warren R (1994) *Lazy leapers: a study of the locomotor ecology of two species of saltory nocturnal lemur in sympatry at Ampijoroa Madagascar*. PhD thesis University of Liverpool Liverpool Great Britain.

Warren R (1997) Habitat use and support preference of two free ranging saltatory lemurs (*Lepilemur edwardsi* and *Avahi occidentalis*). *Journal of Zoology*, **241**, 325–341.

Wilmé L, Goodman SM and Ganzhorn JU (2006) Biogeographic evolution of Madagascar's microendemic biota. *Science*, **312**, 1063–1065.

Wu H-L, Wan Q-H and Fang S-G (2007) Microsatellite analysis of genetic variation and population subdivision for the black muntjac *Muntiacus crinifrons*. *Biochemical Genetics*, **45**, 775–788.

Wyner Y, DeSalle R and Absher R (2000) Phylogeny and character behavior in the family *Lemuridae*. *Molecular Phylogenetics and Evolution*, **15**, 124–134.

Yang Z and Yoder AD (2003) Comparison of likelihood and Bayesian methods for estimating divergence times using multiple gene Loci and calibration points with application to a radiation of cute-looking mouse lemur species. *Systematic Biology*, **52**, 705–716.

Yoder AD (1994) Relative position of the Cheirogaleidae in strepsirhine phylogeny: a comparison of morphological and molecular methods and results. *American Journal of Physical Anthropology*, **94**, 25–46.

Yoder AD (1996) Vilgalys R and Ruvolo M Molecular evolutionary dynamics of cytochrome b in strepsirrhine primates: the phylogenetic significance of third-position transversions. *Molecular Biology and Evolution*, **13**, 1339–1350.

Yoder AD (1997) Back to the future: a synthesis of strepsirhine systematics. *Evolutionary Anthropology*, **6**, 11–22.

Yoder AD and Irwin JA (1999) Phylogeny of the Lemuridae: Effects of Character and Taxon Sampling on Resolution of Species Relationships within *Eulemur*. *Cladistics*, **15**, 351–361.

Yoder AD and Nowak MD (2006) Has Vicariance or Dispersal Been the Predominant Biogeographic Force in Madagascar? Only Time Will Tell. *Annual Review of Ecology Evolution and Systematics*, **37**, 405–431.

Yoder AD and Yang Z (2004) Divergence dates for Malagasy lemurs estimated from multiple gene loci: geological and evolutionary context. *Molecular Ecology*, **13**, 757–773.

Yoder AD, Cartmill M, Ruvolo M, Smith K and Vilgalys R (1996a) Ancient single origin for Malagasy primates. *Proceedings of the National Academy of Sciences U S A*, **93**, 5122–5126.

Yoder AD, Vilgalys R and Ruvolo M (1996b) Molecular evolutionary dynamics of cytochrome b in strepsirrhine primates: the phylogenetic significance of third-position transversions. *Molecular Biology and Evolution*, **13**, 1339–1350.

Zinner D, Roos C, Fausser JL, Groves C and Rumpler Y (2007) Disputed taxonomy classification of sportive lemurs (*Lepilemur*) in NW Madagasacar. *Lemur News*, **12**, 53–56.

Chapter 4

First study

Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species

Published as: Craul M, Zimmermann E, Rasoloharijaona S, Randrianambinina B and Radespiel U (2007) Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species. *BMC Evolutionary Biology 7*, 1, 83.

Data collection:	Craul, Rasoloharijaona, Randrianambinina
Lab work:	Craul
Analysis:	Craul
Manuscript:	Craul, Radespiel, Zimmermann
Supervision:	Radespiel, Zimmermann

4 First study

Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species

4.1 Abstract

Background: The lemurs of Madagascar provide an excellent mammalian radiation to explore mechanisms and processes favouring species diversity and evolution. Species diversity, in particular of nocturnal species, increased considerably during the last decade. However, the factors contributing to this high diversity are not well understood. We tested predictions derived from two existing biogeographic models by exploring the genetic and morphological divergence among populations of a widely distributed lemur genus, the sportive lemur (*Lepilemur* ssp.) along a 560 km long transect from western to northern Madagascar.

Results: By using the phylogenetic analyses of mtDNA sequence data, molecular diagnostic sites, and phenotypic morphometric traits, we uncovered two previously undetected species whose distributions contradict the two existing biogeographic models. Brief species descriptions are provided and a new biogeographic model is proposed (the 'large river model').

Conclusions: According to the 'large river model', large rivers in north and northwestern Madagascar acted as geographical barriers for gene flow and facilitated speciation events on a much smaller spatial scale than previously thought. Thereby, this study does not only show that species diversity in nocturnal Malagasy primates is continuously underestimated but aims to emphasize the need for conservation actions if those species with small ranges shall not face extinction in the near future.

4.2 Background

Malagasy lemurs constitute one of six major radiations of extant primates (Martin, 1990). Lemurs show a remarkable species diversity, both numerically and in terms of adaptations making them an excellent mammalian radiation to explore mechanisms and processes underlying speciation and evolution. During the last decade, species diversity in lemurs increased from 33 to currently 74 (Mittermeier et al., 2006; Olivieri et al., 2006). In relation to the small surface area of Madagascar, diversity of species within this primate radiation is quite high. Individual lemur species tend to have small geographic ranges in comparison to other primates. Because of such limited geographic ranges and the high rate of deforestation, the need for conservation action including genetic monitoring and effective management policies is particularly urgent (Mittermeier et al., 2006; Ganzhorn et al., 1996/7). Two major models have been proposed to explain diversity of Malagasy mammals.

The 'Martin model' divided northern and northwestern Madagascar into four biogeographical zones (circles in Fig. 4-1) (Martin, 1972; Martin et al., 1995). The western zone (W1) covers the area between the two major rivers Tsiribihina and Betsiboka. The northwestern zone 1 covers the area between the two major rivers Betsiboka and Maevarano (NW), the northwestern zone 2 the area between the rivers Maevarano and Mahavavy (X). The northern zone (N) covers the area between the rivers Mahavavy and Fanambana. These riverine barriers were hypothesized to form geographical boundaries to gene flow and consequently favour allopatric speciation. This model of speciation within Madagascar was refined (Martin et al., 1995) and it was shown that it is compatible with a reconstruction of speciation within the families Lemuridae, Cheirogaleidae and Indridae (Pastorini et al., 2003).

The recent 'Wilmé model' explained the process of explosive speciation on the island using a mechanistic model (Wilmé et al., 2006). Madagascar's rivers and associated watersheds with sources at relatively low elevations suggested to be zones of isolation that led to the evolution of locally endemic taxa, whereas those at higher elevations were proposed to have functioned as zones of retreat and dispersion and contain a lower level of microendemism. Wilmé et al. (2006) divided northern and northwestern Madagascar into six centres of endemism (squares in Fig. 4-1). The western zone (zone 8) covers the area between the two major rivers Tsiribihina and Betsiboka, corresponding to Martin's W1. One large northwestern zone, zone 9, corresponded to Martin's NW. Two smaller northwestern zones,

zone 10, between the two rivers Maevarano and Sambirano, and zone 11 between the two rivers Sambirano and Mahavavy were suggested. In addition, two northern zones, one (zone 12), between the river Mahavavy and the continental divide between eastern and western draining watersheds, and another (zone 1), between the continental divide and the river Bemarivo, divided the N-zone of Martin into two partitions.

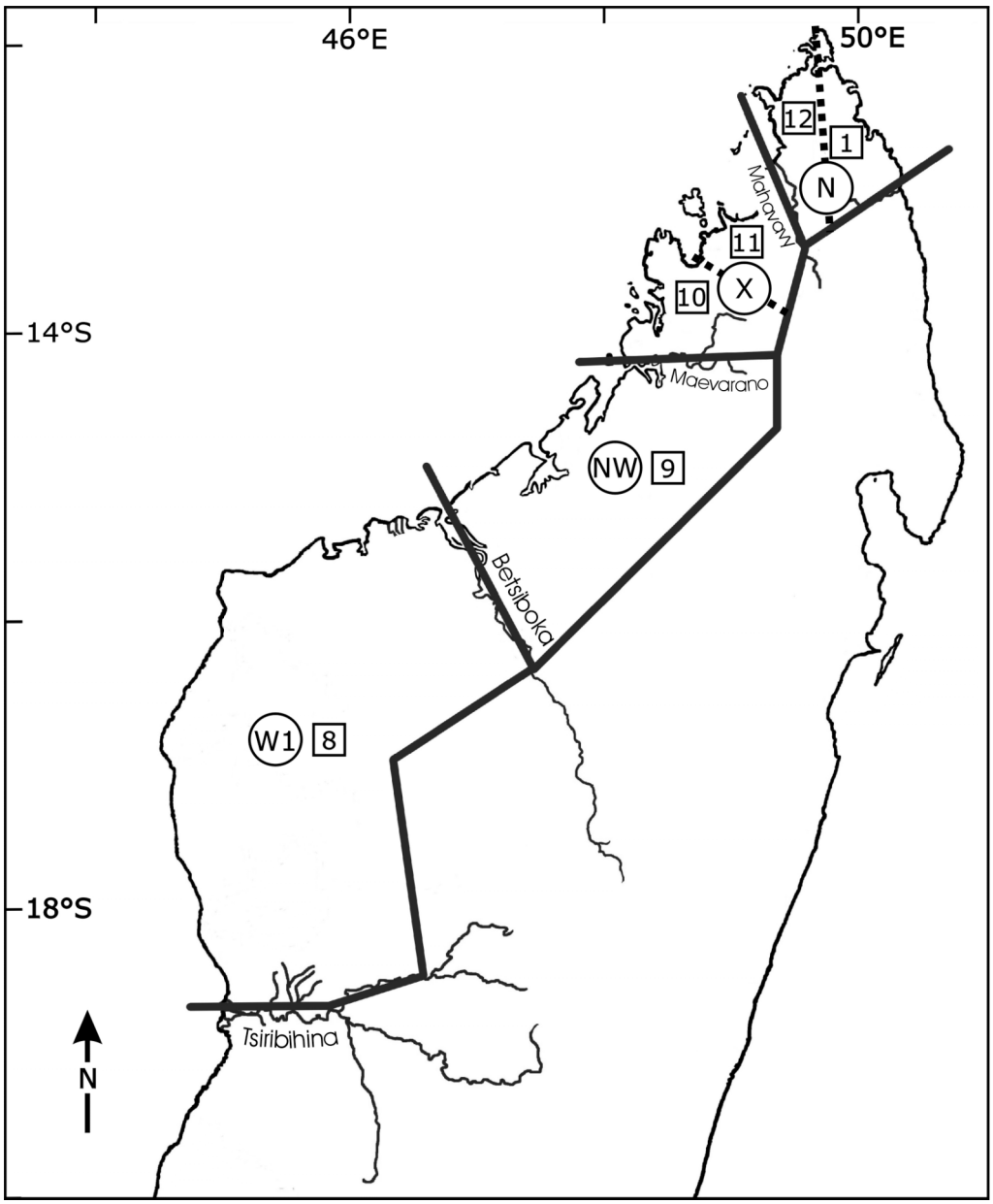

Figure 4-1: Zonation of northwestern Madagascar described by Martin (1972), zones marked with letters, and by Wilmé et al. (2006), zones marked with numbers.

The geographical settings in northwestern and northern Madagascar are perfect to test if allopatric speciation of a widely distributed lemur genus follows one of the models. Each model predicts a different minimum number of species in this region and divergent distributions. Whereas the 'Martin model' predicts four species, the 'Wilmé model' proposes six species.

Sportive lemurs (*Lepilemur* spp.) are an excellent lemur group to test these two models of mammalian distribution in Madagascar, because they occur in almost all forested regions on the island. They are cat-sized vertical clingers and leapers with powerful hind legs. They are nocturnal and totally arboreal. They live in dispersed pairs and have an elaborated vocal repertoire (Rasoloharijaona et al., 2003, 2006; Thalmann & Ganzhorn, 2003). Because differences in pelage colouration and other external characteristics between species are inconspicuous, their early classification (Petit, 1933; Petter & Petter-Rousseaux, 1960) based on morphological features was disputed until comprehensive cytogenetic approaches and molecular studies allowed the recognition of twelve species (Thalmann & Ganzhorn, 2003; Petter et al., 1977; Rumpler & Albignac, 1978; Tattersall, 1982; Andriaholinirina et al., 2006; Rabarivola et al., 2006).

The aim of this study is to test the predictions from the models with the largest available genetic and morphological data set of a large-bodied lemur. We sequenced three mitochondrial genes of particular diagnostic importance for phylogeography (D-loop, Cytochrome *b*, NADH dehydrogenase subunit 4) of individuals captured in 14 different localities that covered a 560 km transect and the area between eight large rivers (Inter-River-Systems, IRS) from western to northern Madagascar. In addition, morphometric data were analysed in order to explore, to which extent genetic differentiation coincides with morphological diversification. As in similar studies (e.g. Wyner et al., 1999; Ravaoarimanana et al., 2004) we favour the phylogenetic species concept (Cracraft, 1983; Davis & Nixon, 1992), where fixed molecular differences among parapatric populations indicate the existence of species barriers.

4.3 Results

4.3.1 Phylogenetic relationships

The 48 sequences available for the D-loop (43 own sequences + five reference sequences), after having cut out the hypervariable part, varied from 388 to 390 bp in length. 128 characters were constant, 201 variable characters were parsimony-uninformative and 66 were parsimony-informative. There were 17 different haplotypes. The 72 sequences available for the partial Cytochrome *b* (43 own sequences + 29 reference sequences) were 352 bp long, with no indels. 211 characters were constant, 17 variable characters are parsimony-uninformative and 124 were parsimony-informative. There were 32 different haplotypes. The 50 sequences available for the partial ND4 (43 own sequences + seven reference sequences) varied from 630 to 631 bp in length. 408 characters were constant, 72 variable characters were parsimony-uninformative and 153 were parsimony-informative characters. There were 19 different haplotypes. Table 4-1 shows the best-fit models for the three loci selected by the hierarchical likelihood ratio test (hLRT) implemented in Modeltest3.5.mac. Based on the single-gene-trees, derived for the new and reference sequences, the samples in this study could be classified as follows: the individuals found in IRS 0 clustered with *L. aeeclis*, the individuals found in IRS I with *L. edwardsi*, the individuals found in IRS IV with *L. sahamalazensis*, the individuals found in IRS V and VI with *L. dorsalis*, the individuals found in IRS VII with *L. ankaranensis*, the individuals from Kirindy with *L. ruficaudatus* and the individuals from Mantadia with *L. mustelinus*. The individuals from IRS II and III did not cluster with any of the reference sequences. No sampled individual clustered with the reference sequences of *L. leucopus*, *L. microdon*, *L. randrianasoli* or *L. septentrionalis*.

Table 4-1: Best-fit mutation model for the mitochondrial loci and the concatenated sequence selected by the hierarchical likelihood ratio test (hLRT) implemented in Modeltest3.5.mac.

Locus	Method	Model	Base	Nst	Alpha	Pinvar	TRatio
D-loop	ML, NJ	HKY+G	0.3066 0.2151 0.1828 0.2955	2	0.1752	0	3.2075
Cyt *b*	ML, NJ	HKY+I+G	0.3092 0.3229 0.1219 0.2460	2	3.5808	0.5469	12.5738
ND4	ML, NJ	HKY+G	0.3372 0.2697 0.1127 0.2804	2	0.2736	0	8.1268
concatenated	ML, NJ	HKY+I+G	0.3124 0.2661 0.1377 0.2838	2	0.8801	0.3833	6.1953

Nst: number of substitution types; Pinvar: assumed proportion of invariable sites; Alpha: shape parameter; TRatio: transition/transversion ratio

In order to reconstruct the phylogenetic relationships within the genus *Lepilemur*, we combined these three loci to one concatenated sequence, 1380 bp in length. 768 characters were constant, 333 variable characters were parsimony-uninformative, and 279 were parsimony-informative. There were 21 different haplotypes. The best-fit model selected by hLRT in Modeltest 3.5.mac was the HKY+I+G model (Table 4-1). Figure 4-2 shows the Neighbour-Joining tree based on the concatenated sequence. All populations within each IRS clustered together, so that each IRS (including the populations Kirindy (West) and Mantadia (East)) built separate terminal clades, supported by high bootstrap values (Fig. 4-2). The phylogram consists of four major clades, a western, a northwestern, a northern clade, and the clade of *L. mustelinus*. *L. mustelinus* branched off first, followed by the western clade that consisted of IRS 0 and the individuals found in Kirindy (West) (bootstrap values between 93 and 96). The northern clade consisted of IRS IV, V, VI, and VII (bootstrap values of 100), and the northwestern clade of IRS I, II, and III (bootstrap values of 100). All so far recognized species formed distinct terminal clades with moderate (*L. ankaranensis*, *L. dorsalis*) to large (*L. mustelinus*, *L. ruficaudatus*, *L. aeeclis*) branch lengths. Branch lengths among IRS I, II, and III in the northwestern clade were in the same scale as these between *L. ankaranensis* and *L. dorsalis*.

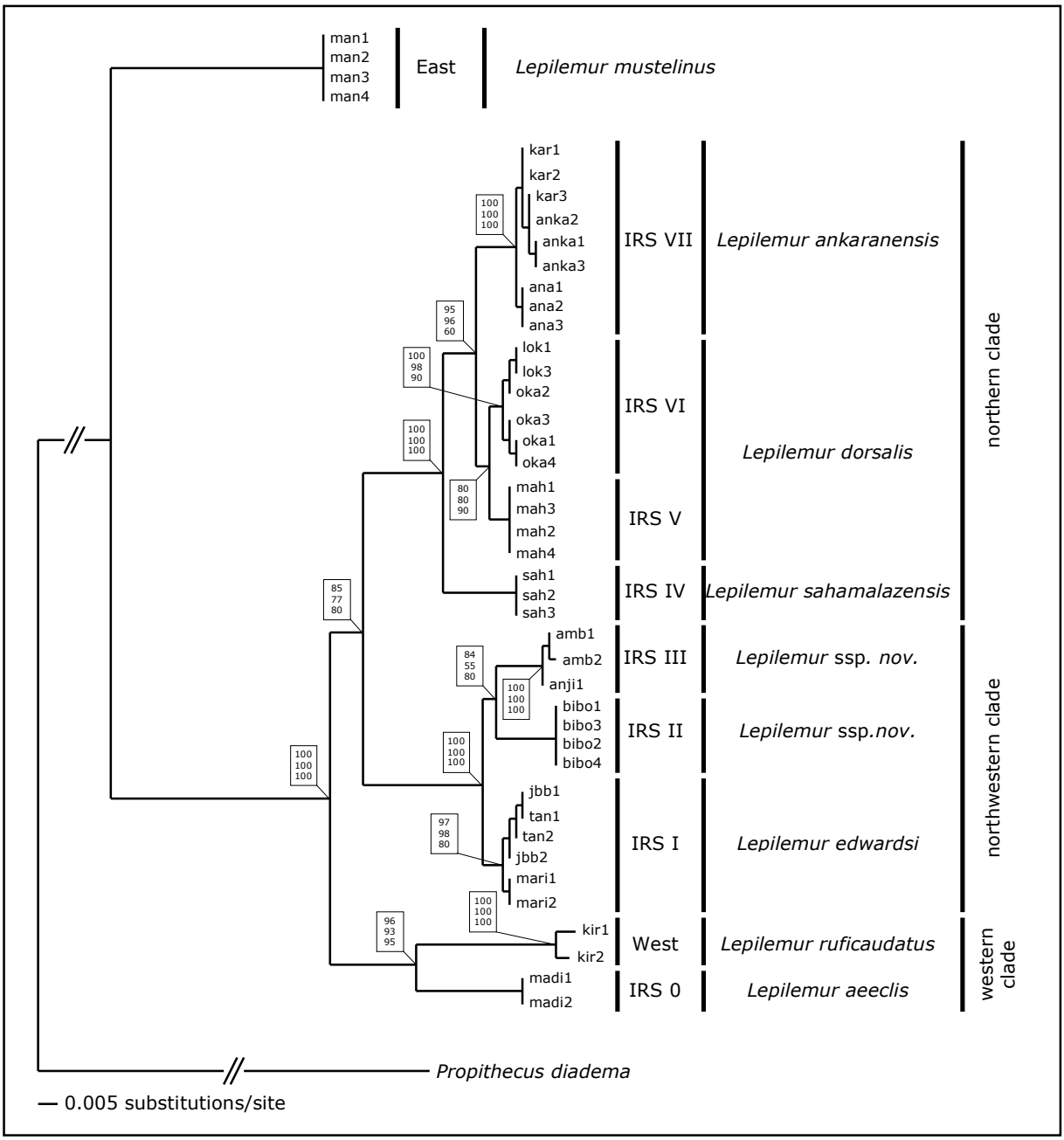

Figure 4-2: Neighbour-Joining tree based on the concatenated sequences of the three loci. The branch lengths indicate the number of substitutions. The numbers at the nodes indicate bootstrap values for internal branches (top: NJ, middle: MP, bottom: ML).

The absolute pairwise distances within an IRS ranged from zero to seven characters (Fig. 4-3). The absolute pairwise distances among IRSs (including Kirindy (West) and Mantadia (East)) varied from 18 to 199 characters. The largest absolute pairwise distance (199 characters) existed between *L. aeeclis* and *L. mustelinus*. The smallest absolute pairwise distance (18 to 23 characters) among IRSs existed between IRS V and IRS VI, both of them were previously supposed to give home to *L. dorsalis*. The relative genetic distance between these two IRSs can be defined as intermediate between the intra-IRS differences (0-7 bp) and the interspecific differences (32-199 bp). This level of differentiation could indicate the presence of two subspecies of *L. dorsalis*. In accordance with the deep phylogenetic splits in the Lepilemur tree among the western, northwestern, and northern clade, absolute pairwise distances were always largest when crossing borders among neighbouring biogeographic zones (*L. aeeclis* to *L. edwardsi* and IRS III to *L. sahamalazensis*, Fig. 4-3). When examining the absolute pairwise distances among the IRSs within the northwestern clade, they were the same size or even larger than between *L. dorsalis* and *L. ankaranensis*, which are accepted species (Fig. 4-3).

Appendix 4-A, 4-B and 4-C shows the molecular diagnostic sites for each terminal clade in each of the three genes. Recognized species had a total number of 1 (*L. dorsalis*) to 73 (*L. mustelinus*) sites that allowed identifying them unmistakably. The two terminal clades containing the individuals of IRS II and III had a total of 11 and 7 diagnostic sites, respectively. The absolute pairwise distances as well as the analysis of the diagnostic sites indicate the presence of two new *Lepilemur* species in northwestern Madagascar, one in IRS II and one in IRS III. Consequently, the geographic range of *L. edwardsi* is much smaller than previously assumed, and limited exclusively to IRS I. By mapping each of these species with respect to their geographical setting (IRS), it can be concluded that all large rivers act as genetic barriers in this genus (Fig. 4-4).

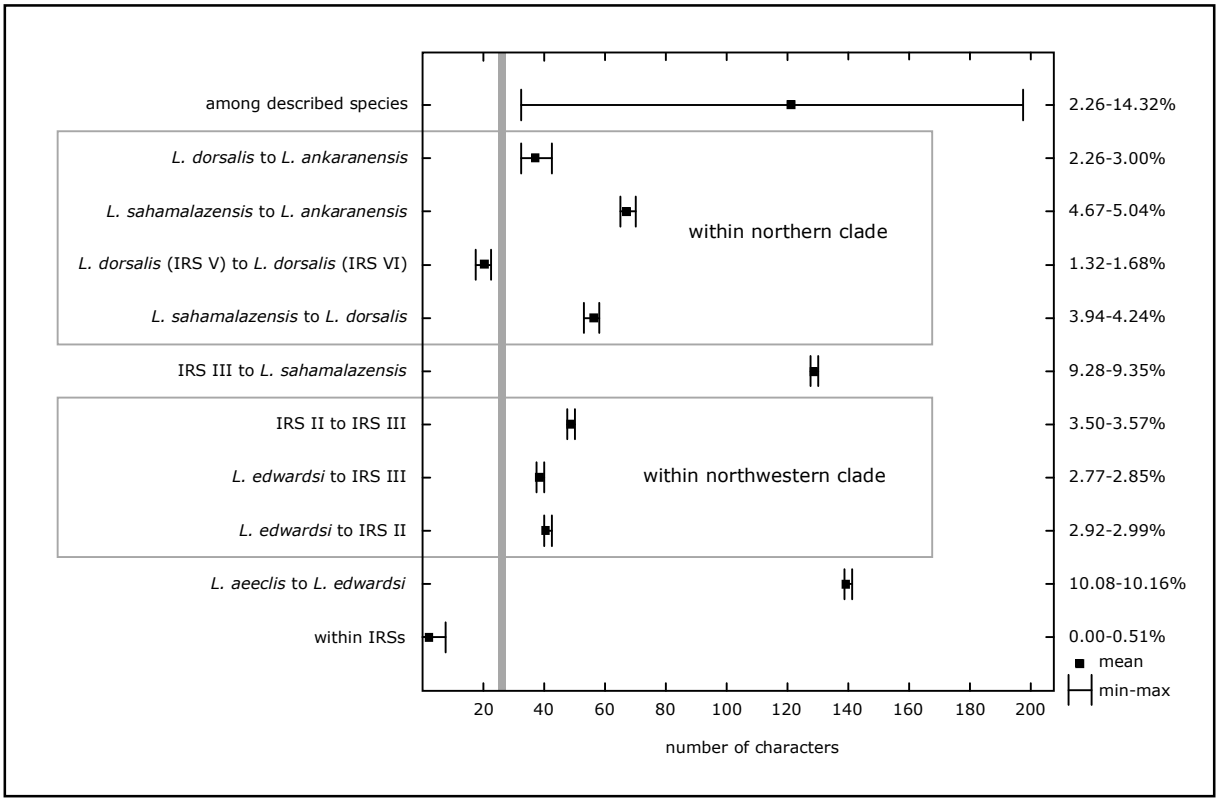

Figure 4-3: Absolute pairwise distances (minimum-maximum and mean) within IRSs, between neighbouring IRSs/species and among the seven already described species.

4.3.2 Morphometry

The means and standard deviations of all morphometric variables for the nine phylogenetically defined species are provided in Table 4-2. All variables showed significant differences for species in the ANOVA. Post-hoc tests revealed that ear length and intraorbital distance was significantly different in 15 of the 28 possible pairs of species and thereby the two most distinct variables, followed by snout length (14/28), weight (12/28), head width, and lower leg length (10/28), tail circumference (9/28), 3rd toe length (8/28), interorbital distance, and hind foot length (6/28), and tail length (4/28). Six variables showed tendencies ($0.05 \leq p < 0.1$) in one to two possible pairs of species. The Post-hoc tests revealed significant differences between the northwestern and northern clade and between the northern clade and *L. mustelinus*. Moreover, it could distinguish between all neighbouring species (established and proposed), except between *L. aeeclis* from the western clade and *L. edwardsi* from the northwestern clade (Table 4-2).

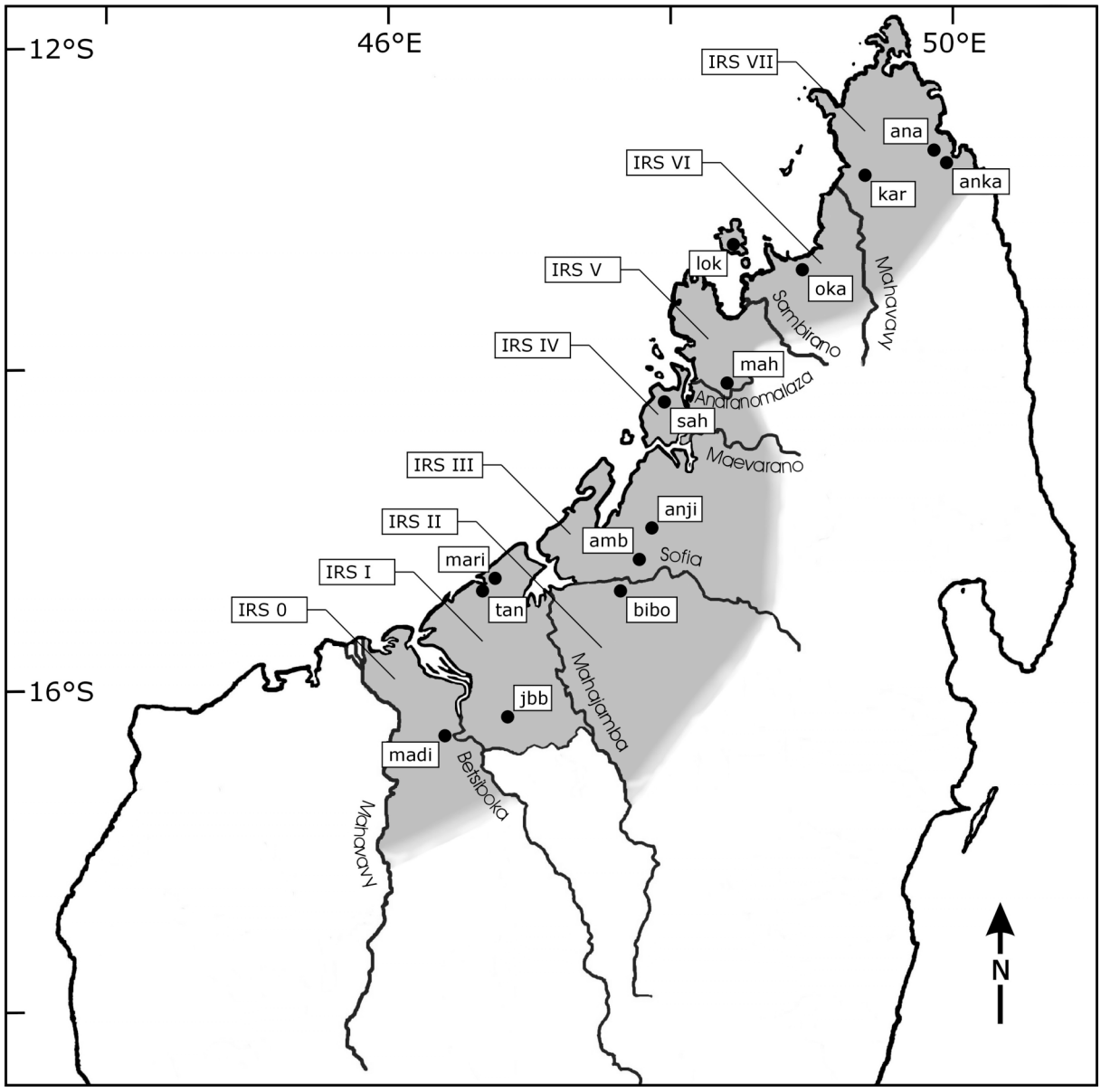

Figure 4-4: Map of study sites, large rivers, and the zonation of the eight Inter-River-Systems (IRSs).

Table 4-2: Descriptive statistics (mean ± *SD*) for 10 morphometric variables from each species.

	L. aeeclis (n=5)		*L. edwardsi* (n=11)		*L.* sp. nov. in IRS II (n=6)		*L.* sp. nov. in IRS III (n=8)		*L. sahamalazensis* (n=7)		*L. dorsalis* (n=30)		*L. ankaranensis* (n=26)		*L. mustelinus* (n=7)		Results of ANOVA		
Variable	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	df	F	*p*
Ear length [mm]	30.72	0.83	31.75	1.93	33.60	0.99	33.41	2.10	26.73	1.79	25.59	1.52	28.79	1.71	31.93	3.11	7	37.081	0.000
Head width [mm]	36.38	0.83	34.93	1.36	37.88	2.24	37.61	2.29	34.00	1.37	34.69	1.72	34.12	1.97	38.36	2.33	7	9.105	0.000
Snout length [mm]	15.50	1.13	17.52	2.25	21.00	0.83	19.34	1.45	15.10	1.05	14.74	1.69	13.97	1.67	18.66	2.14	7	23.894	0.000
Intraorbital distance [mm]	13.60	0.65	14.51	0.79	15.40	1.04	15.29	1.12	13.31	0.96	12.08	0.89	12.34	0.76	12.79	1.17	7	25.088	0.000
Interorbital distance [mm]	36.98	0.80	37.80	1.33	36.05	1.92	36.88	0.82	34.96	1.39	36.82	1.45	35.48	1.13	38.41	1.77	7	7.470	0.000
Lower leg length [mm]	96.30	1.37	96.22	5.37	101.37	3.63	105.44	2.91	99.27	1.40	98.05	5.88	99.74	3.69	113.30	3.94	7	12.874	0.000
Hind foot length [mm]	48.14	2.98	51.96	2.68	50.23	1.43	51.76	3.45	49.00	2.01	49.83	2.31	48.90	2.51	54.94	1.45	7	6.953	0.000
3rd toe length [mm]	21.16	0.90	22.24	1.47	22.37	1.62	23.75	1.60	21.70	0.82	20.61	2.38	20.80	1.13	25.76	2.91	7	9.127	0.000
Tail length [mm]	260.00	16.58	279.73	14.64	253.00	13.58	280.63	15.24	257.57	11.87	263.40	15.53	267.88	17.73	252.57	16.16	7	4.006	0.001
Tail circumference [mm]	34.80	0.84	35.73	3.26	40.33	3.27	35.25	3.58	33.14	4.38	34.40	2.19	34.42	1.60	42.43	6.83	7	9.046	0.000
Body mass [g]	795.20	80.79	934.73	109.06	938.50	116.15	939.50	96.97	673.57	120.13	713.07	93.24	706.31	61.71	964.57	96.27	7	20.680	0.000

Bold lines indicate significant differences ($p \leq 0.05$) and dotted lines indicate a statistical trend ($0.05 \leq p < 0.1$) between the neighbouring species/columns.

The discriminant function analysis used five variables for model calculation, ear length, snout length, lower leg length, interorbital distance, and intraorbital distance. Two functions were computed explaining a significant part of the morphometric variability between the six established and two proposed species (Wilk's $\lambda = 0.006$; $F_{(35,120)} = 8.355$; $p < 0.000$). Table 4-3 shows the classification matrix, with correct classification in 82.5 % of the cross-validated cases. The differences between the classification accuracy of each species ranged from 60 % to 100 %. The individuals of *L. aeeclis*, *L.* sp. nova in IRS III and *L. mustelinus* were correctly classified in 100 % of the cases. The individuals of *L.* sp. nova in IRS II and *L. ankaranensis* were correctly classified in 80 %, and the individuals of *L. edwardsi*, *L. sahamalazensis*, and *L. dorsalis* had the smallest percentage of correct classifications (60 %). All misclassifications occurred within each major clade, indicating again cryptic speciation within the genus *Lepilemur*.

Table 4-3: Classification matrix of the discriminant function analysis.

	% correct	*L. aeeclis*	*L. edwardsi*	*L.* sp. nov. in IRS II	*L.* sp. nov. in IRS III	*L. sahamalazensis*	*L. dorsalis*	*L. ankaranensis*	*L. mustelinus*
L. aeeclis	100.0	5	0	0	0	0	0	0	0
L. edwardsi	80.0	1	4	0	0	0	0	0	0
L. sp. nov. in IRS II	80.0	0	0	4	1	0	0	0	0
L. sp. nov. in IRS III	100.0	0	0	0	5	0	0	0	0
L. sahamalazensis	60.0	0	0	0	0	3	1	1	0
L. dorsalis	60.0	0	0	0	0	0	3	1	1
L. ankaranensis	80.0	0	0	0	0	0	1	4	0
L. mustelinus	100.0	0	0	0	0	0	0	0	5
total	82.5	6	4	4	6	3	5	6	6

4.4 Discussion

4.4.1 Revised phylogeny of the genus *Lepilemur*

Molecular methods, such as DNA sequencing provide powerful tools to understand diversity and phylogeny (Pastorini et al., 2003; Andriaholinirina et al., 2006; Yoder et al., 2003, 2005; Vences et al., 2003; Jansa et al., 2006; Asher & Hofreiter, 2006; Farias et al., 1999; Nagy et al., 2003; Cooper et al., 2001). This could be confirmed by our study in sportive lemurs. The phylogenetic trees distinguished all previously described species. Moreover, it provided evidence for two previously unknown species in northwestern Madagascar. The absolute pairwise distances between all species were in the range of those observed in other lemur genera such as *Mirza* (Kappeler et al., 2005), *Microcebus* (Kappeler et al., 2005; Pastorini et al., 2001; Yoder et al., 2000), *Hapalemur* (Fausser et al., 2002; Pastorini et al., 2002), and *Propithecus* (Pastorini et al., 2003; Mayer et al., 2004). The two new taxa occurred in a single IRS (II and III) each. Their phylogenetic position in the tree, the genetic distances, and the number of diagnostic sites, suggest a separation at the species level. Similar conclusions were drawn with comparable approaches in other taxa (Andriaholinirina et al., 2006; Ravaoarimanana et al., 2004; Asher & Hofreiter, 2006; Kappeler et al., 2005; Yoder et al., 2000).

Besides the molecular evidence, the discriminant function analysis of the morphometric data further supported the species status of the two new *Lepilemur* taxa (IRS II and III) in northwestern Madagascar. Between 80 % (IRS II) and 100 % (IRS III) of the animals were correctly classified into their IRS of origin. The ANOVA of the morphometric data detected significant differences between the northwestern and northern clade and between the northern clade and *L. mustelinus*. Moreover, it could differentiate between the neighbouring species (established and proposed) within the northwestern and northern clade, but it could not distinguish between *L. edwardsi* and *L. aeeclis* that are geographically separated by the river Betsiboka. The differentiation between the northwestern and northern clade, and between the northern clade and *L. mustelinus* is stronger than between the species within these major clades. Although the lack of differentiation between the western and northwestern clade may also partly be due to a sample size effect, it may also suggest cryptic speciation events in the

genus *Lepilemur* not only within major clades as it is known in other taxa (Camargo et al., 2006; Goetze, 2003; Piaggio & Perkins, 2005), but also between major clades.

The revised phylogeny of the genus *Lepilemur* is based on the combination of molecular differences (genetic distances and diagnostic sites) and morphometric traits. Diagnostic sites are also routinely used in DNA barcoding, which is becoming an increasingly important tool in species identification (DeSalle, 2006). Although DNA barcoding requires a large and nearly complete database of sequences to which individuals can be compared (Moritz & Cicero, 2004; Will & Rubinoff, 2004), the diagnostic sites we identified can be seen as first step towards such a database in *Lepilemur*.

4.4.2 Description of two new species

***Lepilemur otto* sp. nov.**

Holotype: Individual 02y04bibo, adult male captured in Ambodimahabibo on the 1st of August 2004 by M. Craul (Fig. 4-5, 4-6, 4-7).

Material: Tissue and hair samples, morphometric measurements as well as photographs of 02y04bibo are stored at the Institute of Zoology of the University of Veterinary Medicine Hannover, Hannover, Germany.

Type locality: Madagascar: Province de Mahajanga, Ambodimahabibo (15°29'54,2"S, 47°28'47,2"E).

Paratype: Individuals 01y04bibo, 03y04bibo, and 04y04bibo were captured in Ambodimahabibo by M. Craul in 2004. Tissue and hair samples, morphometric measurements as well as photographs of each paratype are stored at the Institute of Zoology of the University of Veterinary Medicine Hannover, Hannover, Germany.

Description: The dorsal pelage, including shoulders and the upper and lower arms, is predominantly grey-brown. A dark diffuse line runs from the middle of the upper skull down the spine, ending in the middle or at the lower part of the back, but is never present on the tail. The ventral pelage is generally grey to creamy. The coloration of the tail is grey-brown to deep brown, sometimes with a white tail tip. The face and forehead are essentially grey.

Diagnosis: The sequenced mtDNA of *Lepilemur otto* has 11 diagnostic sites, 8 in the ND4 (positions 42 = G, 57 = T, 123 = G, 255 = A, 306 = C, 630 = A, 631 = T, 632 = C; see Appendix 6-B), and 3 in the D-loop (positions 20=C, 22=A, 23=T; see Appendix 6-C). *L. otto*

differs from its closest relative, *L. edwardsi*, in 2.92-2.99 % and from its sister taxon *L. manasamody* in 3.50-3.57 % in the sequenced mtDNA, respectively. The few morphometric data, which are available at the moment, indicate that *L. otto* has a significant longer snout than the neighbouring species south of the Mahajamba River, *L. edwardsi*. The tail is significant short compared to the neighbouring species north of the Sofia River, *L. manasamody* and to *L. edwardsi*. *L. otto* shows a tendency to have a wider head than *L. edwardsi* and a bigger tail circumference than *L. manasamody*.

Distribution: The known distribution range of *Lepilemur otto* is so far limited to the sample site of Ambodimahabibo. This site is situated in the IRS II, which is limited by the Mahajamba River in the west and the Sofia River in the north. Intensive surveys are now required in this vastly deforested area to obtain additional information about the location and viability of other remaining populations, so that conservation measures can be proposed.

Etymology: The name *Lepilemur otto* was chosen to acknowledge the donation of Dr. Michael Otto for the purpose of research and conservation of Malagasy lemurs.

Vernacular name: Otto's sportive lemur or Lépilemur de Otto.

Figure 4-5: *Lepilemur otto*, portrait of individual 02y04bibo (photo by M. Craul).

Figure 4-6: *Lepilemur otto*, body of individual 02y04bibo (photo by M. Craul).

Figure 4-7: *Lepilemur otto*, back of individual 02y04bibo (photo by M. Craul).

***Lepilemur manasamody* sp. nov.**

Holotype: Individual 16y03amb, adult female captured in Ambongabe on the 20th of September 2003 by M. Craul (Fig. 4-8, 4-9, 4-10).

Material: Tissue and hair samples, morphometric measurements as well as photographs of 16y03amb are stored at the Institute of Zoology of the University of Veterinary Medicine Hannover, Hannover, Germany.

Type locality: Madagascar: Province de Mahajanga, Ambongabe (15°19'38.3"S, 46°40'44.4"E) and Anjiamangirana I (15°09'24.6"S, 47°44'06.2"E).

Paratype: Individuals 14y03amb and 15y03amb were captured in Ambongabe and individuals 07y03anji, 08y03anji, and 09y03anji in Anjiamangirana I by M. Craul in 2003. Tissue and hair samples, morphometric measurements as well as photographs of all paratypes are stored at the Institute of Zoology of the University of Veterinary Medicine Hannover, Hannover, Germany.

Description: The dorsal pelage is predominantly grey-brown, including shoulders, the upper and lower arms. The ventral pelage is generally grey to creamy. The face and forehead are essentially grey. From the middle of the upper skull, a dark diffuse line runs down the spine, ending in the middle of lower part of the back. This line is never present on the tail. The tail is grey-brown to deep brown, sometimes with a white tail tip.

Diagnosis: The sequenced mtDNA of *Lepilemur manasamody* has 7 diagnostic sites, 2 of them in the Cytochrome *b* (positions 86 = G, 140 = G; see Appendix 6-A), 3 in the ND4 (positions 171 = T, 201 = G, 333 = A; see Appendix 6-B), and 2 in the D-loop (positions 75 = G, 156 = G; see Appendix 6-C). *L. manasamody* differs from its sister taxa *L. otto* in 3.50-3.57 % and from *L. edwardsi* in 2.77-2.92 % in the sequenced mtDNA, respectively. The few morphometric data, which are available at the moment, indicate that *L. manasamody* has a significantly longer tail than *L. otto*. *L. manasamody* has significantly longer ears and a longer snout, a significantly wider head and bigger intraorbital distance and is heavier than the neighbouring species to the north, *L. sahamalazensis*. It also shows a tendency to have a smaller tail circumference than *L. otto*.

Distribution: The known distribution range of *Lepilemur manasamody* is so far limited to the sample sites of Ambongabe and Anjiamangirana I. Both sites are situated in the IRS III,

which is limited by the Sofia River in the south and the Maevarano River in the north. Intensive surveys are now required to obtain additional information about the location and viability of the remaining populations, so that conservation measures can be proposed.

Etymology: The name *Lepilemur manasamody* was chosen after the forest region Manasamody, west of Anjiamangirana I between the Sofia and Maevarano River.

Vernacular name: Manasamody sportive lemur or Lépilemur de Manasamody.

Figure 4-8: *Lepilemur manasamody*, portrait of individual 16y03amb (photo by M. Craul).

Figure 4-9: *Lepilemur manasamody*, back of individual 16y03amb (photo by M. Craul).

Figure 4-10: *Lepilemur manasamody*, body of individual 16y03amb (photo by M. Craul).

4.5 Conclusion

Our results showed that all species, except for *L. mustelinus* from the East, grouped in three major clades (western, northwestern, and northern). Taking into account the species diversity within each major clade, however, we can define seven biogeographic zones in northern and northwestern Madagascar. When compared to the predictions derived from the 'Martin model' and the 'Wilmé model', we find several inconsistencies to our data.

The 'Martin model' defined four biogeographic zones from western to northern Madagascar (W1, NW, X and N). They corresponded well to the three deep phylogenetic splits, that gave rise to the western, northwestern, and northern clade in our study. However, the species diversity within each major clade could not be explained by this model.

The 'Wilmé model' defined six biogeographic zones from western to northern Madagascar (numbers 8, 9, 10, 11, 12 and 1). This model may also explain the deep splits

between the three major clades, but it proposed two more. One split is between IRS V and VI, divided by the Sambirano River. This could be confirmed by our study, although it seems not to be a species barrier for sportive lemurs. The level of absolute pairwise distances is intermediate and may rather suggest a variation on a subspecies level. Very recently however, Rabarivola et al. (2006) proposed species status for the individuals in IRS V based on cytogenetics (Rabarivola et al., 2006). They collected samples in IRS V at a locality further north of Mahilaka and the number of chromosomes differed between individuals from IRS V (2N = 24) and the neighbouring *Lepilemur sahamalazensis* (2N = 26) and *Lepilemur dorsalis* (2N = 26). The second additional split indicated by the 'Wilmé model' is that between zone 12 and 1. This split could not be confirmed by our study, since all individuals found in IRS VII (corresponding to Wilmé's zones 12 and 1) clustered together and belonged to the species *L. ankaranensis*. One major discrepancy exists between our findings and the 'Wilmé model'. The 'Wilmé model' predicts one centre of endemism in northwestern Madagascar (zone 9), which should correspond to one *Lepilemur* species in that area. Our study provided evidence, however, for three species of sportive lemurs between the Betsiboka and Maevarano River, each restricted to one of the three IRSs. Thus, we showed that each IRS is represented as a separate terminal clade in the phylogenetic trees, building distinct phylogenetic units. At least six of the seven large rivers act as species barriers for *Lepilemur*. Therefore, we propose a new model, the 'large river model' to explain the biogeography of this large-bodied nocturnal lemur genus. Large rivers acted as insurmountable barriers for gene flow, leading to cryptic speciation within larger biogeographic units. Except for IRS V and VI, the genetic distances among all IRSs reach species level.

The deep splits between the major clades may indicate initial colonization events, with the Betsiboka and Maevarano River playing a major role in long-term and continuous isolation of western, northwestern, and northern Madagascar. The splits within each major clade however, indicate younger cryptic speciation events. Populations, initially belonging to one founder species, entered the IRSs I-III and VI-VII respectively, and were subsequently separated from each other by the rivers Mahajamba and Sofia, and the Andranomalaza. Quaternary paleoclimatic variation may have played another important role in shaping biogeography and speciation events on Madagascar. The climate during periods of glaciations was cooler and drier than today (Wilmé et al., 2006; Haffer, 1969; de Wit, 2003). Rivers with

year-round watercourse could have acted as retreats/refugia in times of aridification. All seven large rivers in northwestern and northern Madagascar should have belonged to this category, since the genetic isolation of the IRSs would otherwise not have persisted over time and signs of repeated introgression should be detectable. Subsequent recolonization of the IRSs should thereby have originated from small and isolated refugia, which further promoted genetic differentiation between the IRSs.

In conclusion, we presented evidence for an unexpected species diversity of sportive lemurs in northwestern and northern Madagascar. Current biogeographic models were not sufficient to explain the underlying processes of speciation. We therefore suggest a new model of biogeographical zonation, the 'large river model'. In this model, biogeographic zones are separated and maintained over time by all large rivers with permanent water bodies that may have provided retreat zones during periods of aridification and may have harboured founder populations for subsequent recolonization. The importance of large rivers as biogeographic barriers was previously emphasized for mouse lemurs (Olivieri et al., 2006), but also for Neotropical primates (Ayres & Clutton-Brock, 1992; Lehman, 2004). Further studies are now needed to test the relevance of this model for other terrestrial taxa, such as the insectivores, rodents, or other lemurs.

4.6 Methods

4.6.1 Fieldwork

A total of 157 *Lepilemur* individuals were captured at 14 different localities along a 560 km transect from western to northern Madagascar (Fig. 4-4, Table 4-4). This region is divided by eight large rivers (over 50 m wide 20 km inlands) into eight Inter-River-Systems (IRS 0 to IRS VII, Fig. 4-4). Six localities were sampled by Mathias Craul (MC) and eight localities were sampled by Solofo Rasoloharijaona (SR) and Blanchard Randrianambinina (BR). At each site we performed daily and nightly surveys to capture the animals. At daytime we used a net to capture the animals out of their sleeping holes and briefly anesthetised them with Ketasel-5 (Selectavet). At night time we anesthetised the animals using a blowpipe (TELINJECT B22T) with Ketasel-5 (Selectavet). Each captured sportive lemur was then characterised with regard to sex, skin colour, reproductive status (testis size or form of vulva), 13 external morphometric measures (ear length, ear width, head length, head width, snout

length, interorbital distance, intraorbital distance, lower leg length, hind foot length, 3rd toe length, body length, tail length, tail circumference) and body mass (Rasoloharijaona et al., 2006; Zimmermann et al., 1998)). In addition, a small biopsy from one or both pinnae was taken as tissue samples. Tissue samples were stored in Queen's lysis buffer (Seutin et al., 1991) for later DNA extraction and genetic analyses.

Table 4-4: Details of study sites.

Locality	Abbreviation	Coordinates	Origin
Madirovalo	Madi	16°22'45.6"S, 46°29'01.9"E	IRS 0
Ampijoroa	JBB	16°17'S, 46°48'E	IRS I
Mariarano	Mari	15°28'50.3"S, 46°41'19.0"E	IRS I
Tananvaovao	Tan	15°28'15.5"S, 46°39'59.4"E	IRS I
Ambodimahabibo	bibo	15°29'54.2"S, 47°28'47.2"E	IRS II
Ambongabe	Amb	15°19'38.3"S, 47°40'44.4"E	IRS III
Anjiamangirana I	Anji	15°09'24.6"S, 47°44'06.2"E	IRS III
Ankarafa	Sah	14°22'47.8"S, 47°45'26.3"E	IRS IV
Mahilaka	Mah	14°17'12.0"S, 48°12'12.0"E	IRS V
Lokobe	Lok	13°23'23.9"S, 48°20'31.0"E	IRS VI
Manehoka	oka	13°25'49.0"S, 48°47'51.0"E	IRS VI
Ankavana	Anka	12°46'55.7"S, 49°22'27.4"E	IRS VII
Ankarana	kar	12°58'05.0"S, 49°08'18.0"E	IRS VII
Analabe	Ana	12°45'13.8"S, 49°30'03.9"E	IRS VII
Kirindy	Kir	20°03'S, 44°37'E	West
Mantadia	Man	18°47'S, 48°25'E	East

4.6.2 Molecular methods and analyses

DNA from the tissue of 37 individuals was isolated with the DNeasy Tissue Kit (Qiagen), or extracted using a standard proteinase K digestion followed by a Phenol/Chloroform protocol (Sambrock et al., 1991) and stored at -20°C. In addition, we analysed the DNA of two

individuals of *Lepilemur ruficaudatus* (Kirindy forest, western Madagascar) provided by Yves Rumpler and of four individuals from Mantadia (eastern Madagascar) sampled previously by SR and BR. We sequenced the mitochondrial genes D-loop, Cytochrome *b* and NADH-dehydrogenase subunit 4 (ND4), because reference sequences from all eleven recognized species were available for these particular markers. The complete D-loop was amplified with the oligonucleotide primers DLp-1.5: 5'-GCA CCC AAA GCT GAR RTT CTA-3' and DLp-5: 5'-CCA TCG WGA TGT CTT ATT TAA GRG GAA-3' (Wyner et al., 1999). Standard PCRs were carried out in a 25µl reaction with a final concentration of 1µM for each primer, 1.5mM for $MgCl_2$, 0.2mM for each dNTP, 1xNH_4 reaction buffer (50mM Tris-HCl pH 8.8, 16mM $(NH_4)_2$ SO_4, 0.1 % Tween® 20), 1.25 units of Taq DNA polymerase, and 1µl of DNA. Successful amplifications were obtained using the following protocol: 35 cycles of denaturing at 94°C for 60 seconds, primer annealing at 47°C for 60 seconds and extension at 72°C for 90 seconds. The partial Cytochrome *b* was amplified with the oligonucleotide primers L14841: 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3' and H15149: 5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3' (Kocher et al., 1989). Standard PCRs were carried out in a 25µl reaction with a final concentration of 1µM for each primer, 1.5mM for $MgCl_2$, 0.2mM for each dNTP, 1xNH_4 reaction buffer (50mM Tris-HCl pH 8.8, 16mM $(NH_4)_2$ SO_4, 0.1 % Tween® 20), 1.25 units of Taq DNA polymerase, and 1µl of DNA. Successful amplifications were obtained using the following protocol: 35 cycles of denaturing at 94°C for 60 seconds, primer annealing at 47°C for 60 seconds and extension at 72°C for 90 seconds. The partial NADH-dehydrogenase subunit 4 was amplified with the oligonucleotide primers LepiP1: 5'-TTG ATG TAG TAT GAC TRT TCC-3' and LepiR1: 5'-GCC AAA CCG ATG GCT GCT TCA CAG GCT GCA AG-3' (Pastorini, 2000). Standard PCRs were carried out in a 25µl reaction with a final concentration of 1µM for each primer, 1.5mM for $MgCl_2$, 0.2mM for each dNTP, 1xNH_4 reaction buffer (50mM Tris-HCl pH 8.8, 16mM $(NH_4)_2$ SO_4, 0.1 % Tween® 20), 1.25 units of Taq DNA polymerase, and 1µl of DNA. Successful amplifications were obtained using the following protocol: 40 cycles of denaturing 95°C for 30 seconds, primer annealing at 60°C for 60 seconds and extension at 72°C for 60 seconds. The PCR products were cleaned with the Invisorb Spin PCRapid Kit (Invitek) or Quick-Clean (Bioline) and checked for successful amplification by running an aliquot on a 1.5 % agarose gel, stained with $1.3x10^{-4}$ mg/ml

ethidium bromide. After cleaning the PCR products, cycle sequencing reactions were carried out using DYEnamic™ ET dye terminator kit (Amersham Biosciences) and the primers indicated above. After a second cleaning with ammonium acetate, provided with the DYEnamic™ ET dye terminator kit, the PCR products were sequenced on a MegaBACE™ 1000 DNA Sequencing System (Amersham Biosciences). The respective sequences were deposited in GenBank (Table 4-5).

Table 4-5: Locality, origin, sample type, and GenBank accession number of analysed individuals for genetic studies. GenBank accession number of sampled individuals will be provided after acceptance of the paper.

Species	Locality	Abbreviation	Origin	Sample type	D-loop	Cyt *b*	ND4
L. ruficaudatus	Kirindy	kir1	west	DNA	ok	ok	ok
L. ruficaudatus	Kirindy	kir2	west	DNA	ok	ok	ok
L. ruficaudatus	Kirindy		west	sequence		DQ109013-DQ109015, DQ109017	AF224596
L. randrianasoli	Andramasay		west	sequence		AY321456	
L. randrianasoli	Andramasay		west	sequence		AY441463, DQ109019, DQ234891-DQ234894	
L. randrianasoli	Ambalarano		west	sequence		DQ234890	
L. aeeclis	Madirovalo	madi1	IRS 0	tissue	ok	ok	ok
L. aeeclis	Madirovalo	madi2	IRS 0	tissue	ok	ok	ok
L. aeeclis	Anjamena		IRS 0	sequence			AF224593
L. aeeclis	Antafia-Anjahamena		IRS 0	sequence		DQ108999-DQ109003, DQ234899	
L. edwardsi	Ampijoroa	jbb1	IRS I	tissue	ok	ok	ok
L. edwardsi	Ampijoroa	jbb2	IRS I	tissue	ok	ok	ok
L. edwardsi	Mariarano	mari1	IRS I	tissue	ok	ok	ok
L. edwardsi	Mariarano	mari2	IRS I	tissue	ok	ok	ok
L. edwardsi	Tananvaovao	tan1	IRS I	tissue	ok	ok	ok
L. edwardsi	Tananvaovao	tan2	IRS I	tissue	ok	ok	ok
L. edwardsi	Ampijoroa		IRS I	sequence		DQ109006	AF224595
L. edwardsi	Andofombombe		IRS I	sequence		DQ109004, DQ109005, DQ234888	
L. sp. nov. IRS II	Ambodimahabibo	bibo1	IRS II	tissue	ok	ok	ok
L. sp. nov. IRS II	Ambodimahabibo	bibo2	IRS II	tissue	ok	ok	ok
L. sp. nov. IRS II	Ambodimahabibo	bibo3	IRS II	tissue	ok	ok	ok

Species	Locality	Abbreviation	Origin	Sample type	D-loop	Cyt *b*	ND4
L. sp. nov. IRS II	Ambodimahabibo	bibo4	IRS II	tissue	ok	ok	ok
L. sp. nov. IRS III	Ambongabe	amb1	IRS III	tissue	ok	ok	ok
L. sp. nov. IRS III	Ambongabe	amb2	IRS III	tissue	ok	ok	ok
L. sp. nov. IRS III	Anjiamangirana I	anji1	IRS III	tissue	ok	ok	ok
L. sahamalazensis	Ankarafa	sah1	IRS IV	tissue	ok	ok	ok
L. sahamalazensis	Ankarafa	sah2	IRS IV	tissue	ok	ok	ok
L. sahamalazensis	Ankarafa	sah3	IRS IV	tissue	ok	ok	ok
L. sahamalazensis	Sahamalaza		IRS IV	sequence		DQ108990-DQ108992, DQ234882, DQ234883	
L. dorsalis	Mahilaka	mah1	IRS V	tissue	ok	ok	ok
L. dorsalis	Mahilaka	mah2	IRS V	tissue	ok	ok	ok
L. dorsalis	Mahilaka	mah3	IRS V	tissue	ok	ok	ok
L. dorsalis	Ambanja		IRS V	sequence		DQ108995-DQ108997, DQ234886, DQ234887	
L. dorsalis	Mahilaka	mah4	IRS V	tissue	ok	ok	ok
L. dorsalis	Lokobe	lok1	IRS VI	tissue	ok	ok	ok
L. dorsalis	Lokobe	lok3	IRS VI	tissue	ok	ok	ok
L. dorsalis	Manehoka	oka1	IRS VI	tissue	ok	ok	ok
L. dorsalis	Manehoka	oka2	IRS VI	tissue	ok	ok	ok
L. dorsalis	Manehoka	oka3	IRS VI	tissue	ok	ok	ok
L. dorsalis	Manehoka	oka4	IRS VI	tissue	ok	ok	ok
L. dorsalis	Nosy Be		IRS VI	sequence		AY441464, DQ108993, DQ108994, DQ108998, DQ234885	
L. ankaranensis	Ankavana	anka1	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Ankavana	anka2	IRS VII	tissue	ok	ok	ok

Species	Locality	Abbreviation	Origin	Sample type	D-loop	Cyt *b*	ND4
L. ankaranensis	Ankavana	anka3	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Ankarana	kar1	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Ankarana	kar2	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Ankarana	kar3	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Analabe	ana1	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Analabe	ana2	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Analabe	ana3	IRS VII	tissue	ok	ok	ok
L. ankaranensis	Ankarana		IRS VII	sequence		DQ109028-DQ109032	AF304597
L. ankaranensis	Analamera		IRS VII	sequence		DQ109022-DQ109024, DQ234884	
L. ankaranensis	Andrafiamena		IRS VII	sequence		DQ109025, DQ109027, DQ234881	
L. septentrionalis	Sahafary		IRS VII	sequence	AJ304651	DQ109020, DQ109021, DQ234900	
L. mustelinus	Mantadia	man1	east	tissue	ok	ok	ok
L. mustelinus	Mantadia	man2	east	tissue	ok	ok	ok
L. mustelinus	Mantadia	man3	east	tissue	ok	ok	ok
L. mustelinus	Mantadia	man4	east	tissue	ok	ok	ok
L. mustelinus	Behasina		east	sequence		DQ109033	
L. mustelinus	near Mantadia		east	sequence		DQ109034	
L. microdon	Vohiparara		east	sequence		DQ109008	
L. microdon	Antarando		east	sequence		DQ109009, DQ109010	
L. microdon	Ambatolampy		east	sequence		DQ234889	
L. leucopus			south	sequence		DQ109007	
P. diadema				sequence	AF354743	AY441452	AF224599

For a comprehensive phylogenetic analysis of the sequence data, we expanded our data set with reference sequences from all eleven recognized species available from GenBank (Table 4-5). As outgroup for phylogenetic tree reconstructions, we selected *Propithecus diadema*. Sequences were aligned using the program CLUSTAL X (Thompson et al., 1997) and checked by eye. Tree reconstructions of each single gene were carried out to phylogenetically classify the sampled individuals within the genus *Lepilemur*. Because of the lack of reference sequences of single individuals for all three genes, further phylogenetic tree reconstructions based on all three genes were performed only with our own data set consisting of 43 sequences. Phylogenetic tree reconstructions were carried out with the maximum-parsimony (MP), neighbour-joining (NJ), and maximum-likelihood (ML) algorithms as implemented in PAUP4.0b10 (Swofford, 1999). Throughout the analyses, all characters were treated as unordered and equally weighted. Gaps were considered as missing data in NJ and ML, but were treated as fifth character in MP analysis. The NJ and ML trees were constructed using the best-fit model selected by the hierarchical likelihood ratio test (hLRT) in Modeltest3.5.mac (Posada & Crandall, 1998). Relative support of internal nodes was provided by bootstrap analyses with 1 000 replications for MP and NJ and 100 replications for ML. Absolute pairwise distances were calculated using PAUP4.0b10 and ARLEQUIN 1.1 to describe the variation among taxa. To determine fixed molecular differences among terminal clades (indicating barriers for gene flow), diagnostic sites for each terminal clade to all others were identified using the program MEGA 3.1 (Kumar et al., 2004).

4.6.3 Statistical analyses of morphometric data

Quantitative analyses of morphometric data were carried out with two different sample sizes. The ANOVA was conducted with 100 individuals. After removing two variables that differed among researchers (1-way ANOVA, STATISTICA 6.0, Statsoft, Inc.), the 11 remaining variables were tested for normality using the Kolmogorov-Smirnov test (Statistica 6.0, StatSoft, Inc.) at a level of $p \leq 0.05$. All were normally distributed. A MANOVA revealed no differences in sex. The variables were then tested for correlation. All variables had an $r < 0.75$ and were therefore defined as sufficiently independent to be used in a discriminant function analysis (Braune et al., 2005). This analysis was limited to five adult individuals per species (established and proposed) in order to equilibrate the samples. The discriminant function

analysis tested only for species differences and for differences between the IRSs. A stepwise forward method (statistic: Wilk's λ) with the criteria $F_{to\ enter} = 3.84$ and $F_{to\ remove} = 2.71$ and a tolerance level of $p \leq 0.01$ was used to calculate the discriminant function model. The computed discriminant functions were used to classify cases with regard to their group membership. All cases were cross-validated by the 'leave-one-out' method, where each case in the analysis is classified by the functions derived from all cases other than that case. The discriminant function analysis was carried out with the program SPSS 13.0 (SPSS, Inc.).

4.7 Footnote

During the review process of this paper, Louis Jr. et al. (2006) described a new sportive lemur species in IRS III. It was named *Lepilemur grewcockorum*. This might be a synonym to *L. manasamody*, as our sampling sites of this species were in the same IRS. However, a joint phylogenetic analysis is still needed to verify the identity of both forms.

4.8 Authors' contributions

MC participated in the design of the study, conducted part of the fieldwork and all lab work, performed the comparative genetic and morphometric analyses and wrote the MS.

EZ initiated, financed and designed the study. She organized and conceptualized the fieldwork, supervised data analyses and critically revised the MS several times and approved its final version.

SR conducted part of the fieldwork including capturing, measuring and sampling *L. ankaranensis*, *L. dorsalis*, *L. mustelinus* and *L. aeeclis*.

BR conducted part of the fieldwork including capturing, measuring and sampling *L. ankaranensis, L. dorsalis*, *L. mustelinus* and *L. aeeclis*.

UR designed the study. She organized and conceptualized the fieldwork, supervised data analyses and critically revised the MS several times and approved its final version.

4.9 Acknowledgements

We like to thank the late B. Rakotosamimanana, as well as C. Andrianarivo, O. Ramilijaona, D. Rakotondravony and the following institutions for their authorization and support of this study: Faculty of Sciences, University of Antananarivo, the Association Nationale pour la Gestion des Aires Protégées (ANGAP), the Ministère des Eaux et Forêts and CAFF/CORE (Commision Tripartite), Madagascar. Many thanks go to G. Olivieri, K. Guschanski, C. Schwitzer, N. Schwitzer, R. Rakotondravony, A. Rabesandratana, J.-A. Rakotonirina, T. Razafimamonjy, B. Rakoloarinelina, and J. Rajanary for their assistance in the field, to H. Breitrück and S. Sippel for their help in the lab and to Y. Rumpler for providing DNA of *Lepilemur ruficaudatus*. The project was funded by the Volkswagen Foundation (VW I, Aktenzeichen I/76828, Zimmermann), and the 'Gesellschaft der Freunde der Tierärztlichen Hochschule Hannover e.V.'. We dedicate this paper to the late Berthe Rakotosamimanana.

4.10 Appendix

Appendix 4-A: Diagnostic sites of the Cytochrome *b* region for each terminal clade.

	3	19	23	24	35	38	39	45	56	68	71	74	86	93	109	137	140	146	155	167	173	179	185	200	204	205	218	224	237	242
L. mustelinus	**A**	**A**	**A**	**C**	**C**	**T**	**A**	**C**	**C**	**C**	**T**	**T**	**A**	**C**	**C**	**T**	**A**	**T**	**C**	**C**	**C**	**T**	**A**	**C**	**G**	**T**	**C**	**C**	**A**	**C**
L. ankaranensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. dorsalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. sahamalazensis	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	.	G	.	.	.	.	.	.	.
L. manasamody	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.
L. edwardsi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.
L. otto	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. ruficaudatus	G	G	G	T	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	T	.	.	.
L. aeeclis	.	.	.	.	.	.	G	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	T

Dashes (-) indicate deletions. Points (.) indicate identical bases.

	248	254	290	295	300	308	314	315	320	325	326	330	333	339	340	341	Sum
L. mustelinus	**A**	**A**	**T**	**C**	**C**	**C**	**T**	**C**	**C**	**T**	**C**	**T**	**C**	**A**	**C**	**A**	
L. ankaranensis	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	1
L. dorsalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
L. sahamalazensis	C	.	.	.	T	.	.	.	.	C	.	.	.	.	.	.	7
L. manasamody	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
L. edwardsi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
L. otto	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
L. ruficaudatus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8
L. aeeclis	.	G	.	T	.	T	.	.	.	.	A	.	.	.	.	G	10

Dashes (-) indicate deletions. Points (.) indicate identical bases.

Appendix 4-B: Diagnostic sites of the ND4 region for each terminal clade.

	10	12	30	39	42	43	48	50	53	54	55	57	63	64	75	78	112	123	131	132	162	171	201	204	214	215	255	267	288
	291	292	296	297	306	307	312	327	333	334	346	381	401	434	446	453	459	461	464	468	469	477	488	492	493	495	497	510	513
L. mustelinus	**A**	**A**	**T**	**G**	**G**	**G**	**C**	**A**	**C**	**A**	**C**	**A**	**T**	**-**	**C**	**T**	**T**	**C**	**C**	**C**	**A**	**A**	**C**	**C**	**T**	**C**	**C**	**A**	**C**
L. ankaranensis	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. dorsalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. sahamalazensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
L. manasamody	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. edwardsi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. otto	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. ruficaudatus	.	G	.	.	.	.	T	G	.	.	.	G	.	.	.	.	.	T	.	.	G	.	.	.	.	.	T	.	T
L. aeeclis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.	T	.	.	.	G	.

Dashes (-) indicate deletions. Points (.) indicate identical bases.

	10	12	30	39	42	43	48	50	53	54	55	57	63	64	75	78	112	123	131	132	162	171	201	204	214	215	255	267	288
	291	292	296	297	306	307	312	327	333	334	346	381	401	434	446	453	459	461	464	468	469	477	488	492	493	495	497	510	513
L. mustelinus	**A**	**A**	**T**	**G**	**G**	**G**	**C**	**A**	**C**	**A**	**C**	**A**	**T**	**-**	**C**	**T**	**T**	**C**	**C**	**C**	**A**	**A**	**C**	**C**	**T**	**C**	**C**	**A**	**C**
L. ankaranensis	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. dorsalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. sahamalazensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.
L. manasamody	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. edwardsi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. otto	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
L. ruficaudatus	.	G	.	.	.	.	T	G	.	.	.	G	.	.	.	.	.	T	.	.	G	.	.	.	.	.	T	.	T
L. aeeclis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	.	T	.	.	.	G	.

Dashes (-) indicate deletions. Points (.) indicate identical bases.

	519	549	558	559	571	574	586	587	590	605	606	610	618	627	630	631	632	Sum
L. mustelinus	**C**	**G**	**A**	**T**	**G**	**T**	**A**	**C**	**T**	**C**	**C**	**A**	**C**	**C**	**T**	**C**	**T**	
L. ankaranensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
L. dorsalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
L. sahamalazensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
L. manasamody	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
L. edwardsi	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1
L. otto	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	C	8
L. ruficaudatus	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	16
L. aeeclis	.	.	.	.	.	.	.	.	.	.	T	.	T	.	.	.	.	10

Dashes (-) indicate deletions. Points (.) indicate identical bases.

Appendix 4-C: Diagnostic sites of the D-loop region for each terminal clade.

	20	22	23	24	75	77	78	80	122	123	151	155	156	157	165	166	181	191	195	208	222	260	272	273	279	281	283	291	358	360	Sum
L. mustelinus	**A**	**G**	**C**	**C**	**C**	**A**	**T**	**A**	**C**	**T**	**C**	**A**	**A**	**A**	**A**	**A**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**C**	**A**	**T**	**T**	**T**	**C**	**C**	
L. ankaranensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
L. dorsalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	1
L. sahamalazensis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	2
L. manasamody	.	.	.	.	G	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2
L. edwardsi	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
L. otto	C	A	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	3
L. ruficaudatus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
L. aeeclis	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	3

Dashes (-) indicate deletions. Points (.) indicate identical bases.

4.11 References

Andriaholinirina N, Fausser JL, Roos C, Zinner D, Thalmann U, Rabarivola C, Ravoarimanana I, Ganzhorn JU, Meier B, Hilgartner R, Walter L, Zaramody A, Langer C, Hahn T, Zimmermann E, Radespiel U, Craul M, Tomiuk J, Tattersall I and Rumpler Y (2006) Molecular phylogeny and taxonomic revision of the sportive lemurs (*Lepilemur*, Primates). *BMC Evolutionary Biology*, **6**, 17.

Asher RJ and Hofreiter M (2006) Tenrec phylogeny and the noninvasive extraction of nuclear DNA. *Systematic Biology*, **55**, 181-194.

Ayres JM and Clutton-Brock TH (1992) River boundaries and species range size in Amazonian primates. *American Naturalist*, **140**, 531–537.

Braune P, Schmidt S and Zimmermann E (2005) Spacing and group coordination in a nocturnal primate, the golden brown mouse lemur (*Microcebus ravelobensis*): the role of olfactory and acoustic signals. *Behavioral Ecology and Sociobiology*, **58**, 587-596.

Camargo A, de Sá RO and Heyer WR (2006) Phylogenetic analyses of mtDNA sequences reveal three cryptic lineages in the widespread neotropical frog *Leptodactylus fuscus* (Schneider, 1799) (Anura, Leptodactylidae). *Biological Journal of the Linnean Society*, **87**, 325-341.

Cooper A, Lalueza-Fox C, Anderson S, Rambaut A, Austin J and Ward R (2001) Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. *Nature*, **409**, 704-707.

Cracraft J (1983) Species concepts and speciation analysis. *Current Ornithology*, **1**, 159-187.

Davis JI and Nixon KC (1992) Populations, genetic variation and the delimitation of phylogenetic species. *Systematic Biology*, **41**, 421-435.

DeSalle R (2006) Species Discovery versus Species Identification in DNA Barcoding Efforts: Response to Rubinoff. *Conservation Biology*, **20**, 1545-1547.

de Wit MJ (2003) MADAGASCAR: Heads It's a Continent, Tails It's an Island. *Annual Review of Earth and Planetary Sciences*, **31**, 213-248.

Farias IP, Orti G, Sampaio I, Schneider H and Meyer A (1999) Mitochondrial DNA phylogeny of the family Cichlidae: monophyly and fast molecular evolution of the neotropical assemblage. *Journal of Molecular Evolution*, **48**, 703-711.

Fausser JL, Prosper P, Donati G, Ramanamanjato JB and Rumpler Y (2002) Phylogenetic relationships between *Hapalemur* species and subspecies based on mitochondrial DNA sequences. *BMC Evolutionary Biology*, **2**, 4.

Ganzhorn JU, Langrand O, Wright PC, O'Connor S, Rakotosamimanana B, Feistner ATC and Rumpler Y (1996/7) The state of lemur conservation in Madagascar. *Primate Conservation*, **17**, 70-86.

Goetze E (2003) Cryptic speciation on the high seas; global phylogenetics of the copepod family Eucalanidae. *Proceedings: Biological Sciences*, **270**, 2321-2331.

Haffer J (1969) Speciation in Amazonian Forest Birds. *Science*, **165**, 131-137.

Jansa SA, Barker FK and Heaney LR (2006) The pattern and timing of diversification of Philippine endemic rodents: evidence from mitochondrial and nuclear gene sequences. *Systematic Biology*, **55**, 73-88.

Kappeler PM, Rasoloarison RM, Razafimanantsoa L, Walter L and Roos C (2005) Morphology, Behaviour and Molecular Evolution of Giant Mouse Lemurs (*Mirza* spp.) Gray, 1870, with Description of a new Species. *Primate Report*, **71**, 3-26.

Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX and Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences USA*, **86**, 6196-6200.

Kumar S, Tamura K and Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinformatics*, **5**, 150-163.

Lehman SM (2004) Distribution and Diversity of Primates in Guyana: Species-Area Relationships and Riverine Barriers. *International Journal of Primatology*, **25**, 73–95.

Louis Jr EE, Engberg SE, Lei R, Geng H, Sommer JA, Randriamampionona R, Randriamanana JC, Zaonarivelo JR, Andriantompohavana R, Randria G, Prosper, Ramaromilanto B, Rakotoarisoa G, Rooney A and Brenneman RA (2006) Molecular and Morphological Analyses of the Sportive Lemurs (Family Megaladapidae: Genus *Lepilemur*) Reveals 11 Previously Unrecognized Species. *Special Publications*, *Museum of Texas Tech University*, **49**, 1-47.

Martin RD (1972) Adaptive radiation and behaviour of the Malagasy lemurs. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **264**, 295-352.

Martin RD (1990) *Primate Origins and Evolution: A Phylogenetic Reconstruction*. London/New Jersey: Chapman Hall/Princeton University Press.

Martin RD (1995) Prosimians: from obscurity to extinction? In: *Creatures of the dark: the nocturnal prosimians* (eds. Alterman L, Doyle GA and Izard MK), pp. 535-563. New York: Plenum Press.

Mayor MI, Sommer JA, Houck ML, Zaonarivelo JR, Wright PC, Ingram C, Engel SR and Louis Jr EE (2004) Specific status of *Propithecus* spp. *International Journal of Primatology*, **25**, 875-900.

Mittermeier RA, Konstant WR, Hawkins F, Louis EE, Langrand O, Ratsimbazafy J, Rasoloarion R, Ganzhorn JU, Rajaobelina S, Tattersall I and Meyers DM (2006) *Lemurs of Madagascar*. 2nd edition, Washington, DC: Conservation International.

Moritz C and Cicero C (2004) DNA barcoding: promise and pitfalls. *PloS Biology*, **2**, 1529-1531.

Nagy ZT, Joger U, Wink M, Glaw F and Vences M (2003) Multiple colonization of Madagascar and Socotra by colubrid snakes: evidence from nuclear and mitochondrial gene phylogenies. *Proceedings: Biological Sciences*, **270**, 2613-2621.

Olivieri G, Zimmermann E, Randrianambinina B, Rasoloharijaona S, Rakotondravony D, Guschanski K and Radespiel U (2006) The ever-increasing diversity in mouse lemurs: three new species in north and northwestern Madagascar. *Molecular Phylogenetics and Evolution*, **43**, 309-327.

Pastorini J (2000) Molecular Systematics of Lemurs. *PhD thesis*. University of Zürich.

Pastorini J, Martin RD, Ehresmann P, Zimmermann E and Forstner MRJ (2001) Molecular phylogeny of the lemur family Cheirogaleidae (Primates) based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, **19**, 45-56.

Pastorini J, Forstner MRJ and Martin RD (2002) Phylogenetic relationships among Lemuridae (Primates): evidence from mtDNA. *Journal of Human Evolution*, **43**, 463-478.

Pastorini J, Thalmann U and Martin RD (2003) A molecular approach to comparative phylogeography of extant Malagasy lemurs. *Proceedings of the National Academy of Sciences USA*, **100**, 5879-5884.

Petit G (1933) Le genre Lepidolemur et sa répartition géographique. *Comptes Rendus de la Société de Biogéographie*, **10**, 33-37.

Petter JJ and Petter-Rousseaux A (1960) Remarque sur la systématique du genre *Lepilemur*. *Mammalia*, **24**, 76-86.

Petter JJ, Albignac R and Rumpler Y (1977) Mammifères lémuriens (Primates prosimiens). In: *Faune de Madagascar*. Paris: C.N.R.S./O.R.S.T.O.M.

Piaggio AJ and Perkins SL (2005) Molecular phylogeny of North American long-eared bats (Vespertilionidae: Corynorhinus); inter- and intraspecific relationships inferred from mitochondrial and nuclear DNA sequences. *Molecular Phylogenetics and Evolution*, **37**, 762-775.

Posada D and Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Rabarivola C, Zaramody A, Fausser JL, Andriaholinirina N and Roos C (2006) Cytogenetic and molecular characteristics of a new species of sportive lemur from Northern Madagascar. *Lemur News*, **11**, 45-49.

Rasoloharijaona S, Rakotosamimanana B, Randrianambinina B and Zimmermann E (2003) Pair-specific usage of sleeping sites and their implications for social organization in a nocturnal Malagasy primate, the Milne Edwards' sportive lemur (*Lepilemur edwardsi*). *American Journal of Physical Anthropology*, **122**, 251-258.

Rasoloharijaona S, Randrianambinina B, Braune P and Zimmermann E (2006) Loud calling, spacing, and cohesiveness in a nocturnal primate, the Milne Edwards' sportive lemur (*Lepilemur edwardsi*). *American Journal of Physical Anthropology*, **129**, 591-600.

Ravaoarimanana IB, Tiedemann R, Montagnon D and Rumpler Y (2004) Molecular and cytogenetic evidence for cryptic speciation within a rare endemic Malagasy lemur, the Northern Sportive Lemur (*Lepilemur septentrionalis*). *Molecular Phylogenetics and Evolution*, **31**, 440-448.

Rumpler Y and Albignac R (1978) Chromosomes studies of the *Lepilemur*, an endemic Malagasy genus of lemurs: Contribution of the cytogenetics to their taxonomy. *Journal of Human Evolution*, **7**, 191-196.

Sambrook J, Fritsch E and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. 2nd edition, Cold Spring Harbor: Cold Spring Harbor press.

Seutin G, White BN and Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, **69**, 82-90.

Swofford DL: PAUP* (1999) *Phylogenetic analysis using parsimony (*and other methods), version 4.0 b2*. Sunderland, MA, Sinauer Associates.

Tattersall I (1982) *The primates of Madagascar*. New York: Columbia University Press.

Thalmann U and Ganzhorn JU (2003) *Lepilemur*, Sportive Lemur. In: *The Natural History of Madagascar* (eds. Goodmann SM and Benstead JP), pp. 1336-1340. Chicago: The University of Chicago Press.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876-4882.

Vences M, Vieites DR, Glaw F, Brinkmann H, Kosuch J, Veith M and Meyer A (2003) Multiple overseas dispersal in amphibians. *Proceedings: Biological Sciences*, **270**, 2435-2442.

Will KW and Rubinoff D (2004) Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics*, **20**, 47-55.

Wilmé L, Goodman SM and Ganzhorn JU (2006) Biogeographic evolution of Madagascar's microendemic biota. *Science*, **312**, 1063-1065.

Wyner YM, Amato G and Desalle R (1999) Captive breeding, reintroduction, and the conservation genetics of black and white ruffed lemurs, *Varecia variegata variegata*. *Molecular Ecology*, **8**, 107-115.

Yoder AD, Rasoloarison RM, Goodman SM, Irwin JA, Atsalis S, Ravosa MJ and Ganzhorn JU (2000) Remarkable species diversity in Malagasy mouse lemurs (primates, Microcebus). *Proceedings of the National Academy of Sciences USA*, **97**, 11325-11330.

Yoder AD, Burns MM, Zehr S, Delefosse T, Veron G, Goodman SM and Flynn JJ (2003) Single origin of Malagasy carnivora from an African ancestor. *Nature*, **421**, 734-737.

Yoder AD, Olson LE, Hanley C, Heckman KL, Rasoloarison R, Russell AL, Ranivo J, Soarimalala V, Karanth KP, Raselimanana AP and Goodman SM (2005) A multidimensional approach for detecting species patterns in Malagasy vertebrates. *Proceedings of the National Academy of Sciences USA*, **102**, 6587-6594.

Zimmermann E, Cepok S, Rakotoarison N, Zietemann V and Radespiel U (1998) Sympatric mouse lemurs in north-west Madagascar: a new rufous mouse lemur species (*Microcebus ravelobensis*). *Folia Primatologica*, **69**, 106-114.

Chapter 5

Second study

Isolation of sixteen autosomal loci and a sex-linked microsatellite locus from the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*)

Published as: Craul M, Dawson DA, Radespiel U, Zimmermann E and Bruford MW (2009) Isolation of sixteen autosomal loci and a sex-linked polymorphic microsatellite locus from the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*). *Molecular Ecology Resources 9*, 333-335.

In press as: Erratum to 'Craul M, Dawson DA, Radespiel U, Zimmermann E and Bruford MW (2009) Isolation of sixteen autosomal loci and a sex-linked polymorphic microsatellite locus from the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*). *Molecular Ecology Resources 9*, 333-335.'

Data collection:	Craul
Lab work:	Craul
Analysis:	Craul, Dawson
Manuscript:	Craul, Dawson, Radespiel
Supervision:	Bruford, Radespiel, Zimmermann

5 Second study

Isolation of sixteen autosomal loci and a sex-linked microsatellite locus from the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*)

5.1 Abstract

We isolated twenty-one microsatellites from the Milne-Edwards' sportive lemur, *Lepilemur edwardsi*. Eighteen microsatellite sequences possessed sufficient flanking DNA for primer design. Seventeen loci amplified and were found to be polymorphic displaying two to 17 alleles in 32 unrelated individuals from a population from the Ankarafantsika National Park in northwestern Madagascar. One locus (*Led-12*) was found to be sex-linked located on the X chromosome and can be used to sex-type 40% of female *Lepilemur edwardsi* lemurs. These 17 loci were characterised to investigate family structure and the phylogeography of *L. edwardsi*.

5.2 Main text of publication

Sportive lemurs (*Lepilemur* spp.) are endemic to Madagascar. They belong to the large-bodied, nocturnal lemurs of the family Lepilemuridae and are found in almost all forested regions of Madagascar. The species diversity, phylogenetic history, and conservation status of *Lepilemur* are all debated (Yoder & Yang, 2004; Craul et al., 2007). All *Lepilemur* species are assigned to the categories 'lower risk', 'vulnerable' or 'endangered' in the International Union for the Conservation of Nature and Natural Resources (IUCN) Red Data Book (www.redlist.org), due to the rapid loss of forest habitats and the high threat imposed by poaching.

Tissue samples were collected from 32 unrelated Milne-Edwards' sportive lemurs from northwestern Madagascar. Genomic DNA was extracted from ear tissue of each individual using the DNeasy Tissue Kit (Qiagen, Germany).

A standard genomic library was constructed (without enrichment) following the protocol provided by the Sheffield Molecular Genetics Facility (http://www.shef.ac.uk/molecol/smgf). Briefly, genomic DNA, pooled from two unrelated female *Lepilemur edwardsi* individuals (14-03 and 05-03), was digested with *Mbo*I (ABgene). The digested DNA was ligated into pUC18-*Bam*HI-BAP vector (Amersham Pharmacia Biotech) and transformed into XL1-Blue competent cells (Stratagene). In total, 29 800 transformant colonies were transferred to a Hybond-N nylon membrane (Amersham Pharmacia Biotech) and screened by hybridisation to the sequences $(CA.GT)_n$, $(GA.CT)_n$ (Amersham Pharmacia Biotech) or $(GATA)_n$ (prepared as in Armour et al., 1994) radiolabelled with ($a^{32}P$)-dCTP. One hundred and nine autoradiograph positives were obtained.

Positive clones were sequenced with the M13 forward primer (5'-TGT AAA ACG ACG GCC AGT-3'). All sequences containing a microsatellite repeat region were sequenced with the M13 reverse primer (5'-CAG GAA ACA GCT ATG AC-3') and consensus sequence files created. Sequencing was performed using ABI BigDye Terminators (Mix version 1.1) on an ABI 3730 Sequencer (PE Applied Biosystems). Of the 109 clones sequenced, 21 contained microsatellite repeat motifs with more than 10 uninterrupted repeats or with multiple shorter interspersed repeats. However, only 17 of 21 possessed suitable flanking regions for primer design and could be amplified via polymerase chain reaction (PCR) (Table 5-1). These 21 repeat-containing sequences were confirmed unique using stand-alone BLASTN 2.2.4

software (Altschul et al., 1997), submitted to the European Molecular Biology Laboratory (EMBL) database (EMBL Accession nos AJ717492 - AJ717512).

PCR primers were designed for 18 sequences using PRIMER version 3 (Rozen & Skaletsky, 2000, Table 5-1). Primers were not designed for three loci that had less than 10 bp of flanking sequence (*Led-02*, *Led-14* and *Led-21*).

A 10 µl PCR reaction was performed with each primer pair containing 50 ng of genomic DNA, 1.0 µM of each primer and 0.25 units *Taq* DNA polymerase (Thermoprime Plus, ABgene) in the manufacturer's buffer [final concentrations 20 mM $(NH_4)_2SO_4$, 75 mM Tris-HCl pH 9.0, 0.01 % (w/v) Tween® 20], including 1.0-3.0 mM $MgCl_2$ (Table 5-1) and 0.2 mM of each dNTP. The 5' end of the forward primer of every pair was fluoro-labelled (Table 5-1). PCR amplification was performed in a Hybaid Touchdown thermal cycler (Thermo Hybaid). The reaction profile used was 94°C for 3 min then 94°C for 30 s, annealing temperatures as described in Table 5-1 for 30 s, 72°C for 30 s during 35 cycles and a final extension at 72°C for 3 min.

PCR amplification was visualised on 2 % agarose gels. Allele sizes were assigned using GENOTYPER 3.6 NT software (PE Biosystems) on an ABI3730 DNA Sequencer. Primers for locus *Led-06* did not amplify any product. Seventeen loci were polymorphic, displaying two to 17 alleles in 32 unrelated individuals (of known sex) belonging to a single population from the Ankarafantsika National Park in northwestern Madagascar (Table 5-1).

Loci were checked for sex linkage by observation of the genotypes of known sex individuals. Heterozygotes were found in both sexes for 16 loci, suggesting that these loci were not sex-linked (X or Y) but for one polymorphic locus (*Led-12*), all of the 12 males tested were homozygous but the 18 females were either heterozygous or homozygous (Table 5-1), suggesting this locus was sex-linked being located on the X chromosome. All sequences were compared to the genome sequence of two other primates (chimp and human) using an ENSEMBL WU-BLAST with the 'distant homologies' settings (http://www.ensembl.org/index.html). The *Led-12* sequence possessed high sequence similarity across the lemur sequence flanking both sides of the repeat region to the chimp/human X chromosome, supporting its suggested X-linked status (E-val = 1.5e-39). Since this locus is sex-linked, we calculated the observed and expected heterozygosity values using the 18 female genotypes only. *Led-12* had an observed heterozygosity of 0.40

(Table 5-1) and therefore can be used to confidently sex-type approximately 40 % of female *Lepilemur edwardsi*. Homozygous genotypes may indicate a homozygous female or a (hemizygous) male so cannot be used to assign sex.

Observed and expected heterozygosity and a test for Hardy-Weinberg Equilibrium (*HWE*) were calculated for each locus using ARLEQUIN 3.01 (Excoffier et al., 2005; Table 5-1). Tests for linkage disequilibrium were conducted using GENEPOP (Raymond & Rousset, 1995). No pairs of loci displayed linkage disequilibrium after correcting for multiple tests (Rice, 1989). One locus (*Led-12*) was not in agreement with *HWE* expectations (Table 5-1). This may be due in part to the high numbers of alleles displayed by each locus when relatively few individuals were genotyped or may be due to null alleles.

These 17 loci will be used to sex-type lemurs and investigate family structure and the phylogeography of *Lepilemur edwardsi*. *Led-12* will also be used to sex-type individuals and may be especially useful to sex-type lemurs sampled from hair traps or fecal samples (when the individual is no longer present so sex cannot be assigned).

5.3 Acknowledgements

Terry Burke kindly provided comments on the manuscript. We thank B. Rakotosamimanana, C. Andrianarivo, O. Ramilijaona, D. Rakotondravony and the following institutions for their authorization and support of this study: Faculty of Sciences, University of Antananarivo, the Association Nationale pour la Gestion des Aires Protégées (ANGAP), the Direction Générale des Eaux et Forêts, Madagascar, and the University of Veterinary Medicine Hannover, Germany. Assistance in the field was provided by G. Olivieri, R. Rakotondranony, A. Rabesandratana, J.-A. Rakotonirina, T. Razafimamonjy, B. Rakoloarinelina, and J. Rajanary. Genotyping and sequencing analysis advice was provided by A. Krupa and A. Leviston. The project was funded by the Volkswagen Foundation (VW I, Aktenzeichen I/76828, Zimmermann), Germany, the Natural Environment Research Council, UK, and the 'Gesellschaft der Freunde der Tierärztlichen Hochschule Hannover e.V.' Germany.

Table 5-1: Characteristics of 17 autosomal and sex-linked polymorphic Milne-Edwards's sportive lemur (*Lepilemur edwardsi*) microsatellite loci.

Locus	Repeat motif	Primer sequence (5' to 3')	Label (F)	T_m (°C)	T_a (°C)	$MgCl_2$	*n*	*A*	Expected product size (bp)†	Observed allele size (bp)	H_o	H_e	p_{HW}	EMBL no.
Led-01	$(GT)_{14}$	(F) TCTCATCAGAGCATGTGTTTTTC (R) TGCCTTCAGAACTTTTTGAATC	HEX	51.2 50.7	54	3.0	22	10	124	108-132	0.76	0.82	0.625	AJ717493
Led-03	$(GT)_{12}$	(F) AACAATAAAAATTCAGTGTGTCACC (R) CCTCATGTTTGTCTTTGACTCAC	HEX	51.3 50.6	56	1.0	31	9	324	320-338	0.90	0.81	0.739	AJ717501
Led-04	$(CA)_5$ AA $(CA)_3$ TA $(CA)_3$CG $(CA)_2$ CG $(CA)_4$	(F) CAGAAATGGAACTTCAACGG (R) AGGGATGGATGTAGATGCTAAG	FAM	49.7 49.5	55	2.0	31	6	207	199-213	0.56	0.64	1.000	AJ717497
Led-05	$(GTCT)_5$CC $(CT)_{12}$	(F) GATCAATCACATGAGCCTTCTG (R) GATCTCCGTGTGCCTGTTAG	HEX	59.7 58.3	60	2.0	32	4	85	82-88	0.25	0.38	0.068	AJ717495
Led-07	$(GT)_9$ CT $(GT)_6$ CT $(GT)_3$	(F) TTCTACAACTTTGGCCTTTGG (R) TTGAGCCCAGGATTTTGAAG	HEX	59.2 60.2	56	1.5	32	2	160	162-164	0.30	0.61	0.115	AJ717498
Led-08	$(GT)_{12}(GA)_{24}$	(F) GTAGCCTCCGGAACAGTGAC (R) GATCTCTCTCTCTCTCTCTCTCTCTC	HEX	59.7 59.8	61	1.0	31	10	132	116-136	0.88	0.82	0.854	AJ717500
Led-09	$(GT)_{19}$	(F) ATCTGCCTGCAGAAGGTC (R) GCTGTGGGAATAGGCACT	HEX	56.3 56.1	60	1.0	32	14	168	136-186	0.65	0.92	0.092	AJ717499
Led-10	$(CT)_7$ $(CA)_6$ GACATA $(CA)_3$ TAGATA $(CA)_{14}$	(F) TGTAATATTATCAAAAATAATCTCTCTCT (R) AGTTCCTAGAGGAATGCTCA	FAM	54.3 54.1	54	1.0	30	8	324	308-338	0.70	0.85	0.298	AJ717502

T_m, melting temperature for each primer (predicted by PRIMER3; Rozen & Skaletsky 2000); T_a, annealing temperature used for PCR; $MgCl_2$, magnesium chloride concentration; *n*, number of unrelated (as known) *Lepilemur edwardsi* individuals genotyped from a population in the Ankarafantsika National Park, northwest Madagascar; *A*, number of alleles; †, based on sequenced allele of one of the two individuals used as the library source; H_e, expected heterozygosity; H_o, observed heterozygosity; p_{HW}, exact *p* for the Hardy-Weinberg equilibrium test; ‡, locus deviates from Hardy-Weinberg equilibrium (*p*-value < 0.05); X, *Led-12*, all 12 males were all homozygous but the 18 females were either heterozygous or homozygous suggesting this locus is X-linked. Observed and expected heterozygosity was therefore calculated using the 18 female genotypes only. *Led-12* was confirmed to be X-linked by observed sequence similarity to chimp and human X chromosome sequence (see text).

Locus	Repeat motif	Primer sequence (5' to 3')	Label (F)	T_m (°C)	T_a (°C)	$MgCl_2$	n	A	Expected product size (bp)†	Observed allele size (bp)	H_o	H_e	p_{HW}	EMBL no.
Led-11	$(GT)_{16}$	(F) TCTCCATTATAGGAAATCCCATTC (R) CACTAATATTCAAGCATCATGAGAAAG	FAM	59.6 59.6	56	1.0	29	7	140	122-144	0.53	0.90	0.108	AJ717503
Led-12‡^{X}	$(CA)_{15}$	(F) GGCACATTTAGGCATTTAGTTTC (R) CAAAAGGAGATGCTTCACCAG	FAM	51.9 51.6	56	2.0	18	3	245	243-247	0.40	0.61	0.028	AJ717504
Led-13	$(GT)_{18}$	(F) AATCTCTGAGCCAGTTTTCTTTC (R) CCCCTCCTAACAGGTTTGG	FAM	50.7 51.0	56	2.0	31	5	173	161-177	0.41	0.48	0.298	AJ717505
Led-15	$(CA)_{14}$	(F) GCCAGGATAGTTATACACATCTGC (R) GACATTGACACTCATCTTCTCTAGG	FAM	51.7 50.8	54	1.0	30	9	157	143-161	0.79	0.81	0.187	AJ717507
Led-16	$(GT)_{16}$	(F) CAGAGCACAGAGGATTTTTAGGGC (R) TGGGGTTTATGGCAACTTTTTTG	FAM	57.3 56.9	58	1.0	30	5	375	375-387	0.42	0.74	0.086	AJ717508
Led-17	$(CT)_{11}$ GT $(CA)_{14}$	(F) GCTGCCTTATATTTTTCATAGCG (R) CGAACCAGTTTCTACTTTCTCGTC	HEX	52.4 53.3	54	1.0	31	7	131	123-135	0.36	0.56	0.109	AJ717509
Led-18	$(GT)_{15}$ TT $(GT)_5$	(F) GCTCTGCACTTCTGTGTTAGG (R) GATCCCTGCAGCTTATTCC	FAM	57.8 57.3	56	1.0	30	17	119	98-138	0.88	0.92	0.410	AJ717510
Led-19	$(CA)_{15}$	(F) CCTGGCTTTATTGTCAGGAAC (R) GAATGAAAACAAAACTTTTTTACTG	FAM	50.7 49.0	49	1.0	30	8	271	261-277	0.68	0.71	0.666	AJ717511
Led-20	$(GA)_{20}$	(F) TGAAACAATAGTCATTCATAGGATG (R) TCCCATCTGTAGAGTTAAAAACC	HEX	50.0 49.1	49	1.0	30	11	219	213-249	0.71	0.80	1.000	AJ717512

T_m, melting temperature for each primer (predicted by PRIMER3; Rozen & Skaletsky 2000); T_a, annealing temperature used for PCR; $MgCl_2$, magnesium chloride concentration; n, number of unrelated (as known) *Lepilemur edwardsi* individuals genotyped from a population in the Ankarafantsika National Park, northwest Madagascar; A, number of alleles; †, based on sequenced allele of one of the two individuals used as the library source; H_e, expected heterozygosity; H_o, observed heterozygosity; p_{HW}, exact p for the Hardy-Weinberg equilibrium test; ‡, locus deviates from Hardy-Weinberg equilibrium (p-value < 0.05); X, *Led-12*, all 12 males were all homozygous but the 18 females were either heterozygous or homozygous suggesting this locus is X-linked. Observed and expected heterozygosity was therefore calculated using the 18 female genotypes only. *Led-12* was confirmed to be X-linked by observed sequence similarity to chimp and human X chromosome sequence (see text).

5.4 References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389-3402.

Armour JAL, Neumann R, Gobert S and Jeffreys AJ (1994) Isolation of human simple repeat loci by hybridization selection. *Human Molecular Genetics*, **3**, 599-605.

Craul M, Zimmermann E, Rasoloharijaona S, Randrianambinina B and Radespiel U (2007) Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species. *BMC Evolutionary Biology*, **6**, 17.

Excoffier L, Laval G and Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47-50.

Raymond M and Rousset F (1995) Genepop (Version 1.2) Population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248-249.

Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223-225.

Rozen S and Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds. Krawetz S and Misener S), pp. 365-386. Humana Press, Totowa, NJ. http://www.genome.wi.mit.edu/genome_software/other/primer3.html

Yoder AD and Yang ZH (2004) Divergence dates for Malagasy lemurs estimated from multiple gene loci: geological and evolutionary context. *Molecular Ecology*, **13**, 757-773.

5.5 Erratum

Craul M, Dawson DA, Radespiel U, Zimmermann E and Bruford MW (2009) Isolation of sixteen autosomal loci and a sex-linked polymorphic microsatellite locus from the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*). *Molecular Ecology Resources 9*, 333-335.

This article was published in *Molecular Ecology Resources* in 2009, since publication we have observed errors in Table 5-1.

In column 8, the number of individuals (*n*) genotyped for *Led-01*, *Led-11*, and *Led-16* to *Led-20* is not correct. In column 11, the observed allele size (bp) for *Led-08*, *Led-11*, *Led-16*, and *Led-20* is not correct. In column 12, the expected heterozygosity (H_e) for all loci is not correct. In column 13, the observed heterozygosity (H_o) for all loci is not correct. In column 14, the exact *p* for the Hardy-Weinberg equilibrium test (p_{HW}) for all loci is not correct.

These errors have been rectified in the Table 5-1 below.

The text did not contain errors and remains unchanged.

We sincerely apologise for these errors.

Table 5-1: Characteristics of 17 autosomal and sex-linked polymorphic Milne-Edwards's sportive lemur (*Lepilemur edwardsi*) microsatellite loci.

Locus	Repeat motif	Primer sequence (5' to 3')	Label (F)	T_m (°C)	T_a (°C)	$MgCl_2$	*n*	*A*	Expected product size (bp)†	Observed allele size (bp)	H_o	H_e	p_{HW}	EMBL no.
Led-01	$(GT)_{14}$	(F) TCTCATCAGAGCATGTGTTTTTC (R) TGCCTTCAGAACTTTTTGAATC	HEX	51.2 50.7	54	3.0	21	10	124	108-132	0.76	0.83	0.260	AJ717493
Led-03	$(GT)_{12}$	(F) AACAATAAAAATTCAGTGTGTCACC (R) CCTCATGTTTGTCTTTGACTCAC	HEX	51.3 50.6	56	1.0	31	9	324	320-338	0.90	0.81	0.108	AJ717501
Led-04	$(CA)_5$ AA $(CA)_3$ TA $(CA)_3$CG $(CA)_2$ CG $(CA)_4$	(F) CAGAAATGGAACTTCAACGG (R) AGGGATGGATGTAGATGCTAAG	FAM	49.7 49.5	55	2.0	31	6	207	199-213	0.55	0.67	0.068	AJ717497
Led-05	$(GTCT)_5$CC $(CT)_{12}$	(F) GATCAATCACATGAGCCTTCTG (R) GATCTCCGTGTGCCTGTTAG	HEX	59.7 58.3	60	2.0	32	4	85	82-88	0.44	0.52	0.123	AJ717495
Led-07	$(GT)_9$ CT $(GT)_6$ CT $(GT)_3$	(F) TTCTACAACTTTGGCCTTTGG (R) TTGAGCCCAGGATTTTGAAG	HEX	59.2 60.2	56	1.5	32	2	160	162-164	0.44	0.49	0.415	AJ717498
Led-08	$(GT)_{12}(GA)_{24}$	(F) GTAGCCTCCGGAACAGTGAC (R) GATCTCTCTCTCTCTCTCTCTCTCTC	HEX	59.7 59.8	61	1.0	31	10	132	118-136	0.87	0.83	0.335	AJ717500
Led-09	$(GT)_{19}$	(F) ATCTGCCTGCAGAAGGTC (R) GCTGTGGGAATAGGCACT	HEX	56.3 56.1	60	1.0	32	14	168	136-186	0.81	0.91	0.059	AJ717499
Led-10	$(CT)_7$ $(CA)_6$ GACATA $(CA)_3$ TAGATA $(CA)_{14}$	(F) TGTAATATTATCAAAAATAATCTCTCTCT (R) AGTTCCTAGAGGAATGCTCA	FAM	54.3 54.1	54	1.0	30	8	324	308-338	0.77	0.85	0.149	AJ717502

T_m, melting temperature for each primer (predicted by PRIMER3; Rozen & Skaletsky 2000); T_a, annealing temperature used for PCR; $MgCl_2$, magnesium chloride concentration; *n*, number of unrelated (as known) *Lepilemur edwardsi* individuals genotyped from a population in the Ankarafantsika National Park, northwest Madagascar; *A*, number of alleles; †, based on sequenced allele of one of the two individuals used as the library source; H_e, expected heterozygosity; H_o, observed heterozygosity; p_{HW}, exact *p* for the Hardy-Weinberg equilibrium test; ‡, locus deviates from Hardy-Weinberg equilibrium (*p*-value < 0.05); X, *Led-12*, all 12 males were all homozygous but the 18 females were either heterozygous or homozygous suggesting this locus is X-linked. Observed and expected heterozygosity was therefore calculated using the 18 female genotypes only. *Led-12* was confirmed to be X-linked by observed sequence similarity to chimp and human X chromosome sequence (see text).

Locus	Repeat motif	Primer sequence (5' to 3')	Label (F)	T_m (°C)	T_a (°C)	$MgCl_2$	n	A	Expected product size (bp)†	Observed allele size (bp)	H_o	H_e	p_{HW}	EMBL no.
Led-11	$(GT)_{16}$	(F) TCTCCATTATAGGAAATCCCATTC (R) CACTAATATTCAAGCATCATGAGAAAG	FAM	59.6 59.6	56	1.0	30	7	140	126-144	0.70	0.77	0.214	AJ717503
Led-12‡[X]	$(CA)_{15}$	(F) GGCACATTTAGGCATTTAGTTTC (R) CAAAAGGAGATGCTTCACCAG	FAM	51.9 51.6	56	2.0	18	3	245	243-247	0.40	0.61	0.039	AJ717504
Led-13	$(GT)_{18}$	(F) AATCTCTGAGCCAGTTTTCTTTC (R) CCCCTCCTAACAGGTTTGG	FAM	50.7 51.0	56	2.0	31	5	173	161-177	0.42	0.48	0.202	AJ717505
Led-15	$(CA)_{14}$	(F) GCCAGGATAGTTATACACATCTGC (R) GACATTGACACTCATCTTCTCTAGG	FAM	51.7 50.8	54	1.0	30	9	157	143-161	0.80	0.81	0.497	AJ717507
Led-16	$(GT)_{16}$	(F) CAGAGCACAGAGGATTTTTAGGGC (R) TGGGGTTTATGGCAACTTTTTTG	FAM	57.3 56.9	58	1.0	31	5	375	369-387	0.65	0.72	0.211	AJ717508
Led-17	$(CT)_{11}$ GT $(CA)_{14}$	(F) GCTGCCTTATATTTTTCATAGCG (R) CGAACCAGTTTCTACTTTCTCGTC	HEX	52.4 53.3	54	1.0	30	7	131	123-135	0.50	0.64	0.052	AJ717509
Led-18	$(GT)_{15}$ TT $(GT)_5$	(F) GCTCTGCACTTCTGTGTTAGG (R) GATCCCTGCAGCTTATTCC	FAM	57.8 57.3	56	1.0	31	17	119	98-138	0.87	0.93	0.189	AJ717510
Led-19	$(CA)_{15}$	(F) CCTGGCTTTATTGTCAGGAAC (R) GAATGAAAACAAAACTTTTTTACTG	FAM	50.7 49.0	49	1.0	32	8	271	261-277	0.69	0.72	0.419	AJ717511
Led-20	$(GA)_{20}$	(F) TGAAACAATAGTCATTCATAGGATG (R) TCCCATCTGTAGAGTTAAAAACC	HEX	50.0 49.1	49	1.0	31	11	219	199-249	0.71	0.82	0.081	AJ717512

T_m, melting temperature for each primer (predicted by PRIMER3; Rozen & Skaletsky 2000); T_a, annealing temperature used for PCR; $MgCl_2$, magnesium chloride concentration; n, number of unrelated (as known) *Lepilemur edwardsi* individuals genotyped from a population in the Ankarafantsika National Park, northwest Madagascar; A, number of alleles; †, based on sequenced allele of one of the two individuals used as the library source; H_e, expected heterozygosity; H_o, observed heterozygosity; p_{HW}, exact p for the Hardy-Weinberg equilibrium test; ‡, locus deviates from Hardy-Weinberg equilibrium (p-value < 0.05); X, *Led-12*, all 12 males were all homozygous but the 18 females were either heterozygous or homozygous suggesting this locus is X-linked. Observed and expected heterozygosity was therefore calculated using the 18 female genotypes only. *Led-12* was confirmed to be X-linked by observed sequence similarity to chimp and human X chromosome sequence (see text).

Chapter 6

Third study

Influence of forest fragmentation on an endangered large-bodied lemur in northwestern Madagascar

In press as: Craul M, Chikhi L, Olivieri G, Rabesandratana A, Zimmermann E and Radespiel U (in press) Influence of forest fragmentation on an endangered large-bodied lemur in northwestern Madagascar. *Biological Conservation*.

Data collection:	Craul, Olivieri, Rabesandratana
Lab work:	Craul
Analysis:	Craul, Chikhi
Manuscript:	Craul, Chikhi, Radespiel
Supervision:	Chikhi, Radespiel, Zimmermann

6 Third study

Influence of forest fragmentation on an endangered large-bodied lemur in northwestern Madagascar

6.1 Abstract

Madagascar's diverse and mostly endemic fauna and flora suffer from recent landscape changes that are primarily caused by high levels of human interventions. The loss and fragmentation of forest habitats are well known consequences of human activities. In this study, we investigate the effects of forest fragmentation on presence, abundance, and genetic diversity in a large-bodied lemur species, *Lepilemur edwardsi*, in northwestern Madagascar. In addition, we characterized the genetic differentiation among populations and demographic changes. We found *L. edwardsi* at only 13 (76.5 %) of 17 visited sites, 11 of which were situated in the Ankarafantsika National Park (ANP). We captured between two and 17 individuals per site. We sequenced the mtDNA D-loop of all samples and genotyped 14 microsatellite loci in two exemplary populations for demographic analyses. A negative influence on forest fragmentation could be detected, since the fragments had a lower genetic diversity than sites in the ANP. Genetic differentiation between populations ranged from low to high but was almost always significant. A typical pattern of isolation-by-distance could not be detected and the data could rather be interpreted as results of random genetic drift. The data furthermore revealed signals of a demographic collapse of about two orders of magnitude in the two exemplary sites. This decline probably started during the last few hundred years of intensified human disturbances and population growth. Given the results of this study, urgent conservation actions are needed and should concentrate on an effective protection of the few remaining populations in order to ensure the long-term survival of *L. edwardsi*.

6.2 Introduction

Habitat loss and fragmentation are among the most pervasive causes of biodiversity loss in the tropics (Laurance et al., 2000). Although they can and do also occur naturally (Watson, 2002), the most important and largest-scale cause of fragmentation is anthropogenic habitat modification leading to a severe loss of habitat surface area (Fahrig, 2003). Fragmentation affects animal populations on several levels and time scales. Direct invasive effects include increased hunting, logging, and burning (Turner, 1996). At the landscape scale, alterations in habitat characteristics may lead to unfavourable environmental conditions such as the increased spatial discontinuity of habitats, which may affect population viability and demography in the long term (Turner, 1996). Negative effects of fragmentation on population sizes have been demonstrated in many species. From a genetic viewpoint, Goossens et al. (2006) have shown that orang-utans from northeastern Borneo have undergone a dramatic population decline that was mainly due to recent habitat loss and fragmentation through human pressure.

Negative effects of habitat fragmentation have also been demonstrated in Malagasy wildlife including lemurs (e.g., Ganzhorn et al., 2003; Scott et al., 2006; Dunham et al., 2008). However, most studies have focused on the number of species surviving in fragments of varying sizes, and very few have investigated the effects of fragmentation on genetic diversity. In a recent study on mouse lemurs (*Microcebus* spp.), Olivieri et al. (2008) have shown that forest loss and fragmentation led to dramatic population declines, which probably took place within the last 500 years. However, population genetic studies concentrating on the effects of forest fragmentation on other lemur species and on large-bodied species in particular are still lacking to our knowledge.

The complex topography and the geographical location of Madagascar, southeast of the African Continent, generated a wide range of climates and environments on the island (Myers et al., 2000; Yoder & Nowak, 2006). The long isolation from the African Continent led to the evolution of an exceptional biodiversity that is characterized by extremely high levels of endemism in almost all taxonomic groups. These unique settings make the island one of the world's highest priority areas for biodiversity conservation (Myers et al., 2000; Goodman & Benstead, 2003; Kremen et al., 2008). However, during the last millennia this extremely diverse and mostly endemic fauna and flora has suffered from major environmental changes,

leading to the extinction of many species (Dewar, 2003). Madagascar has already lost 90 % of its primary vegetation, and forest landscapes are now reduced to a mosaic of fragments of varying size. It is believed that most of these changes occurred since the arrival of the first humans about 2 000 years ago. During the first 1 100 years, the impact on the environment was probably very limited and in particular northwestern Madagascar was hardly occupied (e.g. Wright & Rakotoarisoa, 2003). Critical deforestation has most likely been going on in the last 900 years, with pollen and charcoal profiles indicating substantial local deforestation around archaeological sites. The process of deforestation might have accelerated from the 19th century onwards when human population growth became exponential. Thus, fairly large areas of Madagascar were probably covered by intact forest well into the 20th century (Dewar, 2003), even though only 2.8 % of the original western dry forest has survived into the 1990's (Smith, 1997).

Sportive lemurs belong to the large-bodied, nocturnal lemurs of the family Lepilemuridae (Mittermeier et al., 2006). They are predominantly arboreal, moving among the trees with long jumps powered by their strong hind legs. In the 1990's it was believed that there were seven species across the island, but recent studies have shown that there are at least 25 species (Andriaholinirina et al., 2006; Craul et al., 2007; Louis et al., 2006; Rabarivola et al., 2007; Lei et al., 2008). The distribution of each species is small, since most species are confined to the area between two adjacent large rivers. However, the biology of sportive lemurs is only poorly known in general (Mittermeier et al., 2006). One exception is the Milne-Edwards' sportive lemur.

The Milne-Edwards' sportive lemur (*Lepilemur edwardsi*) lives in dispersed pairs that defend territories of about 1ha (Rasoloharijaona et al., 2003, 2006). Pairs form sleeping groups and share holes or leaf nests in sleeping trees as shelters during the day (Rasoloharijaona et al., 2003). This species shows seasonal reproduction and may have one offspring per year (Randrianambinina et al., 2007). Offspring do not achieve sexual maturity before their second year after birth (Petter-Rousseaux, 1964;). *L. edwardsi* does not show a flight response towards evolutionary new predators such as humans, even at sites where poaching occurs (Rabesandratana & Zimmermann, 2005). The absence of a flight response towards humans, the slow reproductive rate, and the small distribution range indicate that this species may be strongly threatened by habitat fragmentation and poaching.

In the present study we explore the consequences of habitat fragmentation on a large-bodied lemur taxon, *Lepilemur edwardsi*, in northwestern Madagascar. *L. edwardsi* occurs between the Betsiboka and the Mahajamba River (Craul et al., 2007). The habitats of *L. edwardsi* are highly fragmented, with only one relatively large remaining stretch of forest, namely the Ankarafantsika National Park (ANP). In this study, we characterize the genetic diversity and the genetic differentiation in forest fragments of different size, and aim to detect signals of demographic changes in *L. edwardsi*. In particular, we are interested in answering the following questions: i) does the abundance of *L. edwardsi* differ between smaller and larger forest patches, ii) do populations in small fragments show a loss of genetic diversity compared to the populations in the large/continuous forest, the ANP, iii) are nearby populations genetically more similar than distant ones (isolation-by-distance), iv) does an isolated population exhibit a stronger bottleneck signal than a population from the ANP?

6.3 Materials and methods

6.3.1 Study sites and field methods

We visited 17 sites in northwestern Madagascar (Fig. 6-1). The sites were located between the Betsiboka and the Mahajamba River, covering the range of *L. edwardsi*. Across this distribution, 6 sites were located in isolated forest fragments surrounded by savannah, and varied in size from 1.1 to 36.4 km^2, and 11 sites were located in the ANP, which covers about 955 km^2 (Table 6-1). The surface areas were calculated using satellite pictures from the year 2 000. Straight-line distances among sites ranged from 2.56 to 97.7 km and were calculated with ArcView GIS 3.3. At each site we performed 2 to 14 daily and nightly surveys, looking for signs of *Lepilemur*. In addition, we performed 2 to 41 nightly census-walks along 1 000 m trails per site in order to quantify encounter rates (individuals per km) and document vocalisations heard from the distance. At daytime we used a net to capture the animals out of their sleeping holes and briefly anesthetised them with Ketasel-5 (Selectavet). At nighttime we anesthetised the animals using a blowpipe (TELINJECT B22T) with 0,5 ml of 5 % Ketasel-5 (Selectavet). From each captured individual a small biopsy from one or both pinnae was taken as tissue samples. Tissue samples were stored in Queen's lysis buffer (Seutin et al., 1991) for later DNA extraction and genetic analyses.

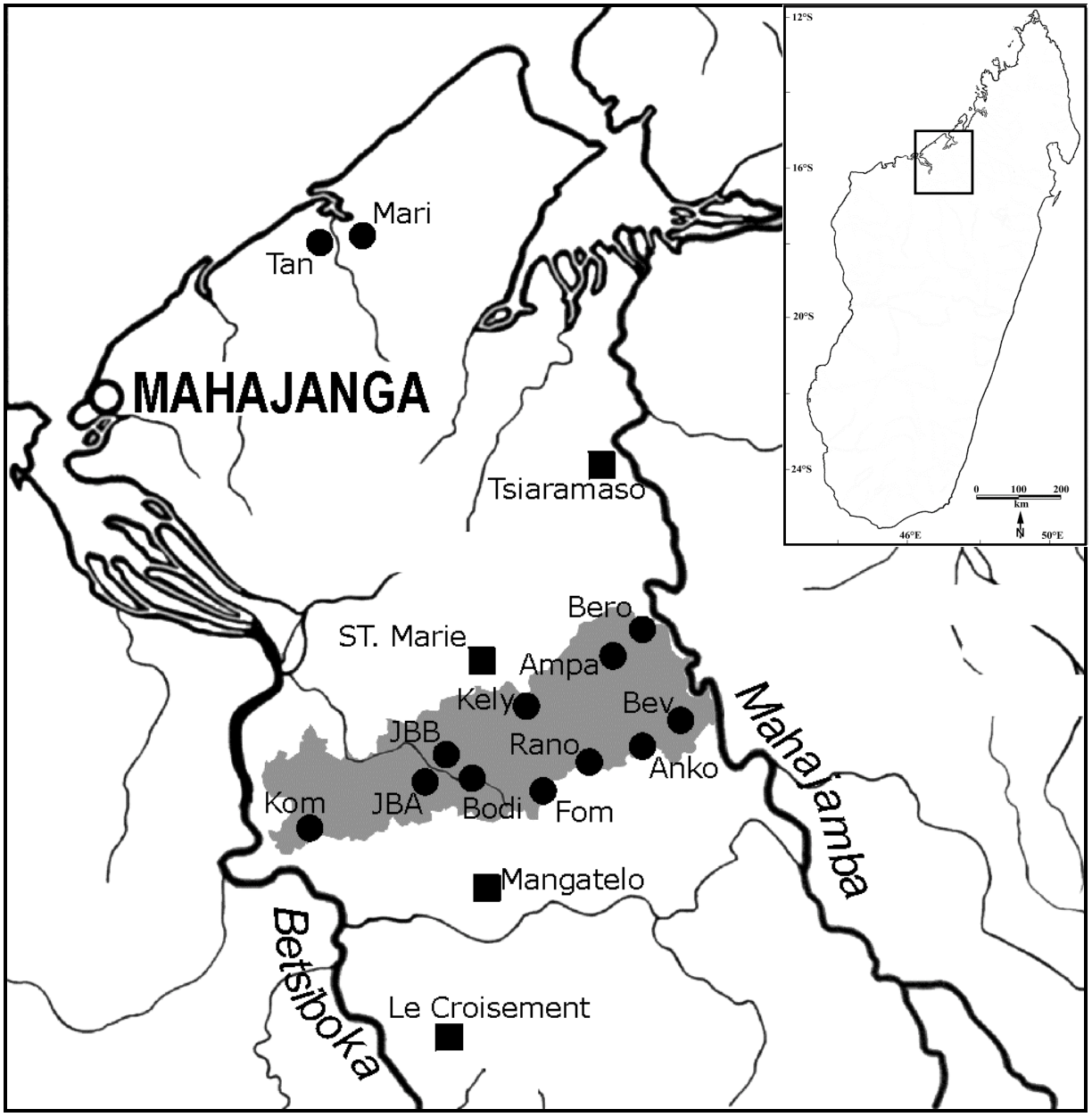

Figure 6-1: Map of the distribution of *L. edwardsi*. Black circles indicate the sites where we found sportive lemurs, and black squares indicate the sites where we did not find sportive lemurs. Ankarafantsika NP in grey.

Table 6-1: Size and location of study sites, evidence for the presence of *L. edwardsi*, number of samples per site, and *L. edwardsi* encounter rates based on the census results.

				Evidence for *Lepilemur* presence from				
Study sites	Coordinates	Origin	Fragment size [km^2]	Census sightings (no. of census walks)	Daily/nightly surveys (no. of d/n surveys)	Vocalisation	*n*	Encounter rate [*n*/km] ± *SD*
Ambanjakely	S 16°11' O 046°54'	ANP	955.0	+ (9)	+ (>3/10)	+	14	0.56 ± 0.81
Ambodimanga	S 16°18' O 046°49'	ANP	955.0	+ (3)	+ (>3/3)	+	8	3.00 ± 0.00
Ampatika	S 16°02' O 047°08'	ANP	955.0	+ (6)	+ (>3/6)	+	9	3.00 ± 2.45
Andofombombe	S 16°19' O 046°55'	ANP	955.0	+ (9)	+ (>3/7)	+	17	2.77 ± 2.47
Andoharano	S 16°17' O 047°00'	ANP	955.0	+ (9)	+ (>3/9)	+	13	0.08 ± 0.28
Ankoririka	S 16°16' O 047°02'	ANP	955.0	+ (11)	+ (>3/13)	+	10	0.77 ± 1.03
Beronono	S 15°59' O 047°09'	ANP	955.0	- (9)	+ (>3/11)	+	6	0.44 ± 0.53
Bevazaha	S 16°13' O 047°08'	ANP	955.0	+ (6)	+ (>3/6)	+	8	0.93 ± 1.43
JBB	S 16°17' O 046°48'	ANP	955.0	+ (12)	+ (>20/>20)	+	11	1.29 ± 0.71
JBA	S 16°18' O 046°48'	ANP	955.0	+ (41)	+ (>20/>20)	+	10	5.50 ± 1.87
Lac Komandria	S 16°21' O 046°39'	ANP	955.0	- (2)	+ (>3/2)	-	2	0
Mariarano	S 15°28' O 046°41'	IFF (north)	32.6	+ (6)	+ (12/14)	+	10	3.50 ± 3.94
Tanambao	S 15°28' O 046°39'	IFF (north)	36.4	+ (7)	+ (10/14)	+	3	5.71 ± 2.56
St. Marie	S 16°12' O 046°53'	IFF	5.4	+ (10)	not done	+	0	0.85 ± 0.07
Le Croisement	S 16°51' O 047°01'	IFF	1.1	- (6)	+ (6/10)	-	0	0
Mangatelo	S 16°24' O 046°58'	IFF	17.7	- (6)	+ (6/6)	-	0	0
Tsiaramaso	S 15°47' O 047°07'	IFF	4.0	- (9)	+ (3/4)	-	0	0

ANP: Ankarafantsika National Park; IFF: isolated forest fragment; +: present; -: absent; *n*: number of individuals; *SD*: standard deviation.

6.3.2 Lab methods

DNA from the tissue was isolated with the DNeasy Tissue Kit (Qiagen), or extracted using a standard proteinase K digestion followed by a Phenol/Chloroform protocol (Sambrook et al., 1989) and stored at -20°C.

We sequenced the mitochondrial D-loop (496 bp) of all captured individuals with the laboratory methods described in Craul et al. (2007). The purified PCR products were sequenced, either using a MegaBACE^{TM} 1000 DNA Sequencing System (Amersham Biosciences) in our lab or an ABI 3730XL automatic DNA sequencer run by Macrogen (http://www.macrogen.com/english/index.html). The respective sequences were deposited in GenBank. In addition, we genotyped all individuals of two populations, Mariarano and Ankoririka, using 14 microsatellite loci. The two populations were chosen as exemplary representatives of two types of forests, one rather disturbed/fragmented and one rather undisturbed/continuous. For genotyping we used the methods described by Craul et al. (2008).

6.3.3 Sequence analyses

Nucleotide diversity (π) and haplotype diversity (*Hd*) was calculated for each sample as measures of genetic diversity with the program DnaSP 4.10.4 (Rozas et al., 2003).

Population structure was analysed using different approaches. Pairwise F_{ST} and Φ_{ST} values were calculated according to Excoffier et al. (1992) and their significance was estimated with 1 000 permutations as implemented in ARLEQUIN 3.01 (Excoffier et al., 2005). We used sequential Bonferroni corrections for all multiple pairwise comparisons. To determine the most appropriate distance measure for the calculation of Φ_{ST} values, we used MODELTEST 3.7mac (Posada & Crandall, 1998). Using the Akaike Information Criterion (AIC), a transversion model (TVM+I) with a gamma correction factor of 0.9526 was suggested as the best model.

In order to test for a correlation between genetic and geographic distances we performed Mantel tests between pairwise F_{ST} and Φ_{ST} values (Rousset, 1997) and the corresponding straight-line geographic distances, and between $F_{ST}/(1-F_{ST})$ and $\Phi_{ST}/(1-\Phi_{ST})$ values and the corresponding straight-line geographic distances. Significance of the correlation was assessed by means of 1 000 permutations as implemented in GENETIX 4.04 (Belkhir et al., 2000).

In order to determine the best possible partition of the samples, we used the method of Dupanloup et al. (2002), implemented in SAMOVA 1.0 (Spatial Analysis of Molecular VAriance). In this approach a simulated annealing algorithm is used to find, for a given number of partitions *k*, a partition or group of partitions that maximizes molecular variance between such groups and minimizes variance within the groups. We let *k* vary between 2 and 12 (i.e. the total number of samples minus 1) and each analysis was repeated 100 times. Finally, a haplotype network based on all sequences was constructed with the program NETWORK 4.5.0.0 (Bandelt et al., 1999) to determine visually if there was geographic structure in the distribution of haplotypes.

Two summary statistics that are known to be affected by demographic events such as bottlenecks and expansions, namely Tajima's *D* (Tajima, 1989) and Fu's *Fs* (Fu, 1997), were computed in order to test for departure from equilibrium and neutrality, potentially indicating historic changes in population size. The significance of these statistics was assessed using 10 000 coalescent simulations based on the observed number of segregating sites in each sample as implemented in ARLEQUIN 3.01.

6.3.4 Microsatellite analyses

All microsatellite analyses were conducted on two populations, one from the ANP (Ankoririka, $n = 10$) and one from a forest fragment in the north (Mariarano, $n = 10$). The genetic diversity was characterized by the number of alleles per locus (n_A), the mean number of alleles per population (*MNA*), the observed heterozygosity (H_o), and the unbiased expected heterozygosity (H_e) (Nei, 1978). Wright's F_{IS} was estimated according to Weir and Cockerham (1984) and its departure from the null hypothesis was tested using 1 000 permutations. All analyses mentioned above were performed in GENETIX 4.01.

In order to determine if there have been any changes in effective population size, three different but complementary approaches were used, as by Olivieri et al. (2008). The first was the method of Cornuet and Luikart (1996) that detects departures from equilibrium and neutrality using summary statistics, namely the number of alleles (n_A) and the expected heterozygosity (H_e). Three mutation models were used: the infinite allele model (IAM), the single stepwise mutation model (SSM), and the two phase mutation model (TPM) with 30 % multi-step mutation events. A Wilcoxon sign-rank test was used as implemented in the

BOTTLENECK 1.2.02 software (Cornuet & Luikart, 1996) to test for significant departure from equilibrium.

The second (Beaumont, 1999) and third (Storz & Beaumont, 2002) method is likelihood-based Bayesian methods that use the information from the full allelic distribution in a coalescent-based framework. The Beaumont (1999) method, implemented in the program MSVAR 0.4.2, assumes that a stable population of size N_1 started to decrease or increase t_a generations ago to the current population size N_0. The change in population size is assumed to be either linear or exponential, and mutations are assumed to occur under a single stepwise mutation model (SSM), with a rate $\Theta = 2N_0\mu$, where μ is the mutation rate per generation. Based on these assumptions, it is possible to estimate the posterior probability distribution of $r = N_0/N_1$ (rate of population size change), $t_f = t_a/N_0$ (time since population started changing in size scaled by N_0), and $\Theta = 2N_0\mu$. A MCMC is used to generate samples from the posterior distribution of these parameters. Although this method allows the quantification of a population increase or decrease, N_0 and N_1 cannot be estimated independently. Similarly, it can only approximate t_a as a time scaled by N_0, which itself remains unknown.

The Storz and Beaumont (2002) method implemented in MSVAR 1.3 overcomes these problems as it quantifies the effective population sizes N_0 and N_1 and the time T (in generations) since the population size change started. However, this approach uses only the exponential model, and prior distributions for N_0, N_1, T and μ are assumed to be lognormal. The means and standard deviations of these prior lognormal distributions are themselves drawn from priors (or hyper priors) distributions. We note that this method represents an improvement on the Beaumont method only at a certain price. First, it only allows for an exponential model of population size change, whereas the Beaumont method allows for both a linear and an exponential model. Second, it requires that priors are given for N_0, N_1. T, and μ. The two approaches are thus complementary. For both the Beaumont (1999) and the Storz and Beaumont (2002) methods, at least nine independent runs were performed for each population, using different parameter configurations, starting values and random seeds. The values we used for the different priors and the length of each MCMC are available from the authors upon request. The first 10 % of the outputs (or burn-in) were discarded to avoid bias in parameters estimation due to starting conditions. Convergence of the different chains was visually checked and tested using the Gelman and Rubin (1992) statistic.

6.4 Results

6.4.1 Presence and abundance in the study sites

L. edwardsi was only found in 13 of the 17 prospected sites (76.5 %). They were present in all ANP sites (100 %) but only in 2 of the 6 forest fragments (33.3 %, Table 6-1). Therefore, the vast majority of fragments in the range of *L. edwardsi* did not harbour sportive lemurs anymore.

Abundance, quantified as sighted individuals per km (nightly census), varied greatly (0.00-5.71 individuals/km) but did not differ significantly between the continuous ANP ($n = 11$) and forest fragments ($n = 3$) (Mann-Whitney U test: $U = 27.50$, $p = 0.591$): in the National Park the abundance varied between 0.00 and 5.50 individuals per km and the abundance in the two forest fragments layed within the range of 0.00-5.71 (Table 6-1). We caught a total of 121 individuals, between 2 and 17 individuals at each site (Table 6-1).

6.4.2 Genetic diversity and demographic change within sites

L. edwardsi had 1 to 6 haplotypes per site (Table 6-2). The two fragments Mariarano and Tanambao showed no diversity, i.e., all D-loop sequences were identical, both within and between sites. In contrast, regarding the sites in the ANP, *Hd* and π varied between 0.378-1.000 and 0.0009-0.0059, respectively.

All microsatellite loci were polymorphic with 2 to 8 alleles each. However, *Led05* was monomorphic in Ankoririka, as were *Led07* and *Led16* in Mariarano (Table 6-3). The *MNA* value and the average H_e in Ankoririka was slightly higher than in Mariarano. The average H_o values did not differ significantly from H_e.

Table 6-2: Genetic diversity measures of the D-loop sequences for each site and inference of demographic events based on Tajima's *D*, and Fu's *Fs* test.

Origin	Size [km^2]	Population	*n*	*H*	*Hd* ± *SD*	π ± *SD*	*D*	*Fs*
ANP	955.0	kely	14	2	0.528 ± 0.064	0.0011 ± 0.0011	1.434	1.251
ANP	955.0	bodi	8	2	0.429 ± 0.169	0.0009 ± 0.0010	0.334	0.536
ANP	955.0	Ampa	9	3	0.556 ± 0.165	0.0031 ± 0.0023	0.078	1.152
ANP	955.0	fom	17	4	0.684 ± 0.096	0.0027 ± 0.0020	0.363	0.457
ANP	955.0	rano	13	6	0.821 ± 0.082	0.0053 ± 0.0034	-1.118	-0.491
ANP	955.0	Anko	10	5	0.822 ± 0.097	0.0059 ± 0.0038	0.025	0.200
ANP	955.0	Bero	6	3	0.600 ± 0.215	0.0020 ± 0.0018	-1.233	-0.189
ANP	955.0	Bev	8	3	0.714 ± 0.123	0.0029 ± 0.0023	0.966	0.875
ANP	955.0	JBB	11	4	0.764 ± 0.100	0.0024 ± 0.0019	0.587	-0.353
ANP	955.0	JBA	10	3	0.378 ± 0.181	0.0012 ± 0.0007	-1.562	-0.459
ANP	955.0	Kom	2	2	1.000 ± 0.500	0.0041 ± 0.0050	0.000	0.693
IFF (north)	32.6	Mari	10	1	0.0	0.0	-	-
IFF (north)	36.4	Tan	3	1	0.0	0.0	-	-
total		13	121	23	0.892 ± 0.017	0.0049 ± 0.0003		

ANP: Ankarafantsika National Park; IFF: isolated forest fragment; *n*: number of individuals; *H*: number of haplotypes; *Hd*: haplotype diversity; π: nucleotide diversity; *SD*: standard deviation; *D*: Tajima's *D*; *Fs*: Fu's *Fs*.

Table 6-3: Genetic diversity of the microsatellite loci for the sampling sites Ankoririka (Anko) and Mariarano (Mari).

		Led-01	*Led-03*	*Led-04*	*Led-05*	*Led-07*	*Led-08*	*Led-09*	*Led-12*	*Led-13*	*Led-15*	*Led-16*	*Led-17*	*Led-18*	*Led-19*	*MNA*	average
	n_A	6	5	2	1	2	3	8	2	4	6	2	3	7	5	4.00	
	H_e	0.850	0.816	0.337	0.000	0.100	0.529	0.874	0.479	0.700	0.758	0.100	0.611	0.805	0.763		0.552
Anko	H_o	0.875	0.700	0.400	0.000	0.100	0.111	0.900	0.300	0.800	0.700	0.100	1.000	0.900	0.800		0.549
	F_{IS}	-0.032	0.149	-0.200		0.000	0.800	-0.032	0.386	-0.152	0.080	0.000	-0.698	-0.125	-0.051		0.005
		ns	ns	ns		ns	*	ns	ns	ns	ns	ns	***	ns	ns		ns
	n_A	5	6	3	2	1	6	6	2	3	3	1	5	8	3	3.86	
	H_e	0.549	0.795	0.468	0.268	0.000	0.747	0.779	0.190	0.653	0.653	0.000	0.732	0.875	0.653		0.526
Mari	H_o	0.667	0.900	0.400	0.300	0.000	0.400	0.800	0.200	0.600	0.800	0.000	1.000	0.875	0.500		0.532
	F_{IS}	-0.231	-0.141	0.153	-0.125		0.478	-0.029	-0.059	0.085	-0.241		-0.396	0.000	0.244		-0.012
		ns	ns	ns	ns		**	ns	ns	ns	ns		*	ns	ns		ns

n_A: number of alleles; H_e: unbiased expected heterozygosity (Nei, 1978); H_o: observed heterozygosity; ns: not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$

Based on the D-loop data, no population showed significant values of Tajima's *D* and Fu's *Fs*, i.e. there was no clear deviation from the neutrality and/or stationarity hypotheses (Table 6-2). Based on the microsatellite data, we also found no clear evidence for past population bottlenecks in Mariarano or Ankoririka with the program BOTTLENECK 1.2.02 (results not shown). On the other hand, the two MCMC-based methods (Beaumont, 1999; Storz & Beaumont, 2002) revealed clear signals of a past bottleneck. The Gelman and Rubin convergence test indicated that the Beaumont (1999) and the Storz and Beaumont (2002) MCMC runs had reached equilibrium and could hence be used for inference. With the Beaumont (1999) method we found a clear signal of population collapse for both sites under both a linear and an exponential model for population size change (results not shown). The posterior modes of N_0/N_1 indicated a decrease of the effective population size of about two orders of magnitude, but the width of the distribution indicated some uncertainty of this value. Using the Storz and Beaumont (2002) method, we estimated the past (N_1) and present (N_0) effective population size and dated the population collapse (Fig. 6-2a, 6-2b). The posterior distributions of *log* (N_0) and *log* (N_1) showed hardly any overlap. The posterior modes of *log* (N_0) for Ankoririka and Mariarano were 1.97 and 1.94, respectively, which corresponds to 94 and 87 individuals. These values contrasted with the modes of *log* (N_1), which were 4.10 and 4.07, respectively, corresponding to more than 10 000 individuals. These figures should not be taken literally, but they suggest an about 100-fold reduction of the population size. As can be seen in Figure 6-2a and 6-2b, the priors used (dotted and dashed lines) differed strongly from each other and from the posteriors (solid lines). On the contrary, the posteriors did not differ much from each other regardless of the priors, a further indication that the data do contain significant and consistent information on a population collapse. In both populations the posterior distributions for the time since the population decrease started had a mode around two in *log* scale, which corresponds to 100 years ago. We note however, that the distribution is wide (Ankoririka: 25 % quartile: 27-78 years ago, 75 % quartile: 756-1 323 years ago; Mariarano: 25 % quartile: 19-66 years ago, 75 % quartile: 544-1 055 years ago).

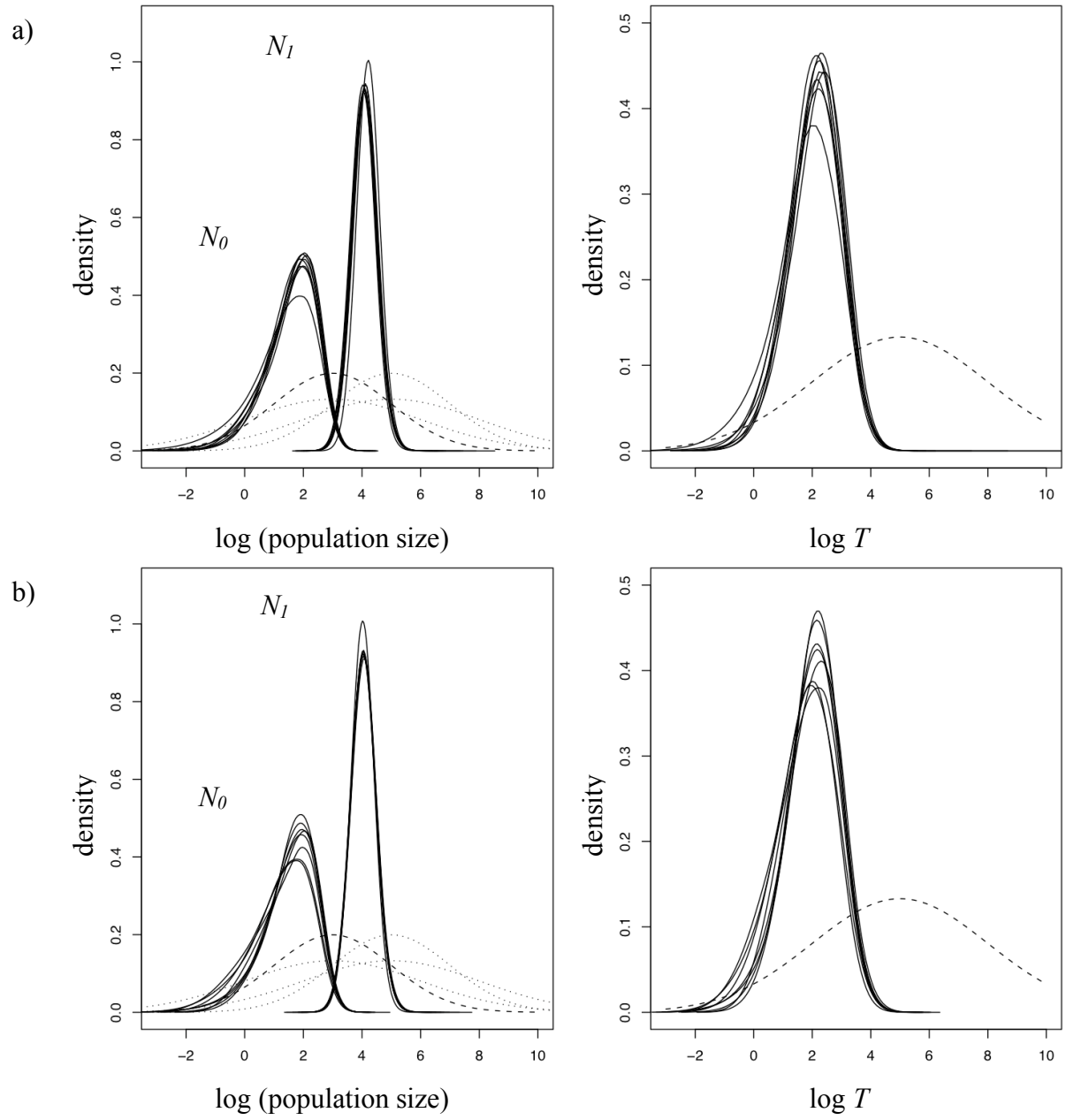

Figure 6-2: Posterior distribution of the present (*log* N_0) and past (*log* N_1) population size and the time in years since population collapse (*log* T) for a) Ankoririka and b) Mariarano. The dotted lines represent the prior distributions for N_1, and the dashed lines represent the prior distributions for N_0 or T.

6.4.3 Genetic differentiation among sites

The pairwise Φ_{ST} and F_{ST} values ranged between -0.08 and 0.96, and -0.09 and 1.00, respectively (Appendix 6-A). Most pairwise Φ_{ST} and F_{ST} comparisons were significant (60 of 78 and 61 of 78, respectively). After sequential Bonferroni correction, 53.85 % and 52.56 %

of these comparisons remained significant (Appendix 6-A). No signal of isolation-by-distance could be detected in the complete data set ($Z = 4077.17$, $r = 0.0517$, $p = 0.071$). When excluding the Mariarano and Tanambao sites, Mantel tests showed a surprising but significant negative correlation between Φ_{ST} and linear geographical distance ($Z = 939.10$; $r = -0.291$; $p = 0.014$), and between $\Phi_{ST}/(1-\Phi_{ST})$ and linear geographical distance ($Z = 1994.66$; $r = -0.234$; $p = 0.049$).

The SAMOVA analysis produced grouping patterns that always explained more than 47 % of the molecular variation (Appendix 6-B). The two fragments in the north, Mariarano and Tanambao were always together in one group. The remaining genetic grouping pattern was relatively stable across the different k values but it did not correspond to an obvious geographic pattern. For instance, for $k = 6$, 7 and 8, the five sites Ambanjakely, Ambodimanga, Andoharano, Beronono and JBA were always grouped together. This group cannot be explained by geographic proximity, since two sites are located in the west, two in the centre, and one in the east of the ANP. The highest amount of molecular variance among groups was detected at $k = 8$, with Mariarano and Tanambao as one group, Ambanjakely, Ambodimanga, Andoharano, Beronono, and JBA as another group, and the six remaining populations as single groups.

The 121 sequences grouped in 23 different haplotypes. Neighbouring haplotypes were quite similar to each other, usually separated by one to two mutations only (Fig. 6-3). Seven haplotypes were shared among sites. The two neighbouring fragments in the north, Mariarano and Tanambao, had both the same haplotype. Within the ANP, the most common haplotype was shared among five populations, Ambanjakely, Ambodimanga, Andoharano, Beronono, and JBA, explaining why they were grouped together by the SAMOVA analysis. Two further haplotypes were shared among four populations. Another two haplotypes were shared among three populations. Only one haplotype was shared between two populations. Haplotype sharing among ANP populations was not related to geographic proximity, as it occurred between neighbouring and distant sites equally likely. Altogether, this suggests that *L. edwardsi* populations are clearly differentiated from each other, but that no simple geographical pattern can explain this yet. The fact that these analyses are limited to one locus (mtDNA), which is clearly not highly variable, may explain that difficulty.

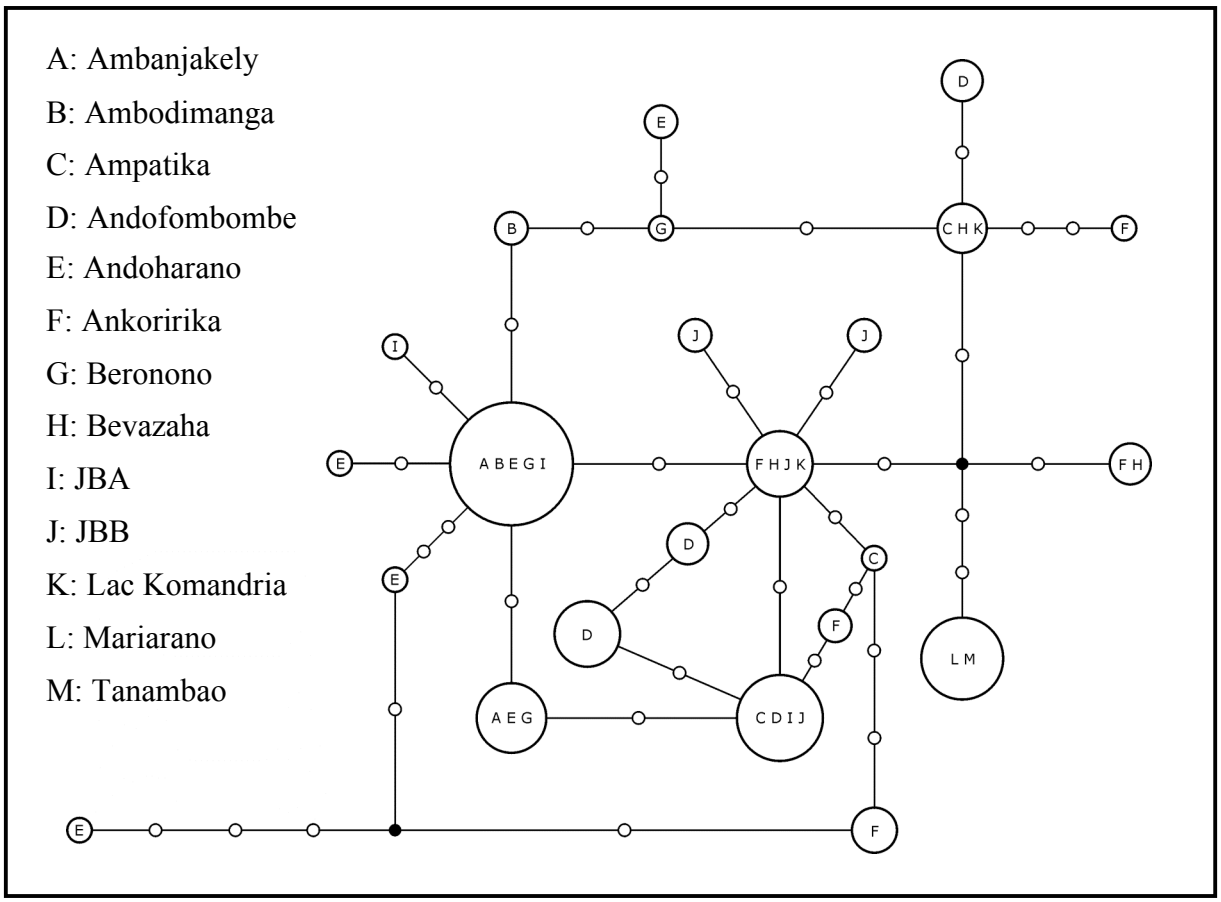

Figure 6-3: Haplotype network of the D-loop sequences of *L. edwardsi*. Each node corresponds to one mutation step. The sizes of the circles are proportional to the number of individuals sharing that haplotype. The letters correspond to the populations.

6.5 Discussion

6.5.1 Fragmentation, abundance, and genetic diversity

The first main result of this study is the alarming lack of *L. edwardsi* from many sites outside the ANP. *L. edwardsi* was only found in one third of the visited fragments. They could be reliably encountered only in the largest remaining forest in the region, the ANP. The smallest fragment with presence of *Lepilemur* was St. Marie (5.4 km^2). In all smaller fragments, we did not find any sign of *Lepilemur*. Even worse, sportive lemurs were also missing in one larger fragment, Mangatelo (17.7 km^2). When the ANP was excluded, the number of *L. edwardsi* fell from 121 to 13 individuals sampled in two out of six visited fragments. The fact that *Lepilemur* could be observed and sampled in all locations visited in the ANP

suggests that *Lepilemur* was once ubiquitous in northwestern Madagascar. In contrast, a similar sampling scheme performed by Olivieri et al. (2008) on *Microcebus* spp. in the same geographical areas revealed the continuous presence of mouse lemurs in all eight visited sites. It is not likely that these divergent results are based on different detection probabilities for both genera, since *Lepilemur* spp. is larger and more stationary than mouse lemurs and therefore easy to detect during transect walks if present. Whether the discrepancy between these two genera is merely the consequence of differential vulnerability to habitat fragmentation or a combined effect of fragmentation coupled with increased hunting pressure in isolated and mostly unprotected fragments (Olivieri et al., 2005), cannot be answered at present and requires further investigations.

In the ANP populations genetic diversity was overall large in contrast to the fragments, where there was no diversity at all. This is not surprising since a similar significant loss of genetic diversity in small fragments has already been described in golden-brown mouse lemurs (Guschanski et al., 2007). However, we must note that even in the ANP some samples exhibited low genetic diversity. This suggests that fragmentation and size reduction may have affected the smallest sites via the effects of genetic drift in isolated populations, but that this process may also affect some regions of the park.

The amount of genetic diversity in the two exemplary samples with microsatellite multilocus genotypes was also not very high with some loci exhibiting no diversity and more than half possessing less than four alleles. The H_e values, exhibited by the microsatellite data, were quite variable from locus to locus. While the average was rather low (H_e = 0.549 in the ANP and 0.532 in Mariarano) it is worth noting that the highest values were well within the range found in other species (e.g. Goossens et al., 2000 in chimps; Turner et al., 2004 in cyprinid fish). Overall, our data indicate a medium or low level of microsatellite diversity in both populations, even though the signal of diversity reduction is not as obvious as in the mtDNA. This is probably due to the lower effective population size of mtDNA, which make it more sensitive to bottlenecks. Also, it is known that during the first stages of a bottleneck, H_e is not as strongly affected as the number of alleles (Cornuet & Luikart, 1996). The reason for this is that it is mostly rare alleles that are lost during the first stages of a bottleneck, and they do not impact much on the computation of H_e.

Despite the lack of mtDNA diversity and the limited diversity observed in microsatellites,

the tests based on summary statistics (the Tajima and Fu tests for mtDNA and the BOTTLENECK tests for the microsatellites) did not reveal any clear signal of a past population bottleneck. This either suggests that a) the species is not variable enough because its (stationary) effective size is naturally low or b) these methods lack power to detect a bottleneck that could be either limited in importance, too ancient or too recent (Cornuet & Luikart, 1996). The fact that the Bayesian approaches detected a major population collapse in both populations suggests that the second hypothesis is more likely (i.e. a lack of power). Indeed, summary statistics do not use the genetic information very efficiently as was originally noted by Felsenstein (1992). The analyses suggested a population decrease of about two orders of magnitude and that this decrease took place most likely in the last few hundred years. It is remarkable that even the exemplary population in the ANP (Ankoririka) showed a collapse. This suggests that the original forest in that area was far bigger only a few centuries ago and that deforestation can generate signals of population decrease even in a population that lives in a relatively large forest fragment. Another possibility is that the forest only appears to be continuous, but is already influenced by fragmentation, either at a scale larger than the park, or within the park, with some savannah already representing a barrier to movement for *L. edwardsi*.

Our results are similar to those of Olivieri et al. (2008). Olivieri and colleagues found patterns of genetic differentiation caused by genetic drift and fragmentation in *Microcebus ravelobensis*. They also found signals of a strong recent bottleneck in all analysed populations, both within the ANP and in the isolated fragments. These similarities suggest that deforestation and forest fragmentation affect smaller- and large-bodied lemur species in similar ways even though life histories and the social organisation may differ considerably among those species. Indeed, as noted above the main difference lies in the fact that *L. edwardsi* has already completely disappeared from most isolated fragments. This definitely confirms that the whole process that has taken place over the last centuries poses an enormous threat to all species with small-scale distributions.

6.5.2 Fragmentation and genetic differentiation

The D-loop data suggested a high level of genetic differentiation among sites, but it did not reveal a clear geographic structure. Several populations were consistently associated with

each other, but genetic association did not reflect geographic proximity. This pattern may be explained as the result of random genetic drift (Goodman et al., 2001; Schafer et al., 2001), and by the fact that we only could apply it to a single locus (mtDNA) that had already lost most diversity. This random loss of alleles may also explain why we found a negative correlation between genetic and geographic distance within the ANP. Future data on other loci could possibly help to further illuminate the genetic structure of the ANP populations and to test this explanation. However, some conservation guidelines may already be made.

6.5.3 Implications for conservation

We have shown that *L. edwardsi* populations of the ANP still possess genetic diversity. Conservation management for this species should thus concentrate on this remaining diversity. This study furthermore suggests that *Lepilemur* spp. in general is threatened by three main factors. First, very small distribution ranges coupled with large-scale deforestation led to very small remaining habitable forest areas, restricting strongly total individual numbers. Second, *Lepilemur* spp. is highly vulnerable to hunting, which has probably contributed substantially to their disappearance from many unprotected fragments. Third, the genetic diversity of the remaining populations is threatened by habitat fragmentation across their distributions. Signals of past population bottlenecks can already be seen even in seemingly large forests. The combination of these three factors could soon lead to their disappearance outside protected zones. In the case of *L. edwardsi*, conservation actions should include the continuous legal protection of the Ankarafantsika National Park and the installation of a newly protected zone in the Mariarano/Tanambao region. In addition, careful translocations could be another option to conserve the remaining genetic intraspecific diversity (Jones et al., 1999; de Thoisy et al., 2001; Strum, 2005) and to recolonize newly protected habitats that were previously depleted by hunting.

6.6 Acknowledgements

We would like to thank CAFF/CORE, the ‘Direction Générale des Eaux et Forêts’ and ANGAP (in particular C. Andrianarivo) for giving us permission to conduct the fieldwork in Madagascar. For their support and long lasting collaboration, we would like to thank the late B. Rakotosamimanana, D. Rakotondravony, and O. Ramilijaona. We are very thankful to S. Mbotizafy, J.-A. Rakotonirina, B. Randrianambinina, S. Rasoloharijaona, and T.

Razafimamonjy for their help in collecting the samples. For their assistance in the lab, we would like to thank H. Breitrück and S. Sippel. Some of the calculations were performed with the help of P. Fernandes, using the High Performance Computing Resource at the Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal. We thank Vitor Sousa for numerous helpful discussions during the visit of M. Craul in Oeiras. We furthermore thank two anonymous reviewers for their valuable comments on the manuscript. The study was financially supported by the Volkswagen Foundation (VW I/76828 Zimmermann, VW I/81906 Radespiel), by the 'Gesellschaft der Freunde der Tierärztlichen Hochschule Hannover e. V.' (travel grant to M.C.), and by the DAAD (PROCOPE program D/0707573 to U.R.) and the Egide PHC (PROCOPE program N^{o} 17831XM to L.C.).

6.7 Appendix

Appendix 6-A: Genetic distances (Φ_{ST} below diagonal, F_{ST} above diagonal) among study populations of *L. edwardsi*.

	Site	*n*	kely	bodi	Ampa	fom	rano	Anko	Bero	Bev	JBA	JBB	Kom	Mari	Tan
ANP	kely	14	-	0.30 (*)	0.55 ***	0.66 ***	0.04 ns	0.55 ***	0.07 ns	0.64 ***	0.55 ***	0.13 ns	0.68 (*)	0.91 ***	0.87 (**)
ANP	bodi	8	0.29 (*)	-	0.65 ***	0.71 ***	0.02 ns	0.52 ***	0.05 ns	0.64 ***	0.63 ***	-0.02 ns	1.00 (*)	1.00 ***	1.00 (**)
ANP	Ampa	9	0.55 ***	0.58 ***	-	0.33 ***	0.35 ***	0.24 (**)	0.46 (**)	0.23 (*)	0.06 ns	0.53 ***	0.10 ns	0.75 ***	0.61 (**)
ANP	fom	17	0.65 ***	0.67 ***	0.33 **	-	0.50 ***	0.47 ***	0.60 ***	0.48 ***	0.40 ***	0.64 ***	0.42 (*)	0.77 ***	0.70 ***
ANP	rano	13	0.05 ns	0.00 ns	0.34 ***	0.49 ***	-	0.35 ***	-0.09 ns	0.35 ***	0.37 ***	0.00 ns	0.19 ns	0.64 ***	0.51 (**)
ANP	Anko	10	0.55 ***	0.49 ***	0.24 (**)	0.47 ***	0.33 ***	-	0.42 ***	0.25 (*)	0.30 ***	0.47 ***	0.06 ns	0.63 ***	0.47 (**)
ANP	Bero	6	0.06 ns	-0.05 ns	0.45 (***)	0.60 ***	-0.08 ns	0.41 ***	-	0.45 ***	0.01 *	0.49 ***	0.49 (*)	0.89 ***	0.79 (*)
ANP	Bev	8	0.64 ***	0.56 ***	0.22 (*)	0.48 ***	0.34 ***	0.25 (**)	0.45 (***)	-	0.53 ***	0.28 ns	-0.01 ns	0.68 ***	0.50 (**)
ANP	JBA	10	0.13 ns	0.06 ns	0.53 ***	0.64 ***	0.01 ns	0.47 ***	-0.03 ns	0.53 ***	-	0.55 ***	0.50 ns	0.78 ***	0.68 (**)
ANP	JBB	11	0.55 ***	0.58 ***	0.06 ns	0.40 ***	0.36 ***	0.29 ***	0.48 ***	0.28 ***	0.50 ***	-	0.50 (*)	0.90 ***	0.85 (**)
ANP	Kom	2	0.70 (**)	0.64 (*)	0.08 ns	0.43 (*)	0.21 ns	0.10 ns	0.42 (*)	-0.30 ns	0.60 ns	0.24 ns	-	1.00 (*)	1.00 ns
North	Mari	10	0.93 ***	0.96 ***	0.81 ***	0.82 ***	0.70 ***	0.70 ***	0.91 ***	0.79 ***	0.93 ***	0.84 ***	0.92 (*)	-	0.00 ns
North	Tan	3	0.90 (**)	0.92 (**)	0.70 ***	0.76 **	0.59 (**)	0.56 (**)	0.83 (*)	0.66 (**)	0.88 (**)	0.75 (**)	0.77 ns	0.00 ns	-

ANP: Ankarafantsika National Park, ns: not significant, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, (): not significant after sequential Bonferroni correction.

Appendix 6-B: SAMOVA 1.0 results for d-loop. Parentheses indicate which sites were best grouped together for each *k*.

		Percentage of variation					
k	Groups	Among groups	Among populations	Within populations	Φ_{ST}	Φ_{SC}	Φ_{CT}
1	(Mari, Tan) (Ampa, Anko, Bero, Bev, bodi, fom, JBA, JBB, kely, Kom, rano)	54.72	19.82	25.46	0.74542*	0.43776*	0.54721*
2	(Mari, Tan) (Ampa, Anko, Bero, Bev, bodi, JBA, JBB, kely, Kom, rano) (fom)	47.05	19.88	33.07	0.66931*	0.37544*	0.47053*
3	(Mari, Tan) (Bero, Bev, bodi, JBA, kely, rano) (Ampa, fom, JBB, Kom) (Anko)	48.71	12.94	38.34	0.61658*	0.25239*	0.48174*
4	(Mari, Tan) (Bero, bodi, JBA, kely, rano) (Ampa, fom, JBB, Kom) (Anko) (Bev)	53.47	7.35	39.18	0.60819*	0.15801*	0.53467*
5	(Mari, Tan) (Bero, bodi, JBA, kely, rano) (Ampa, JBB, Kom) (Anko) (Bev) (fom)	58.80	1.10	40.10	0.59899*	0.02672*	0.58798*
6	(Mari, Tan) (Bero, JBA, kely, rano) (Ampa, JBB, Kom) (Anko) (Bev) (bodi) (fom)	57.32	1.13	41.55	0.58453*	0.02657*	0.57319*
7	(Mari, Tan) (Bero, bodi, JBA, kely, rano) (Ampa) (Anko) (Bev) (fom) (JBB) (Kom)	59.49	-0.09	40.60	0.59397*	-0.00223*	0.59488*
8	(Mari, Tan) (Bero, JBA, kely) (Ampa, Kom) (Anko) (Bev) (bodi) (fom) (JBB) (rano)	57.46	-0.98	43.52	0.56484*	-0.02297*	0.57462*
9	(Mari, Tan) (Bero, rano) (JBA, kely) (Ampa) (Anko) (Bev) (bodi) (fom) (JBB) (Kom)	56.56	0.05	43.39	0.56609*	0.00123*	0.56555*
10	(Mari, Tan) (Bero, kely) (Ampa) (Anko) (Bev) (bodi) (fom) (JBA) (JBB) (Kom) (rano)	56.79	-0.50	43.71	0.56286*	-0.01157*	0.56786*
11	(Mari, Tan) (Ampa) (Anko) (Bero) (Bev) (bodi) (fom) (JBA) (JBB) (kely) (Kom) (rano)	56.36	-0.84	44.48	0.55519*	-0.01920*	0.56357

*: $p < 0.05$

6.8 References

Bandelt HJ, Forster P and Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37-48.

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. *Genetics*, **153**, 2013-2029.

Belkhir K, Borsa P, Chikhi L, Raufaste N and Bonhomme F (2000) GENETIX 4.01, logiciel sous Windows^{TM} pour la génétique des populations. Laboratoire Génome, Populations, Interactions, Université de Montpellier II, Montpellier.

Cornuet JM and Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001-2014.

Craul M, Zimmermann E, Rasoloharijaona S, Randrianambinina B and Radespiel U (2007) Unexpected species diversity of malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species. *BMC Evolutionary Biology*, **7**, 83.

de Thoisy B, Vogel I, Reynes J-M, Pouliquen J-F, Carme B, Kazanji M and Vie J-C (2001) Health evaluation of translocated free-ranging primates in French Guiana. *American Journal of Primatology*, **54**, 1–16.

Dewar RE (2003) Relationship between human ecological pressure and the vertebrate extinctions. In: *The Natural History of Madagascar* (eds. Goodman SM and Benstead JP), pp. 119-122. The University of Chicago Press, Chicago.

Dunham AE, Erhart EM, Overdorff DJ and Wright PC (2008) Evaluating effects of deforestation, hunting and El Niño events on a threatened lemur. *Biological Conservation*, **141**, 287-297.

Dupanloup I, Schneider S and Excoffier L (2002). A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571-2581.

Excoffier L, Smouse P-E and Quattro J-M (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Excoffier L, Laval G and Schneider S (2005) Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47-50.

Fahrig L (2003) Effects of habitat fragmentation on biodiversity. *Annual Review of Ecology, Evolution and Systematics*, **34**, 487–515.

Felsenstein J (1992) Estimating effective population size from samples of se-quences: a bootstrap Monte Carlo integration method. *Genetical Research*, **60**, 209–220.

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147**, 915-925.

Ganzhorn JU, Goodman SM and Dehgan A (2003) Effects of forest fragmentation on small mammals and lemurs. In: *The Natural History of Madagascar* (eds. Goodman SM and Benstead JP), pp. 1228-1234. The University of Chicago Press, Chicago.

Gelman A and Rubin DB (1992) Inference from interactive simulation using multiple sequences. *Statistical Science*, **7**, 457-511.

Goodman SM and Benstead JP (2003) The Natural History of Madagascar. The University of Chicago Press, Chicago and London.

Goodman SJ, Tamate HB, Wilson R, Nagata J, Tatsuzawa S, Swanson GM, Pemberton JM and McCullough DR (2001) Bottlenecks, drift and differentiation: the population structure and demographic history of sika deer (*Cervus nippon*) in the Japanese archipelago. *Molecular Ecology*, **10**, 1357-1370.

Goossens B, Latour S, Vidal C, Jamart A, Ancrenaz M and Bruford MW (2000) Twenty new microsatellite loci for use with hair and faecal samples in the chimpanzee (*Pan troglodytes troglodytes*). *Folia Primatologica*, **71**, 177-180.

Goossens B, Chikhi L, Ancrenaz M, Lackman-Ancrenaz I, Andau P and Bruford MW (2006) Genetic signature of anthropogenic population collapse in orang-utans. *PLoS Biology*, **4**, 285-291.

Guschanski K, Olivieri G, Funk S and Radespiel U (2007) MtDNA reveals strong genetic differentiation among geographically isolated populations of the golden brown mouse lemur, *Microcebus ravelobensis*. *Conservation Genetics*, **8**, 809–821.

Jones C (1999) A Method to Determine When Active Translocation of Nonhuman Primates Is Justified. *Journal of Applied Animal Welfare Science*, **2**, 229–238.

Kremen C, Cameron A, Moilanen A, Phillips S, Thomas C, Beentje H, Dransfield J, Fisher B, Glaw F, Good T, Harper G, Hijmans R, Lees D, Louis Jr. EE, Nussbaum R, Raxworthy C, Razafimpahanana A, Schatz G, Vences M, Vieites D, Wright P and Zjhra M (2008) Aligning conservation priorities across taxa in madagascar with high-resolution planning tools. *Science*, **320**, 222–226.

Laurance SG, Vasconcelos HL and Lovejoy TE (2000) Rain forest fragmentation kills big trees. *Nature*, **404**, 836.

Lei R, Engberg S, Andriantompohavana R, McGuire S, Mittermeier R, Zaonarivelo J, Brenneman R and Louis Jr. EE (2008) Nocturnal Lemur Diversity at Masoala National Park. *Special Publications, Museum of Texas Tech University*, **53**, 1-48.

Louis Jr EE, Engberg SE, Lei R, Geng H, Sommer JA, Randriamampionona R, Randriamanana JC, Zaonarivelo JR, Andriantompohavana R, Randria G, Prosper, Ramaromilanto B, Rakotoarisoa G, Rooney A and Brenneman RA (2006) Molecular and Morphological Analyses of the Sportive Lemurs (Family Megaladapidae: Genus *Lepilemur*) Reveals 11 Previously Unrecognized Species. *Special Publications, Museum of Texas Tech University*, **49**, 1-47.

Mittermeier RA, Konstant WR, Hawkins F, Louis Jr. EE, Langrand O, Ratsimbazafy J, Rasoloarison R, Ganzhorn JU, Rajaobelina S, Tattersall I and Meyers DM (2006) Lemurs of Madagascar, 2nd edn. Conservation International Tropical Field Guide Series.

Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GAB and Kent J (2000). Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853-858.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583-590.

Olivieri G, Sousa V, Chikhi L and Radespiel U (2008). From genetic diversity and structure to conservation: Genetic signature of recent population declines in three mouse lemur species (*Microcebus* spp.). *Biological Conservation*, **141**, 1257-1271.

Posada D and Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.

Rabarivola C, Zaramody A, Fausser JL, Andriaholinirina N and Roos C (2006) Cytogenetic and molecular characteristics of a new species of sportive lemur from Northern Madagascar. *Lemur News*, **11**, 45-49.

Rabesandratana A and Zimmermann E (2005) Regional biogeograpgy and conservation status of the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*) in the Ankarafantsika National Park northwestern Madagascar. 5. Göttinger Freilandtage: Primate Diversity: Past, Present and Future.

Randrianambinina B, Mbotizafy S, Rasoloharijaona S, Ravoahangimalala R and Zimmermann E (2007) Seasonality in reproduction of *Lepilemur edwardsi*. *International Journal of Primatology*, **28**, 783–790.

Rasoloharijaona S, Rakotosamimanana B, Randrianambinina B and Zimmermann E (2003) Pair-specific usage of sleeping sites and their implications for social organization in a nocturnal Malagasy primate, the Milne Edwards' sportive lemur (*Lepilemur edwardsi*). *American Journal of Physical Anthropology*, **122**, 251-258.

Rasoloharijaona S, Randrianambinina B, Braune P and Zimmermann E (2006) Loud calling, spacing, and cohesiveness in a nocturnal primate, the Milne Edwards' sportive lemur (*Lepilemur edwardsi*). *American Journal of Physical Anthropology*, **129**, 591-600.

Rousset F (1997) Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, **145**, 1219-1228.

Rozas J, Sanchez-DelBarrio J-C, Messeguer X and Rozas R (2003) DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, **19**, 2496–2497.

Sambrook J, Fritsch E and Maniatis T (1989) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor press, Cold Spring Harbor.

Schafer MA, Hille A and Uhl GB (2001) Geographical patterns of genetic subdivision in the cellar spider *Pholcus phalangioides* (Araneae). *Heredity*, **86**, 94-102.

Scott DM, Brown D, Mahood S, Denton B, Silburn A and Rakotondraparany F (2006) The impact of forest clearence on lizard, small mammal and bird communities in the arid spiny forest, southern Madagascar. *Biological Conservation*, **127**, 72-87.

Seutin G, White BN and Boag PT (1991) Preservation of avian blood and tissue samples for DNA analyses. *Canadian Journal of Zoology*, **69**, 82–90.

Smith PS (1997) Deforestation, fragmentation, and reserve design in western Madagascar. In: *Tropical forest remnants: Ecology, managment, and conservation of fragmented communities* (eds. Laurance WF and Bierregaard RO), pp. 415-441 The University of Chicago Press, Chicago and London.

Storz JF and Beaumont MA (2002) Testing for genetic evidence of population expansion and contraction: An empirical analysis of microsatellite DNA variation using a hierarchical Bayesian model. *Evolution*, **56**, 154-166.

Strum S (2005) Measuring success in primate translocation: A baboon case study. *American Journal of Primatology*, **65**, 117–140.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585-595.

Turner IM (1996) Species loss in fragments of tropical rain forest: a review of the evidence. *Journal of Applied Ecology*, **33**, 200-209.

Turner TF, Dowling TE, Broughton RE and Gold JR (2004) Variable microsatellite markers amplify across divergent lineages of cyprinid fishes (subfamily Leusicinae). *Conservation Genetics*, **5**, 279-281.

Watson DM (2002) A conceptual framework for studying species composition in fragments, islands and other patchy ecosystems. *Journal of Biogeography*, **29**, 823–834.

Weir BS and Cockerham CC (1984) Estimating F-Statistics for the analysis of population structure. *Evolution*, **38**, 1358-1370.

Wright HT and Rakotoarisoa JA (2003) The rise of Malagasy societies: New developments in the archaeology of Madagascar. In: *The Natural History of Madagascar* (eds. Goodman SM and Benstead JP), pp. 112-119. The University of Chicago Press, Chicago.

Yoder AD and Nowak MD (2006) Has vicariance or dispersal been the predominant biogeographic force in Madagascar? Only time will tell. *Annual Review of Ecology Evolution and Systematics*, **37**, 405-431.

Yoder AD and Nowak MD (2006) Has vicariance or dispersal been the predominant biogeographic force in Madagascar? Only time will tell. *Annual Review of Ecology Evolution and Systematics*, **37**, 405-431.

Yoder AD and Nowak MD (2006) Has vicariance or dispersal been the predominant biogeographic force in Madagascar? Only time will tell. *Annual Review of Ecology Evolution and Systematics*, **37**, 405-431.

Chapter 7
General discussion

The three main topics in this thesis address a variety of questions concerning the biogeography, phylogeny, and conservation genetics of sportive lemurs in northwestern Madagascar. Nine species (*L. aeeclis*, *L. edwardsi*, *L. otto*, *L. grewcockorum*, *L. sahamalazensis*, *L. mittermeieri*, *L. dorsalis*, *L. ankaranensis*, and *L. septentrionalis*) were shown to inhabit western to northern Madagascar, which we divided into eight Inter-River-Systems (IRSs). In the following, I will briefly summarize the main findings for each of the three main areas of interest mentioned in the introduction and discuss them in view of the latest developments in the respective field.

7.1 *Lepilemur* biogeography

Biogeography is the study of the distribution of biodiversity over space and time. It aims to reveal where organisms live, at what abundance (Cox & Moore, 2005), and has often been divided into ecological biogeography and historical biogeography (Lomolino et al., 2005). Classical biogeography has been expanded by the development of molecular systematics, creating a new discipline known as phylogeography. This development allowed scientists to test theories about the origin and dispersal of populations, such as island endemics. The work presented in this thesis contributes to a better understanding of the historical biogeography in the genus *Lepilemur* in northwestern Madagascar. The two models (Martin, 1995; Wilmé et al., 2006) explain the deep splits between the three major clades, but could not explain the species diversity within each major clade. We, therefore, propose a new model, the 'large river model' to explain the biogeography of this large-bodied nocturnal lemur genus. Most sportive lemur distributions could be shown to be much smaller than previously assumed. For example, *L. edwardsi* was only found in IRS I and does not occur in all three IRSs that constitute northwestern Madagascar. Instead, in IRS II and III, I described two new species, *L. otto* and *L. manasamody* respectively that are each limited to a single IRS. Independently, an American-Malagasy research team has constructed a molecular phylogeny including mtDNA sequences of sportive lemurs in IRS III. As a result, Louis et al. (2006) also described a new sportive lemur species in IRS III, which they named *L. grewcockorum.* Later on, *L. manasamody* and *L. grewcockorum* turned out to be synonymous names for the same species, in which case *L. grewcockorum* would have priority, since it was printed prior to our publication. Our study showed furthermore that the individuals we sampled in IRS IV

belonged to a previously described species, *L. sahamalazensis.* We suggested that the individuals sampled north (IRS VI (mainland and the island Nosy Bé)) and south (IRS V) of the Sambirano River may belong to two subspecies of the previously described *L. dorsalis* known from that region. Finally, in the far north, IRS VII, we sampled individuals, which belong to the previously described *L. ankaranensis* known from that region. For all sportive lemur species mentioned above, it can be stated that wide rivers generally act as species barriers. There seems to be only one exception to this pattern. We could confirm a genetic divergence between the animals from IRS V and VI, but it seemed not to reach species level but only subspecies level. Very recently however, Rabarivola et al. (2006) proposed species status for individuals in IRS V, named *L. mittermeieri*, based on cytogenetics. They collected samples at a locality further north (76.8 km) of our study site, Mahilaka and the number of chromosomes differed between the sampled individuals and the neighbouring species, *L. sahamalazensis* and *L. dorsalis.* The situation in IRS V and VI is further complicated by the study of Louis et al. (2006) in which they describe a new species of sportive lemurs from Nosy Bé, which they named *L. tymerlachsoni*. Louis et al. (2006) refer to the samples in IRS V as *L. dorsalis.* Andriaholinirina et al. (2006) and Craul et al. (2007), however, found no evidence that the samples from Nosy Bé and in IRS VI are taxonomically different. They refer to the samples from Nosy Bé and IRS VI as *L. dorsalis.* The study of Zinner et al. (2007) indicates distinct species in IRS V and VI, respectively. The problems in defining the species and distribution boundaries in and between IRS V and IRS VI can only be solved by an even finer-scaled sampling scheme. In addition, since Gray (1871) has given the type locality of *L. dorsalis* simply as 'NW Madagascar', it is difficult or even impossible to know, without testing samples from the type specimen, which of the recently described new species might be synonyms of the type specimen.

These results and other recent studies furthermore show that the taxonomic clarifications within the genus *Lepilemur* are far from being completed. Most recent publications increased the number of known extant *Lepilemur* species. Rabarivola et al. (2006), Andriaholinirina et al. (2006), Louis et al. (2006), Craul et al. (2007), and Lei et al. (2008) have, in total, described 15 new sportive lemur species all over the island. Unfortunately, their geographic ranges and biogeographic history are still far from being fully understood. In particular, their sampling scheme makes it difficult to test the influence of large rivers as species barriers,

since not all IRSs have yet been sampled, and for those that have been sampled, often only one locality is known. The importance of large rivers as biogeographic barriers was previously emphasized for mouse lemurs (Olivieri et al., 2007) and also for Neotropical primates (Ayres & Clutton-Brock, 1992; Lehman, 2004).

However, large rivers do not always act as species barrier for *Lepilemur.* Craul et al. (2008) suggested, that altitudinal stratification might be another important factor limiting the migratory potential of sportive lemurs in the East. In the case of *L. seali*, the two large rivers Antainambalana and Rantabe could not be confirmed as a species barrier, as *L. seali* could be found north and south of those rivers, respectively. These findings differ not only from the study of Louis et al. (2006), but also from the predictions made by Wilmé et al. (2006). Wilmé et al. (2006) suggested the Antainambalana watershed as a zone of retreat and dispersion during times of paleoclimatic periods of fluctuating aridification. However, this scenario should subsequently have led to separate centres of endemism on either side of the river. At least for the genus *Lepilemur*, this model could not be confirmed by this study. The lack of isolating effects of the Antainambalana River is surprising, as this river is still about 30 m wide even 100 km inland (http://eartHgooglEcom) and has been shown to separate even larger congeneric taxa, such as *Varecia Varecia variegata* from *V. V. rubra* (Goodman & Ganzhorn, 2004).

Altitudinal stratification has previously been suggested as important factor limiting the migratory potential of lemur species. Goodman and Ganzhorn (2004) suggested that the potential for river crossings depends on the elevational range of a given species in comparison to the altitude of the headwaters of the river. The headwaters of the Antainambalana River do not exceed 1,500 m (source at 1,450 m, Goodman & Ganzhorn, 2004). The elevational range of *L. seali* is not yet known, but its congeners *L. mustelinus* and *L. microdon* have been observed at altitudes higher than 1,600 m (Goodman & Rasolonandrasana, 2001; Goodman & Ganzhorn, 2004). If *L. seali* would possess the same altitudinal tolerance, they could have migrated around the headwaters of the Antainambalana River. The same scenario may be true for the sportive lemurs in IRS V and VI, where they could have migrated around the headwaters of the Sambirano River.

7.2 *Lepilemur* phylogeny

Phylogenetics deals with the identification and understanding of the evolutionary relationships among taxa, both living (extant) and dead (extinct). Evolutionary theory states that similarity among taxa is usually attributable to descent from a common ancestor. Thus, the relationships established by phylogenetic systematics often describe the evolutionary history of species and hence their phylogenetic origin. Molecular data is of particular importance in order to reveal the origin and evolution of sportive lemurs. The phylogenetic results of the molecular analyses should enable us to understand the biogeographic pattern in northwestern Madagascar in a phylogenetic context.

The molecular phylogeny we reconstructed included one to four individuals of 14 sampled sites and reference sequences from all so far known species. All populations within each IRS clustered together, so that each IRS (including the populations Kirindy (West) and Mantadia (East)) formed separate terminal clades. The phylogram consists of four major clades, a western, a northwestern, a northern clade, and the clade of *L. mustelinus*. *L. mustelinus* branched off first, followed by the western clade that consisted of IRS 0 and the individuals found in Kirindy (West). The northern clade consisted of the individuals from IRS IV, V, VI and VII, and the northwestern clade of all specimen from IRS I, II, and III. The terminal clades, which represent the different species, where highly supported and stable across all phylogenetic methods. However, the relationship between them was not always the same in all phylogenetic reconstructions, and furthermore different from the molecular phylogenies published from other sportive lemur studies (Andriaholinirina et al., 2006; Louis et al., 2006; Lei et al., 2008; Zinner et al., 2007). Altogether, 16 trees have been published using different genes and algorithms (Andriaholinirina et al., 2006; Craul et al., 2007; Louis et al., 2006; Lei et al., 2008; Zinner et al., 2007). Incongruent results between phylogenies can be a result of i) violations of the orthology assumption, ii) stochastic errors related to the sequence length, and/or iii) systematic error leading to tree reconstruction artefacts generated by the presence of a non-phylogenetic signal in the data (Jeffroy et al., 2006). The first point could be a problem when comparing species of different genera. The second point may be relevant, but could be buffered by an analysis including more genes and a combination of nuclear and mitochondrial DNA. However, the third problem, systematic error, is not expected to disappear with additional data (Felsenstein, 1978). On the contrary, Jeffroy et al.

(2006) showed that systematic biases are more problematic in genomic trees and can result in highly supported but wrong phylogenies. However, we are far from using whole genomes in sportive lemurs. Sequencing more loci would certainly help obtaining a robust consensus especially if based on nuclear genes and the complete genome of the mitochondria.

In spite of the divergence obtained between trees, two clustering patterns appeared in all of our molecular phylogenetic reconstructions and had good support. Moreover, they made sense from a geographical point of view. First, the monophyly of sportive lemurs, having one common ancestor. Second, the three separate major geographic clusters, western, northwestern and northern. Combining these results to the observed distributions of the different species, we developed a possible evolutionary scenario for the given sportive lemur biodiversity. During the cooler and drier periods of glaciations, sportive lemurs may have been forced to use the forest corridors around rivers supplied by orographic precipitations for reaching a forested refugium in higher altitudes. Continuous gene flow could have been maintained between subpopulations until relatively recently. Following the postglacial expansion of the forests, the sportive lemurs would have been able to recolonize a variety of IRSs from their central highland refugium. The time-span since then, however, was not long enough to allow speciation to take place. Since then, sportive lemurs could not migrate between neighbouring retreat-dispersion watersheds and were therefore trapped in their respective IRS between dried or flowing rivers. The genetic differentiation through vicariance could continue. In other words, the sportive lemur ancestors already lived and speciated in the current distribution. Post-Pleistocene expansion may also have occurred but was restricted to the single IRS. MtDNA sequences have frequently been used to uncover such migration patterns that took place during the Pleistocene (e.g. Goldberg & Ruvolo, 1997; Collins & Dubach, 2000). Unfortunately, these genetic signatures can be covered by more recent demographic events such as population fragmentation and declines.

13 external body measurements were compared between the sampled sportive lemur species. Between two and five of these measurements were significantly different between geographically neighbouring species. Similarly, the field observation revealed that some species were easily identified while others were more difficult to differentiate. For example, *L. sahamalazensis* was easy to distinguish from its neighbours *L. dorsalis* and *L. grewcockorum.* However, *L. edwardsi*, *L. otto*, and *L. grewcockorum* all showed high

levels of intraspecific morphometric variations, which made it difficult to confidently classify them in the field. Such species that are at least superficially morphologically indistinguishable and have previously been classified as one species are called cryptic species.

Speciation must not always be accompanied by morphological changes. It can be based on changes in behaviour, communication, ecology, or reproductive traits that lead to continuous reproductive isolation (Coyne & Orr, 2004). Since humans rely mostly on the visual sense, most species descriptions that had been published prior to the sequencing age were based on morphological differences. However, in many other taxa, auditory characters, for example, are far more important characters for species separation. This is presumably also the case for the nocturnal sportive lemurs that mostly use vocal communication for individual and species recognition (Braune, 2006). Therefore, species might differ at this level among each other, but would not be easily recognized as such in traditional taxonomic studies. As a consequence, the number of species is easily underestimated, as it was traditionally the case for sportive lemurs. Presently, cryptic species are revealed in many taxa and habitats (Bickford et al., 2007).

The identification and description of cryptic species have important implications for conservation, because the existence of further cryptic species in already endangered nominal species creates two problems: i) a species that is already considered endangered or threatened might be composed of multiple cryptic species that are even rarer than previously thought; and ii) the different species might require different conservation strategies (Schoenrogge et al., 2002). The last years have indeed revealed that several already endangered species are in fact cryptic species complexes, making them a collection of even more critically endangered sportive lemur species with fewer numbers and smaller distributions. For example, mitochondrial DNA and karyotypic evidence indicates that one of the four described subspecies of the endangered northern sportive lemur, *Lepilemur septentrionalis*, is a distinct species with a very restricted range (Ravaoarimanana et al., 2004). As an example for other taxonomic groups, a recent molecular study has revealed at least 14 frog species within two nominal species (Stuart et al., 2006), a revelation that could have consequences for conservation. Whereas the cryptic frog species complex had broad geographical ranges, actual species in those complexes have much more limited distributions, making each species more prone to extinction.

Recently, molecular techniques (primarily DNA sequencing) have transformed our ability to describe and define biological diversity. Although these methods are not a universal remedy for species delimitation, molecular data are important and useful when combined with other types of data. Studies using non-morphological characters to discriminate otherwise indistinguishable species are being published at an increasing rate. Bioacoustic studies in nonhuman primates have shown that loud calls can be reliably used as non-invasive diagnostic tools for discriminating cryptic taxa, for their monitoring in the field as well as for the reconstruction of their phylogeny. For example, Mendez-Cardenas et al. (2008) showed that loud calls separated geographically isolated populations of sportive lemurs specifically. The phylogenetic analysis using parsimony yielded 11 out of 17 acoustic characters as phylogenetically informative. The topology of the acoustic tree coincided less with geographic distances than with genetic tree topology. In conclusion, preventing habitat loss is therefore a great challenge for the conservation of biodiversity, and prioritizing habitats for conservation often relies on the estimation of species richness and endemism.

This study does not only present evidence for an unexpected species diversity of sportive lemurs in northwestern and northern Madagascar, but emphasizes the need for conservation actions if those species with very small distributions shall not face extinction in the very near future.

7.3 *Lepilemur* conservation genetics

Based on biological and climatic studies, as well as historic records, we only begin to understand the abiotic and biotic characteristics of Madagascar's habitats, the lemurs' ecological adaptations to these unique habitats, the extent of forest fragmentation and hunting, and the differential vulnerability of extant lemur species to these pressures (Godfrey & Irwin, 2007). It is a complex set of interactions affecting an initially rich but vulnerable fauna. One of the most dramatic Holocene extinction events occurred on the island of Madagascar. At the time of human arrival, about 2 000 years ago, the primate communities of Madagascar were ecologically and taxonomically diverse. There were 8 families of Malagasy lemurs (Godfrey & Irwin, 2007). That humans were the driving force of extinction is undisputed, although the extent of the human impacts is continuously debated. Most of the subfossil fauna was present on the island when humans arrived (Burney et al., 2004). Many species were still present at

the end of the first millennium and some appear to have even survived into the second half of the second millennium (Burney et al., 2004). In recent time, however, deforestation and forest fragmentation are proceeding at an extremely rapid pace (Green & Sussman, 1990).

Fragmentation is considered to be one of the most important threats to biodiversity in tropical forest ecosystems (Smith & Hellmann, 2002). While early studies concentrated on the effect of habitat loss based on the hypotheses of island biogeography (Laurance & Bierregaard, 1997), recent approaches are broader and investigate all processes in fragmented landscapes that have negative effects on native populations (Fahrig, 2003). Plant and animal species that inhabit tropical forests have developed complex ecological interactions with one another over millions of years, and have thereby shaped these forests. There is a sudden interruption of these interactions when species go locally extinct or reduced to very low abundances (Dausmann et al., 2008).

A general concern for the conservation of an endangered species in its natural habitat is the maintenance of genetic variation within populations, particularly when the remaining populations become fragmented and reduced in size. The loss of genetic variation could lead to short-term reduction of fitness, such as survival, reproductive output, or growth rates, and to a reduced ability to adapt to long-term environmental changes (Allendorf & Leary, 1986; Primack, 1993; Lacy, 1997; Frankham & Ralls, 1998). Changes in population size and density may also modify behavioural mechanisms, such as sex-biased dispersal patterns, inbreeding avoidance or local mate or resource competition, with negative consequences for gene flow within and between populations (Greenwood, 1980; Johnson & Gaines, 1990; Bohonak, 1999). Changes in allele frequencies can occur between subsequent generations, being an indicator for demographic changes in some species (Luikart et al., 1999).

Primates are readily hunted in many parts of the tropics (Perez, 1987; Godfrey & Irwin, 2007; Dunham et al., 2008). Intense hunting can increase the risk of extinction. Theoretically, larger body size and diurnality should increase vulnerability to human predation because these traits cause animals to be easier targets. Also the life history may affect the vulnerability of populations to hunting pressure, because species with slow life histories may have low reproductive resilience and therefore less ability to recover from a high hunting pressure (Godfrey & Irwin, 2007).

It has been shown, for example, that hunting has a negative effect on the relatively large *Propithecus edwardsi* (Dunham et al., 2008). Their life history makes them sensitive to disruptions in adult survival rates. Hunting, which is likely to target larger animals, has a strong potential to limit the viability of populations. Lemur surveys by Lehman et al. (2005, 2006) suggest declines due to hunting. In seven of eight different sites they found no *P. edwardsi* individuals. Although the villagers explained that sifakas were common in the forests within the past one to ten years, it is possible that heavy hunting from blowguns, darts, and slingshots have already dramatically reduced individual numbers in these areas (Lehman & Ratsimbazafy, 2001).

We only found *Lepilemur* spp. in 13 of 17 prospected sites (76.5 %). They were present in all sites (100 %) of the Ankarafantsika NP but only in two of the six forest fragments (33.3 %). Therefore, the vast majority of fragments did not harbour sportive lemurs anymore. They could be reliably encountered only in the largest remaining forest, the Ankarafantsika National Park. The broad-scale disappearance of *Lepilemur* from isolated forest fragments is dramatic. In contrast, a similar sampling scheme performed by Olivieri et al. (2008) on *Microcebus* spp. in the same geographical area revealed the continuous presence of *Microcebus* in all 28 visited sites. The discrepancy between these two genera is most likely the consequence of differential vulnerability to habitat fragmentation coupled with increased hunting pressure in isolated and mostly unprotected fragments (Olivieri et al., 2005).

It is becoming increasingly important to monitor the consequences of anthropogenic changes on natural populations. Although many national and international organizations have established guidelines and strategies for monitoring biological diversity (Holthausen et al., 2005; Kurtz et al., 2001), little use has been made of the benefits of molecular markers. Meanwhile, new laboratory and statistical techniques enable us to perform a proper genetic monitoring of wild populations (Luikart et al., 2003; Manel et al., 2003; Beaumont & Rannala, 2004; Herbert et al., 2004). For example, in Australia, microsatellite DNA has provided a feasible and cost-effective strategy for monitoring long-term changes in brush-tailed rock-wallaby *Petrogale penicillata* abundance (Piggott et al., 2006). Another study by Rudnick et al. (2005) used naturally shed feathers of Eastern imperial eagles *Aquila heliaca* in a microsatellite analysis to identify individuals, monitor population turnover, and estimate annual survivorship over a four-year period. The results showed annual variations in turnover

rates and a reduction in cumulative survival. Such information cannot be obtained easily using traditional approaches.

In addition, monitoring population genetic parameters can provide insights into demographic and evolutionary processes in natural and captive populations that are difficult or impossible to obtain using traditional methods (e.g. Barrett et al., 2005). This type of monitoring can evaluate population characteristics (e.g. effective population size, N_e, or connectivity), even from archived material. This enables monitoring to assess historical conditions (Pertoldi et al., 2005; Poulsen et al., 2006). For example, N_e has been estimated for brown bears in Yellowstone National Park by analyzing samples from the 1910s, 1960s, and 1990s (Miller & Waits, 2003). N_e estimates were periods (1910-1960s and 1960s-1990s), providing no evidence of a recent population decline. Other studies have also used a combination of contemporary and historical samples to obtain multiple temporal estimates of N_e (Nielsen et al., 2006; Ardren & Kapuscinski, 2003).

Effective conservation often depends on the identification of management units and timely information regarding the effects of natural and anthropogenic factors on movement and gene flow (Palsboll et al., 2007). For example, genetic monitoring of leopard frog *Rana pipiens* populations revealed that the genetic structure was stable over 11 to 15 generations (Hoffman & Blouin, 2004). A study of cod *Gadus morhua* populations from the Baltic and North Sea using both historical and contemporary samples also found high temporal stability (Poulsen et al., 2006). However, other studies have found opposite results. Genetic monitoring of Scottish red deer *Cervus elaphus* demonstrated that genetic structure in females declined at a steady rate over a 24-year period (Nussey et al., 2005). Based on demographic data collected over the same time period, the authors concluded that this decline was due to a combination of increasing population size and decrease in polygyny.

Genetic monitoring is also a useful tool for evaluating the cumulative effects of habitat fragmentation (Schwartz et al., 2007). An allozyme and microsatellite-based study of California valley oak *Quercus lobata* pollen suggests that there was a decline in the effective number of fathers contributing pollen to the next generation between 1944 and 1999 (Sork et al., 2002). The authors propose that this was the result of stand thinning and that it might lead to genetic isolation.

In this study we detected a major population collapse not only in forest fragments, but

also in the Ankarafantsika NP. The analyses revealed a population decrease of probably 100-fold in size, which took place most likely in the last few hundred years. It is remarkable that even the population in the NP showed a collapse. This suggests that only a few centuries ago the original forest in that area was far bigger and that deforestation can generate signals of population decrease even in a population that lives in a relatively large forest fragment. Another possibility is that the forest only appears continuous to us, but is already influenced by fragmentation, with some savannah already representing a barrier to movements of *Lepilemur* specimen.

Our results are similar to those of Olivieri et al. (2008). Olivieri et al. (2008) found patterns of genetic differentiation caused by genetic drift and fragmentation in *Microcebus ravelobensis.* They also found signals of a strong recent bottleneck for all the populations analysed, both within the ANP and in isolated fragments. These similarities suggest that deforestation and forest fragmentation affect small- and large-bodied lemur species in similar ways even though life histories and the social organisation may differ. Genetic drift and fragmentation affected more strongly the small fragments, where a significant loss of diversity has already occurred. The alarming fact is that *Lepilemur* has already completely disappeared from most isolated fragments. This definitely confirms that the whole process that has taken place over the last centuries poses an enormous threat to all species with small-scale distributions and relatively large body size.

However, it still remains challenging for scientists of different fields such as biology, geography or geology to synergistically advance their respective fields of work. Effective conservation decisions based on currently available data have to be made rapidly, so it is indispensable to enlarge the tools that help quickly and efficiently to collect information. During the last decades, conservation genetics has proliferated and been integrated and adapted for conservation.

The D-loop data from this study suggested a high level of genetic differentiation among sites, but it did not reveal a clear geographical structure. Several populations were consistently associated with each other, but association did not reflect geographic proximity. This pattern may be explained as the result of random genetic drift, and by the fact that we only included one mtDNA locus that, moreover, may have already lost most genetic diversity. This random loss of alleles may also explain why we found a negative correlation between

genetic and geographic distance within the ANP. It was not possible within this study to genotype all populations using the 14 microsatellites, but a better understanding of the spatial patterns will require that. This will be done in the future. It is indeed urgent to acquire more information on the genetic diversity at the microsatellite level. These new data will probably not be available in the very near future, but some conservation recommendations may already be given.

7.4 Implications for conservation actions

Our work allowed defining the distributions of several *Lepilemur* species, which is a basic requirement to determine their conservation status. Moreover, within one sportive lemur species (*L. edwardsi*), we were able to quantify the remaining genetic diversity using two kinds of markers, D-loop sequences and 14 microsatellites, within and between several populations. We applied a wide range of analytical methods, including some established and some new ones, and were able to detect demographic changes that took place in the recent history of the populations.

This study suggests that *Lepilemur* spp. is threatened by three main factors. First, the very small distribution ranges coupled with large-scale deforestation. Second, *Lepilemur* spp. is highly vulnerable to hunting. Third, the genetic diversity of the remaining populations is threatened by habitat fragmentation. The combination of these three factors could soon lead to their disappearance outside protected zones. Given the fact that the distributions of some species do not currently include any effectively protected zone, urgent conservation actions are now needed in order to prohibit their extinction in the near future. We suggest, therefore, a focus on conservation strategies that include reduction of hunting pressures and protection of intact habitats, which are important to adult survival and fecundity. In the case of *L. edwardsi*, this could be achieved by the continuous legal protection of the Ankarafantsika National Park and by the protection of the Mariarano/Tanambao region. In addition, programs to promote awareness and education in the villages and schools surrounding the forest fragments should be established. While protection of the fauna and flora is of lower priority to most local people, only a respectful and sustainable use of the nature can assure a worthy future for upcoming generations. Therefore, education programs help to promote appreciation of the values and functions of the forest ecosystem, especially their important ecological roles, and

to promote awareness in order to achieve conservation of the forest by the local inhabitants. The largest benefit for the local community from conservation and education programs will be empowerment to control and manage their natural resources in a sustainable manner. In such programs the government could also transfer natural resource management rights to the local communities. This allows the communities themselves to manage their forests and makes them responsible for enforcing forest-use rules. An active dialogue would need to be maintained with local stakeholders to help ensure that protected area limits are proposed and accepted by local and regional authorities. Conservation and education programs are absolutely essential to improve living standards and quality of life of the local inhabitants in positive coexistence with the fragile and unique local environment.

7.5 References

Allendorf F, and Leary R (1986) Heterozygosity and fitness in natural populations of animals. In: *Conservation Biology: the Science of Scarcity and Diversity* (ed. Soule M), pp. 57–76. Sinauer Associates, Sunderland, MA.

Andriaholinirina N, Fausser J-L, Roos C, Zinner D, Thalmann U, Rabarivola C, Ravoarimanana I, Ganzhorn JU, Meier B, Hilgartner R, Walter L, Zaramody A, Langer C, Hahn T, Zimmermann E, Radespiel U, Craul M, Tomiuk J, Tattersall I, and Rumpler Y (2006) Molecular phylogeny and taxonomic revision of the sportive lemurs (*Lepilemur*, Primates). *BMC Evolutionary Biology*, **6**, 17.

Ardren WR and Kapuscinski AR (2003) Demographic and genetic estimates of effective population size (*Ne*) reveals genetic compensation in steelhead trout. *Molecular Ecology*, **12**, 35–49.

Ayres JM and Clutton-Brock TH (1992) River boundaries and species range size in Amazonian primates. *American Naturalist*, **140**, 531–537.

Barrett LG, He T, Lamont BB, and Krauss SL (2005) Temporal patterns of genetic variation across a 9-year-old aerial seed bank of the shrub *Banksia hookeriana* (Proteaceae). *Molecular Ecology*, **14**, 4169–4179.

Beaumont MA and Rannala B (2004) The Bayesian revolution in genetics. *Nature Reviews, Genetics*, **5**, 251–261.

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, Ingram KK, and Das I (2007) Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, **3**, 148–155.

Bohonak AJ (1999) Dispersal, gene flow, and population structure. *Quaterly Review of Biology*, **74**, 21–45.

Braune P (2006) *Acoustic variability and its biological significance in nocturnal lemurs.* PhD thesis, Gottfried Wilhelm Leibniz Universität Hannover, Hannover.

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

Collins AC, and Dubach JM (2000) Biogeographic and ecological forces responsible for speciation in *Ateles. International Journal of Primatology*, **21**, 421–444.

Cox B, and Moore PD (2005) *Biogeography: An Ecological and Evolutionary Approach.* Blackwell Publishing, Malden, MA.

Coyne JA and Orr HA (2004) *Speciation.* Sunderland MA.

Craul M, Zimmermann E, Rasoloharijaona S, Randrianambinina B, and Radespiel U (2007) Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species. *BMC Evolutionary Biology*, **7**, 83.

Craul M, Radespiel U, Rasolofoson DW, Rakotondratsimba G, Rakotonirainy O, Rasoloharijaona S, Randrianambinina B, Ratsimbazafy J, Ratelolahy F, Randrianamboavaonjy T and Rakotozafy L (2008) Large rivers do not always act as species barriers for *Lepilemur* sp. *Primates*, **49**, 211–218.

Dausmann KH, Glos J, Linsenmair KE and Ganzhorn JU (2008) Improved recruitment of a lemur-dispersed tree in Malagasy dry forests after the demise of vertebrates in forest fragments. *Oecologia*, **157**, 307–316.

Dunham AE, Erhart EM, Overdorff DJ and Wright PC (2008) Evaluating effects of deforestation, hunting and El Niño events on a threatened lemur. *Biological Conservation*, **141**, 287–297.

Fahrig L (2003) Effects of habitat fragmentation on biodiversity. *Annual Review of Ecology, Evolution and Systematics*, **34**, 487–515.

Felsenstein J (1978) Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology*, **27**, 401–410.

Frankham R and Ralls K (1998) Inbreeding leads to extinction. *Nature*, **392**, 441–442.

Godfrey LR and Irwin MT (2007) The evolution of extinction risk: past and present anthropogenic impacts on the primate communities of Madagascar. *Folia Primatologica*, **78**, 405–419.

Goldberg TL and Ruvolo M (1997) The geographic apportionment of mitochondrial genetic diversity in east African chimpanzees, *Pan troglodytes schweinfurthii. Molecular Biology and Evolution*, **14**, 976–984.

Goodman SM and Rasolonandrasana BPN (2001) Elevational zonation of birds, insectivores, rodents and primates on the slopes of the Andringitra Massif, Madagascar. *Journal of Natural History*, **35**, 285-305.

Goodman SM and Ganzhorn JU (2004) Biogeography of lemurs in the humid forests of Madagascar: the role of elevational distribution and rivers. *Journal of Biogeography*, **31**, 47–55.

Green G and Sussman R (1990) Deforestation History of the Eastern Rain Forests of Madagascar from Satellite Images. *Science*, **248**, 212–215.

Greenwood P (1980) Mating systems, philopatry and dispersal in birds and mammals. *Animal Behaviour*, **28**, 1140–1162.

Hoffman EA and Blouin MS (2004) Historical data refute recent range contraction as cause of low genetic diversity in isolated frog populations. *Molecular Ecology*, **13**, 271–276.

Holthausen R, Czaplewski R, DeLorenzo D, Hayward G, Kessler W, Manley P, McKelvey K, Powell D, Ruggiero L, Schwartz M, van Horne B and Vojta C (2005) Strategies for Monitoring Terrestrial Animals and Habitats. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fort Collins, CO.

Jeffroy O, Brinkmann H, Delsuc F and Philippe H (2006) Phylogenomics: the beginning of incongruence? *Trends in Genetics*, **22**, 225–231.

Johnson M and Gaines M (1990) Evolution of Dispersal: Theoretical Models and Empirical Tests Using Birds and Mammals. *Annual Review of Ecology and Systematics*, **21**, 449–480.

Kurtz J, Jackson L and Fisher W (2001) Strategies for evaluating indicators based on guidelines from the Environmental Protection Agency's Office of Research and Development. *Ecological Indicators*, **1**, 49–60.

Lacy R (1997) Importance of genetic variation to the viability of mammalian populations. *Journal of Mammalogy*, **78**, 320–335.

Laurance WF and Bierregaard Jr RO (1997) *Tropical forest remnants: ecology, management, and conservation of fragmented communities*.

Lehman SM (2004) Distribution and Diversity of Primates in Guyana: Species-Area Relationships and Riverine Barriers. *International Journal of Primatology*, **25**, 73–95.

Lehman SM (2006) Conservation biology of malagasy strepsirhines: A phylogenetic approach. *American Journal of Physical Anthropology*, **130**, 238–253.

Lehman SM and Ratsimbazafy R (2001) Biological assessment of the Fandriana-Marolambo forest corridor, Madagascar. *Lemur News*, **6**, 8.

Lehman SM, Ratsimbazafy J, Rajaonson A and Day S (2005) Decline of *Propithecus diadema edwardsi* and *Varecia variegata variegata* (Primates: *Lemuridae*) in south-east Madagascar. *Oryx*, **40**, 1–4.

Lei R, Engberg S, Andriantompohavana R, McGuire S, Mittermeier R, Zaonarivelo J, Brenneman R and Louis Jr EE (2008) Nocturnal Lemur Diversity at Masoala National Park. *Special Publications of the Museum of Texas Tech University*, **53**, 1–48.

Lomolino MV, Riddle BR and Brown JH (2005) *Biogeography*. Sinauer Associates, Sunderland, MA.

Louis Jr EE, Engberg S, Lei R, Geng H, Sommer J, Randriamampionona R, Randriamanana J, Zaonarivelo J, Andriantompohavana R, Randria G, Prosper Ramaromilanto B, Rakotoarisoa G, Rooney A and Brenneman R (2006) Molecular and Morphological Analyses of the Sportive Lemurs (Family Megaladapidae: Genus *Lepilemur*) Reveals 11 Previously Unrecognized Species. *Special Publications, Museum of Texas Tech University*, **49**, 1–47.

Luikart G, Cornuet J-M and Allendorf FW (1999) Temporal Changes in Allele Frequencies Provide Estimates of Population Bottleneck Size. *Conservation Biology*, **13**, 523–530.

Luikart G, England PR, Tallmon D, Jordan S and Taberlet P (2003) The power and promise of population genomics: from genotyping to genome typing. *Nature Reviews, Genetics*, **4**, 981–994.

Manel S, Schwartz M, Luikart G and Taberlet P (2003) Landscape genetics: combining landscape ecology and population genetics. *Trends in Ecology and Evolution*, **18**, 189–197.

Martin RD, Alterman L, Doyle GA and Izard MK (1995) Prosimians: from obscurity to extinction? In: *Creatures of the dark: the nocturnal prosimians* (eds. Alterman L, Doyle GA, Izard MK), pp. 535–563. New York: Plenum Press.

Mendez-Cardenas M, Randrianambinina B, Rabesandratana A, Rasoloharijaona S and Zimmermann E (2008) Geographic variation in loud calls of sportive lemurs (*Lepilemur* ssp) and their implications for conservation. *American Journal of Primatology*, **70**, 828–838.

Miller CR and Waits LP (2003) The history of effective population size and genetic diversity in the Yellowstone grizzly (*Ursus arctos*): implications for conservation. *Proceedings of the National Academy of Sciences U S A*, **100**, 4334–4339.

Nielsen E, Hansen M and Mensberg K-L (2006) Underwater but not out of sight: genetic monitoring of effective population size in the endangered North Sea houting (*Coregonus oxyrhynchus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **63**, 780–787.

Nussey DH, Coltman DW, Coulson T, Kruuk LEB, Donald A, Morris SJ, Clutton-Brock TH and Pemberton J (2005) Rapidly declining fine-scale spatial genetic structure in female red deer. *Molecular Ecology*, **14**, 3395–3405.

Olivieri G, Craul M and Radespiel U (2005) Inventaire des lémuriens dans 15 fragments de forêt de la province de Mahajanga. *Lemur News*, **10**, 11–16.

Olivieri G, Zimmermann E, Randrianambinina B, Rasoloharijaona S, Rakotondravony D, Guschanski K and Radespiel U (2007) The ever-increasing diversity in mouse lemurs: three new species in north and northwestern Madagascar. *Molecular Phylogenetics and Evolution*, **43**, 309–327.

Olivieri G, Sousa V, Chikhi L and Radespiel U (2008) Genetic signature of recent population declines in the endangered golden-brown mouse lemur (*Microcebus ravelobensis*). *Biological Conservation*, **141**, 1257–1271.

Palsboll PJ, Berube M and Allendorf FW (2007) Identification of management units using population genetic data. *Trends in Ecology and Evolution*, **22**, 11–16.

Pertoldi C, Loeschcke V, Randi E, Madsen A, Møller Hansen M, Bijlsma R and van de Zande L (2005) Present and past microsatellite variation and assessment of genetic structure in Eurasian badger (*Meles meles*) in Denmark. *Journal of Zoology*, **265**, 387-394.

Poulsen NAA, Nielsen EE, Schierup MH, Loeschcke V and Gronkjaer P (2006) Long-term stability and effective population size in North Sea and Baltic Sea cod (*Gadus morhua*). *Molecular Ecology*, **15**, 321–331.

Primack R (1993) *Essentials of Conservation Biology* Sinauer Associates, Sunderland, MA.

Rabarivola C, Zaramody A, Fausser J-L, Andriaholinirina N and Roos C (2006) Cytogenetic and molecular characteristics of a new species of sportive lemur from Northern Madagascar. *Lemur News*, **11**, 45–49.

Ravaoarimanana IB, Tiedemann R, Montagnon D and Rumpler Y (2004) Molecular and cytogenetic evidence for cryptic speciation within a rare endemic Malagasy lemur, the Northern Sportive Lemur (*Lepilemur septentrionalis*). *Molecular Phylogenetics and Evolution*, **31**, 440–448.

Rudnick JA, Katzner TE, Bragin EA, Rhodes Jr OE and DeWoody JA (2005) Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan. *Molecular Ecology*, **14**, 2959–2967.

Schoenrogge K, Barr B, Wardlaw J, Napper E, Gardner M, Breen J, Elmes G and Thomas J (2002) When rare species become endangered: cryptic speciation in myrmecophilous hoverflies. *Biological Journal of the Linnean Society*, **75**, 291–300.

Schwartz MK, Luikart G and Waples RS (2007) Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution*, **22**, 25–33.

Smith J and Hellmann J (2002) Population persistence in fragmented landscapes. *Trends in Ecology and Evolution*, **17**, 397–399.

Sork VL, Davis FW, Smouse PE, Apsit VJ, Dyer RJ, Fernandez-M JF and Kuhn B (2002) Pollen movement in declining populations of California Valley oak, *Quercus lobata*: where have all the fathers gone? *Molecular Ecology*, **11**, 1657–1668.

Stuart BL, Inger RF and Voris HK (2006) High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. *Biology Letters*, **2**, 470–474.

Wilmé L, Goodman S and Ganzhorn JU Biogeographic evolution of Madagascar's microendemic biota. *Science*, **312**, 1063–1065.

Zimmermann E, Cepok S, Rakotoarison N, Zietemann V and Radespiel U (1998) Sympatric mouse lemurs in north-west Madagascar: a new rufous mouse lemur species (*Microcebus ravelobensis*). *Folia Primatologica*, **69**, 106–114.

Zinner D, Roos C, Fausser J-L, Groves C and Rumpler Y (2007) Disputed taxonomy classification of sportive lemurs (*Lepilemur*) in NW Madagasacar. *Lemur News*, **12**, 53–56.

Acknowledgements

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those, who have made this dissertation possible.

My first gratitude is to PD Dr. Ute Radespiel and Prof. Elke Zimmermann. I have been amazingly fortunate to have supervisors, who gave me the freedom to explore on my own, and at the same time the guidance to recover when my steps faltered. They encouraged me to question thoughts and express ideas. Their patience and support helped me overcome many crisis situations and finish this dissertation.

Many friends have helped me stay sane throughout these years. Their support and care helped me overcome setbacks and stay focused on my study. I greatly value their friendship and I deeply appreciate their belief in me.

Most importantly, none of this would have been possible without the love and patience of my family. My family, to whom this dissertation is dedicated to, has been a constant source of love, concern, support, and strength all these years.

I appreciate the financial support from the VW Foundation, the Natural Environment Research Council (NERC), the 'Gesellschaft der Freunde der TiHo e.V.', and Dr. Michael Otto.

I would like to thank CAFF/CORE, the 'Direction Générale des Eaux et Forêts' and ANGAP (in particular Chantal Andrianarivo), for the authorization and support during fieldwork in Madagascar. I thank the late Prof. Berthe Rakotosamimanana for always welcoming me. I also thank Dr. Daniel Rakotondravony and Prof. Olga Ramilijaona for their help in providing permits.

I thank M.W. Bruford and Terry Burke for giving me the opportinuty to work at the SMGF, D.A. Dawson, A. Krupa, and A. Leviston for helping me during lab work and analyses in Sheffield, and Raj and April Whitlock for welcoming me there.

I am also thankful to the technical staff who maintained all the machines in the lab so efficiently that I never had to worry about anything. Thanks to Hella Breitrück and Sabine Sippel.

I am also grateful to the former and current staff at the Institute of Zoology, for their various forms of support during my work.

I thank Dr. Lounès Chikhi for receiving me first in Lisbon, then in Toulouse. His insightful comments and constructive criticisms at different stages of my research were thought provoking and they helped me focus my ideas. I am grateful to him for holding me to a high research standard.

I am grateful to Vitor Sousa for his encouragement and practical advice. I am also thankful to him for discussing and commenting on my views and helping me understand and enrich my ideas.

Many thanks go to Dr. Blanchard Randrianambinina and Dr. Solofo Rasoloharijaona for their incredible support in Madagascar. Their help and friendship throughout the study was amazing, and without it this work would hardly have been possible.

Many people participated in the fieldwork. A special thanks goes to Gillian Olivieri for her excellent company during the study. Katja Guschanski, Paul Koenig, Chantal Peverelli, Jean Rajanary, Benjamin Rakoloarinelina, Jean-Aimé Rakotonirina, Todisoa Razafimamonjy, Christoph Schwitzer and Nora Schwitzer were all of great help. I am also very thankful to Andriatahina Rabesandratana for the samples in and around Ankarafantsika.

My last and deepest thanks goes to Anja Pietsch. She has helped me in so many ways during the last year that I can't possibly mention them all. I want to thank her for always having listened to my problems and for her constant encouragements, and for Krümel.

Curriculum Vitae

Mathias Craul

Birthdate	22.11.1973
Birthplace	Hannover, Germany
Nationality	German

Currently

since 2003 — **PhD thesis**

Institute of Zoology, University of Veterinary Medicine Hannover, Germany

PhD topic: 'Molecular phylogenetics and conservation genetics of sportive lemurs (*Lepilemur* spp.) in northwestern Madagascar'

Supervision: PD Dr. Ute Radespiel, Prof Dr. E. Zimmermann

Education

1997 – 2002 — **Diploma in Biology**

Gottfried Wilhelm Leibniz University Hannover, Germany

Diploma thesis: 'Female mate choice in the Gray Mouse Lemur (*Microcebus murinus*)'. Institute of Zoology, University of Veterinary Medicine Hannover, Germany

Supervision: Prof. Dr. Elke Zimmermann, Dr. Ute Radespiel

1994 – 1997 — Undergraduate studies on Physics

Gottfried Wilhelm Leibniz University Hannover, Germany

1984 – 1994 — Secondary School Ratsgymnasium Hannover, Germany

Qualification: **A levels**

1984 – 1985 — Secondary School Birkenstrasse, Hannover, Germany

1980 – 1983 — Primary School Hemmingen, Hemmingen-Westerfeld, Germany

Publications

Craul M, Zimmermann E and Radespiel U (2004) First experimental evidence for female mate choice in a nocturnal primate. *Primates 45*, 271–274.

Olivieri G, Craul M and Radespiel U (2005) Inventaire des lémuriens dans 15 fragments de forêt de la province de Mahajanga. *Lemur News 10*, 11–16.

Andriaholinirina N, Fausser J-L, Roos C, Zinner D, Thalmann U, Rabarivola C, Ravoarimanana I, Ganzhorn JU, Meier B, Hilgartner R, Walter L, Zaramody A, Langer C, Hahn T, Zimmermann E, Radespiel U, Craul M, Tomiuk J, Tattersall I and Rumpler Y (2006) Molecular phylogeny and taxonomic revision of the sportive lemurs (*Lepilemur*, Primates). *BMC Evolutionary Biology 6*, 17.

Craul M, Zimmermann E, Rasoloharijaona S, Randrianambinina B and Radespiel U (2007) Unexpected species diversity of Malagasy primates (*Lepilemur* spp.) in the same biogeographical zone: a morphological and molecular approach with the description of two new species. *BMC Evolutionary Biology 7*, 1, 83.

Craul M, Radespiel U, Rasolofoson DW, Rakotondratsimba G, Rakotonirainy O, Rasoloharijaona S, Randrianambinina B, Ratsimbazafy J, Ratelolahy F, Randrianamboavaonjy T and Rakotozafy L (2008) Large rivers do not always act as species barriers for *Lepilemur* sp. *Primates 49*, 3, 211–218.

Radespiel U, Olivieri G, Rasolofoson DW, Rakotondratsimba G, Rakotonirainy O, Rasoloharijaona S, Randrianambinina B, Ratsimbazafy JH, Ratelolahy F, Randriamboavonjy T, Rasolofoharivelo T, Craul M, Rakotozafy L and Randrianarison RM (2008) Exceptional diversity of mouse lemurs (*Microcebus* spp.) in the Makira region with the description of one new species. American Journal of Primatology 70, 11, 1033–1046.

Craul M, Dawson DA, Radespiel U, Zimmermann E and Bruford MW (2009) Isolation of sixteen autosomal loci and a sex-linked polymorphic microsatellite locus from the Milne-Edwards' sportive lemur (*Lepilemur edwardsi*). *Molecular Ecology Resources 9*, 333-335.

Craul M, Chikhi L, Olivieri O, Rabesandratana A, Zimmermann E and Radespiel U (in press) Influence of forest fragmentation on an endangered large-bodied lemur in northwestern Madagascar. *Biological Conservation*.