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GENETIC DIVERSITY IN A LITTLE KNOWN LEMUR SPECIES FROM THE NORTH OF MADAGASCAR (*Microcebus tavaratra*)

Isa Gameiro Aleixo Pais

Mestrado em Biologia da Conservação

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Dissertação orientada pelos Prof. Doutor Lounès Chikhi, IGC

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Resumo

Madagáscar é um dos mais importantes hotspots de biodiversidade do nosso planeta (Goodman and Benstead, 2003; Myers *et al.,* 2000) mas a sua biodiversidade única tem sido, e ainda é, ameaçada por alterações climáticas e por pressões antropogénicas (Ganzhorn *et al.,* 2001; Allun *et al.,* 2008). Os lémures, como animais dependentes da floresta, são particularmente afectados por estas mudanças no habitat. Embora a razão pela qual estas alterações climáticas ocorreram ainda não seja muito clara, a história de Madagáscar revela que muitas das espécies existentes extinguiram-se no último milénio (Dewar 2003; Lawler 2010).

Contrariamente ao esperado, esta diminuição no número de espécies não é notória nos lémures, o que tem levado a debates acerca de uma possível "inflação taxonómica" (Tattersall, 2007). Isto é particularmente verdade para as espécies nocturnas de pequeno e médio porte como é o caso do lémure rato (*Microcebus* spp.) e do lémure desportivo (*Lepilemur* spp.), que têm visto o seu número de espécies reconhecidas passar de duas (para a espécie *Microcebus*) e sete (para a espécie *Lepilemur*) para 18 e 26 respectivamente (Louis *et al.,* 2006; Olivieri *et al.,* 2007, Mittermeier *et al.,* 2010). Esta característica poderá explicar o rápido crescimento do número de espécies descritas, mas também dá ênfase à vulnerabilidade das espécies em relação à destruição do seu habitat (Yoder *et al.,* 2002, Weisrock *et al.,* 2010), em particular os lémures rato que parecem ser influenciados pela presença de espaços não florestados entre os fragmentos de floresta (Radespiel *et al., 2008*).

Os lémures rato (género *Microcebus*) encontram-se distribuídos por toda a ilha de Madagáscar (Louis *et al*., 2006; Olivieri *et al.,* 2007) ocupando diversos habitats (Vigilant and Guschanski, 2009). Devido ao facto de este género ser microendémico, estas espécies revelam grande importância para estudos comparativos em vários pontos de Madagáscar. Na grande maioria das regiões já foram realizados censos, mas ainda existem áreas que não foram estudadas e em muitas

destas regiões apenas um ou dois indivíduos foram amostrados. Este é o caso da região de Daraina onde apenas foram recolhidas amostras de dois indivíduos da espécie lémure rato nos fragmentos de floresta de Bekaraoka e Bobankora. Os dois espécimens foram identificados como pertencentes à espécie *Microcebus tavaratra*. Contudo espécies coabitantes de lémure rato são conhecidas por habitarem várias regiões de Madagáscar e não é claro que Daraina apenas abrigue uma espécie em particular, uma vez que ainda não foi realizada nenhuma amostragem para as regiões a sul de Daraina.

Este trabalho insere-se num projecto que pretende estudar várias espécies de lémures da zona de Daraina, no norte de Madagáscar, e tem como principal objectivo identificar a diversidade genética existente em populações de lémure rato presentes em três fragmentos de floresta desta região. Daraina é uma área com indústrias mineiras e florestais que provavelmente contribuem em grande parte para a desflorestação do habitat (Vargas *et al.,* 2002, Goodman *et al.,* 2006, Quéméré *et al.,* 2010), e consequentemente, espécies que dependem da floresta como é o caso dos lémures, são particularmente afectadas por estas acções (Banks *et al.,* 2005; Sott *et al.,* 2006).

Embora haja evidência da presença do lémur rato tavaratra (*Microcebus tavaratra*) em Daraina, não é certo que os indíviduos amostrados para este estudo pertençam a esta espécie. Deste modo é importante determinar se todos os indivíduos pertencem à mesma espécie e se esta é a anteriormente descrita por Weisrock et al. (2010). Para tal, ADN de 72 indivíduos amostrados no campo foi extraído e amplificado para 3 loci do ADN mitochondrial (cyt b, COII e d-loop). Em seguida, procedeu-se à sequenciação do ADN amplificado e as sequências obtidas foram alinhadas no programa BIOEDIT© version 7.0.9.0 (Hall, 1999). Sequências de 5 espécies de *Microcebus* foram adquiridas da base de dados GenBank para serem utilizadas como referência às nossas amostras. Para construir as árvores filogenéticas necessárias de modo a determinar a espécie amostrada, foi necessário calcular o modelo evolutivo que melhor se adequa aos nossos dados.

Este foi calculado usando o jModelTest$^{©}$ 0.1.1 (Posada, 2005). Este programa testou 40 modelos para encontrar aquele que tinha menos perda de informação quando comparado com os dados reais. As árvores filogenéticas foram construídas para cada locus no programa MEGA$^{©}$ version 5 (Tamura *et al.,* 2011) usando o modelo evolutivo seleccionado. Os resultados obtidos demonstraram que as nossas amostras encontram-se agrupadas juntamente com as sequências de referência de *M. tavaratra*, o que sugere que os indivíduos amostrados pertencem, provavelmente, a esta espécie.

Frequentemente, o processo de identificação das espécies é também utilizado para obter informação ao nível da diversidade genética das populações. No caso destes indivíduos, foi utilizado o ADN mitocondrial anteriormente sequenciado e ADN nuclear genotipado para cinco loci polimórficos. Diversidade haplotídica e nucleotídica foram calculadas para os loci *cyt b*, COII e d-loop (mtDNA) e todos estes índices encontravam-se dentro dos valores apresentados noutros estudos de genética populacional, à excepção de uma população, Binara. Para estes indíviduos não foi encontrada qualquer diversidade genética para nenhum dos loci, um resultado interessante visto que o número mínimo de indíviduos sequenciados (N=10) estava no limite do número sugerido por Markolf e tal, 2011 (10 indivíduos por cada fragmento amostrado). Para além disso, os locais de captura dos indivíduos neste fragmento eram os mais dispersos de todas as populações amostradas. Para este marcador genético (mtDNA), a população de Bekaraoka apresentou as taxas de variação mais altas de entre as três populações com valores mínimos de Hd=0.709±0.003, e π=0.003±0.002. Esta população foi também a única que mostrou evidências de partilha de haplótipos com as outras duas populações, resultados facilmente observados nas redes de haplótipos calculadas.

No entanto, a diversidade genética encontrada ao nível do ADN nuclear apresentou resultados contrários aos anteriormente obtidos. Nesta análise, a população de Binara demonstrou

variabilidade genética alta, tendo o maior número de allelos (7.8 comparativamente a 6.8 e 5.8 para Solaniampilana e Bekaraoka, respectivamente). Nenhuma das populações demonstrou um desvio do equilíbrio de Hardy-Weinberg, embora os valores de p não sejam estatisticamente significativos.

Foi ainda realizado o teste AMOVA para ambos os tipos de marcadores genéticos e enquanto no ADN mitocondrial foi detectada variação entre e dentro de cada população (54.48 - 64.81% e 35.19 – 45.52%, respectivamente), para o ADN nuclear apenas foi encontrada uma variação muito alta dentro de cada população (94.16%). Um outro teste de estrutura populacional foi realizado no programa STRUCTURE 2.3.3 (2010), mas mais uma vez não foi detectada nenhuma estrutura a nível da diversidade nuclear para as populações amostradas neste estudo.

A estrutura populacional, o efeito de gargalo, a selecção natural, o ciclo de vida, o sistema de acasalamento e a estrutura social são factores que podem ter um efeito na genética das populações (Bazin et al., 2006). Neste estudo foi detectada variação genética entre e dentro de cada população mas é complicado atribuir essa diversidade a eventos específicos, uma vez que a nossa amostragem abrange apenas três fragmentos de floresta e 72 indivíduos, e não existe muita informação disponível para a espécie de *Microcebus* presente na região de Daraina.

Contudo, estudos como este são importantes para a conservação de espécies pois permitem-nos perceber como é que as populações respondem a alterações climáticas e/ou pressões antropogénicas, e desta forma desenvolver planos de conservação coerentes e consistentes. Estas acções requerem um trabalho conjunto entre investigadores, comunidades locais, ONG's (Organizações não-governamentais), universidades e entidades governamentais (Dunham *et al.,* 2008, Quéméré *et al.,* 2009).

Resumo

Estudos sobre a delimitação das espécies e genética populacional providenciam-nos informação sobre a evolução das espécies assim como a sua resposta natural às pressões antropogénicas. Ainda assim, para muitas espécies, esses efeitos ainda não foram estudados e necessitam de mais investigação (Hanski *et al.,* 1998). Este é o caso dos lémures rato (género *Microcebus*) que habitam as florestas de Daraina. Foi sequenciado o ADN mitocondrial de 72 indivíduos *Microcebus,* cujas amostras tinham sido recolhidas em três fragmentos de floresta, de modo a determinar se eram todos membros da mesma espécie, a *M. tavaratra,* uma vez que dois outros indivíduos desta espécie já tinham sido identificados nesta região (Weisrock *et al.,* 2010). É importante perceber como a diversidade genética é distribuída tanto para os genes mitocondriais como para os nucleares (microsatélites), uma vez que o rio Manankolana e outros aspectos ecológicos de Daraina já demonstraram desempenhar um papel importante na estrutura genética de algumas populações (Quéméré *et al.,* 2010; Radespiel *et al.,* 2008).

Os resultados sugerem que todos os indivíduos pertencem à espécie *M.tavaratra* e que é mantida uma grande variabilidade genética nas populações de Bekaraoka e Solaniampilana. Contudo, na floresta de Binara, a falta de diversidade genética em todos os mtADN loci é surpreendente. Por outro lado, os dados dos microsatélites demonstraram que afinal a população de Binara exibe variabilidade genética assim como as outras duas populações. Como tal, não foi possível identificar nenhuma estrutura populacional ao nível nuclear. Embora seja provável que este seja o estudo mais abrangente geograficamente sobre os Microcebus de Daraine, os resultados sugerem que é necessária mais investigação. Estudos como este são importantes para que se implementem planos de conservação coerentes e consistentes para as espécies em risco.

Palavras-chave: Madagáscar, identificação de espécies, *M.tavaratra*, mtDNA, microsatélites.

Abstract

Species delimitation and population genetic studies can provide information on the evolution of species, as well as their response to natural or anthropogenic pressures. However, for many species these effects have not yet been studied and need further research (Hanski *et al.,* 1998). This is the case of mouse lemurs (genus *Microcebus*) living in the forests of Daraina.

We will sequence the mitochondrial DNA of 72 *Microcebus* individuals sampled in three forest fragments, in order to determine if they are all members of the same species, the *M. tavaratra* species previously identified in 2 individuals from this region (Weisrock *et al.,* 2010). We would also like to understand how is genetic diversity distributed within and between populations for both mitochondrial and nuclear genes (microsatellites), as the Manankolana river and other natural features in Daraina have shown to play a role in the genetic structure of some populations (Quéméré *et al.,* 2010; Radespiel *et al.,* 2008). Finally, we will try to identify genetic clusters, despite the limited number of nuclear loci and populations, and determine if these clusters correspond to specific features of the habitat.

The results suggest that all individuals belong to the *M. tavaratra* species and high genetic variation is maintained in populations from Bekaraoka and Solaniampilana. However, the lack of genetic diversity in the Binara forest across all mtDNA loci is surprising. Microsatellite data though, showed that the Binara population actually exhibited genetic variability and thus, no clustering could be defined at the nuclear level.

Despite being probably the most geographically widespread study on *Microcebus* from Daraina, our results suggest that more research should be carried out. Nevertheless, studies like this one are important for the implementation of appropriate and effective conservation plans of threatened species.

Key words: Madagascar, species identification, M.tavaratra, mtDNA, microsatellites

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0. Structure of the project

The present thesis is divided in six chapters.

Chapter 1 is a brief introduction to the main theme considered in this thesis. Herein, we aim to relate this study with all subjects presented throughout the work with basis on up to date knowledge in this field. A description of the species studied as well as the study site is also found in this section.

In chapter 2, the methodology necessary to obtain the data for this work is described in detail..

The analysis performed for the taxonomy and genetic diversity of mouse lemurs is described in section 3.

A description of the results obtained from the phylogenetic analysis, quantification of genetic diversity and study of population structure are presented in this fourth chapter.

Finally, in chapter 5, all the results obtained are discussed and related with other studies, and final conclusions are attained. In this chapter we also discuss the main contributions of the work, give future guidelines and identify open questions that deserve further investigation in the context of the conservation of lemurs in Madagascar.

Chapter 6 cites all the references used in the writing of this thesis.

1. Introduction

Biodiversity is variably distributed across the World with some regions featuring exceptional concentrations of endemic species and experiencing exceptional loss of habitat (Myers, 1990; Pressey *et al.,* 1993; Prendergast *et al.,* 1999; Ginsberg 1999 in Goodman and Benstead 2003). Biodiversity is currently under threat due to habitat loss and fragmentation (Meyers *et al.,* 2000; Ganzhorn *et al.,* 2003) caused by both natural and anthropogenic factors (Watson 2002; Fahrig 2003). These Biodiversity hotspots are being identified as priority areas for Conservation. Madagascar is characterized by a high level of endemism in plants (3,2% of global species total – 300,000) and animals (2,8% of global species total – 27,298) (Goodman and Benstead 2003; Myers *et al.,* 2000).

The identification of new species, the quantification of the species diversity and differentiation are fundamental for defining conservation strategies. Differentiation among populations affects species overall genetic diversity and this differentiation is usually measured by different statistics which use allelic frequencies from microsatellite and sequence DNA data. The analysis of genetic data of natural populations has the ultimate goal of understanding species evolution and diversification. Moreover, population genetic studies used to assess the distribution of diversity within and between populations are essential to understand which factors, anthropogenic or natural, are the responsible for such diversity.

In this thesis, I will therefore address several questions (i) are individuals sampled across three forest fragments of one particular region of Madagascar members of the same species, (ii) is this (or one of these) species the same species as *M. tavaratra* identified by Weisrock *et al.,* (2010) in previous studies, (iii) how is genetic diversity distributed within and between populations for both mitochondrial and nuclear genes (microsatellites), (iv) can we identify genetic clusters despite the limited number of nuclear loci, (v) do these clusters, if any correspond to specific features? However, given the limited number of individuals and locations sampled we do not expect to be able to answer the last questions with more than preliminary results. A better understanding on these issues should contribute to a better understanding on the current status of the lemur species diversity in Madagascar and should highlight aspects of this lemur population diversity.

I should also finish this introduction by indicating that part of my Masters work was also carried out to set up and test protocols. For instance I have (I) tested and validated a DNA extraction protocol on faecal material from *P. tattersalli* (ii) amplified microsatellites for several populations of *P. coronatus*, and *P. coquereli*, (iii) tested and validated several extraction protocols to identify the cheapest and most efficient one, and (iv) tested and validated a sexing protocol on *M. tavaratra* (biopsy), *P. coronatus* (faeces) and *P. verreauxi* (faeces).

Altogether the present study is part of a wider project which aims at understanding the effects of habitat loss and fragmentation across lemur species within the same region and in which I am involved. It builds on a previous work carried out by Quéméré *et al.* (2010 a, b) on the golden-crowned sifaka (*Propithecus tattersalli*) whose distribution is limited to the Daraina region (Quéméré *et al.,* 2009, 2010a, b). The work presented here is thus a first step towards a wider phylogenetic and multispecies landscape and population genetics region-wide study. The Daraina region is particularly interesting because, besides being fragmented, it is ecologically very variable. Anthropogenic activities, such as gold mining and wood exploitation, are factors that may contribute to habitat loss and fragmentation in this region (Vargas *et al.,* 2002; Goodman *et al.,* 2006; Quéméré *et al.,* 2010 a, b), but a recent study has shown that the major barrier to gene flow in *P. tattersalli* was the Manankolana river that crosses the region. Other factors appeared to be the presence of savannah and geographic distance whereas the national road, also crossing the region, did not appear to have such a major impact (Radespiel *et al.,* 2008).

1.1. Conservation in Madagascar

Madagascar is one of the most important Biodiversity hotspots in the World (Goodman and Benstead, 2003; Myers *et al.,* 2000) but its unique biodiversity has been, and still is, highly threatened by major environmental changes and anthropogenic pressures (Ganzhorn *et al.,* 2001; Allnut *et al.,* 2008). While the exact causes of these environmental changes are not fully clear, the history of Madagascar reveals that many species have become extinct in the last millennia (Dewar,

2003; Lawler *et al.,* 2001). Harper *et al.* (2007), have recently estimated that only 10%−15% of the original forests of Madagascar remain and that deforestation continues at a rate of 1% per year, hence contributing to further habitat loss and fragmentation (Burney *et al.,* 2004; Smith *et al.,* 1997), and potentially driving species to extinction (Goodman and Benstead, 2003). Despite the existence of a few continuous forest reserves in Madagascar, most forest mammals survive in small, and increasingly isolated habitat patches (Smith *et al.,* 1997). Lemurs in particular, as forest-dwelling animals, are strongly affected by these changes (Godfrey and Irwin 2007). In order to preserve such a unique and diverse ecosystem, conservation actions must be developed (Rands *et al.,* 2010). This requires both local and global strategies which must involve local communities, NGOs (non-governmental organizations), universities and governmental agencies. Together, these entities may define priorities regarding deforestation, mining and logging, to reduce habitat loss and fragmentation in Madagascar (Dunham *et al.,* 2008, Quéméré *et al.,* 2009).

The number of studies on the impact of habitat loss and fragmentation on the genetic diversity of lemur populations has increased in the last years, in part due to increased sampling efforts, new molecular techniques and population genetics analyses (e.g. Olivieri *et al.,* 2008; Quéméré *et al.,* 2010[a]; Schneider *et al.,* 2010). However, for many other species from the same regions these effects have not yet been studied and are still poorly understood, making long-term consequences difficult to infer and study (Hanski *et al.,* 1998; Ewers and Didham, 2006).

In parallel to this need for multispecies studies within the same region there are still many issues that need to be solved at higher taxonomical levels. Indeed, it is important to improve our understanding on the taxonomic units present in the regions of interest. This is particularly true in Madagascar where the number of recognised species has significantly increased in the last fifteen years in several groups of vertebrates (Vences *et al.,* 2009; Mittermeier *et al.,* 2010). In lemurs this has led to debates regarding a possible "taxonomic inflation" (Tattersall, 2007) This is particularly true for nocturnal small and medium-sized species such as mouse lemurs (*Microcebus* spp.) and sportive lemurs (*Lepilemur* spp.), which have seen their number of recognized species go from

two (for *Microcebus*) and seven (for *Lepilemur*), to 18 and 26, respectively (Louis *et al.,* 2006[a]; Olivieri *et al.,* 2007, Mittermeier *et al.,* 2010).

1.2. Lemurs of Madagascar: mouse lemurs

Molecular analysis suggest that out of the 18 mouse lemurs species officially recognised (Mittermeier *et al.,* 2008, 2010), several do show extremely limited geographic distributions (micro-endemism) (Vences *et al.,* 2009), with rivers playing an apparent role as barriers of gene flow (Wilmé *et al.,* 2006). This characteristic may explain the rapid increase in the number of species described but also emphasizes the species vulnerability to habitat destruction (Yoder *et al.,* 2002, Weisrock *et al.,* 2010), particularly as mouse lemurs appear to be influenced by the presence of non forested areas between forest fragments (Radespiel *et al., 2008*).

Mouse lemurs (genus *Microcebus*) are the world's smallest primates with average adult body size ranging from 30 to 72 g (Rasoloarison *et al.,* 2000; Yoder *et al.,* 2000). They are strictly nocturnal solitary foragers that live in dispersed social organizations (Heckman *et al.,* 2007; Olivieri *et al.,* 2008) and are sympatric with other nocturnal species and in some areas, with other *Microcebus* species (Mittermeier *et al.,* 2010). They are found in most habitat types of Madagascar, thus showing a widely distribution across the island (Louis *et al.,* 2006[a]; Olivieri *et al.,* 2007) (Figure 1), but their home range is relatively small ($0.01 - 0.02$ km^2) (Kappeler and Rasoloarison, 2003). Basic aspects of the ecology and behaviour of some *Microcebus* species still remain to be studied. The IUCN Red List conservation status for the *Microcebus* genus varies across species from Endangered to Least Concern, and for many data is deficient. Nevertheless, *M. tavaratra* is classified as Endangered (EN), having habitat loss due to illegal logging, mining for sapphires, uncontrolled brush fires and charcoal production as the principal threat (Mittermeier *et al.,* 2010).

Mouse lemurs are a potentially interesting model to study the genetic consequences of habitat fragmentation over short evolutionary periods (Chikhi and Bruford, 2005) because they are

present in all types of habitats, and have short generation times, making them sensitive to (and hence ideal for inferring) recent demographic events due to anthropogenic or climatic changes. The fact that most mouse lemurs are thought to be microendemic (Gligor *et al.,* 2009), makes them vulnerable to deforestation and poaching (Yoder *et al.,* 2000; Mittermeier *et al.,* 2010) but also suggests that comparative studies can be carried out across Madagascar.

Recently, Weisrock *et al.* (2010) analysed genetic data from 286 individuals across Madagascar in order to clarify the phylogenetic relationships between mouse lemurs. While most regions were covered, there were still some gaps in the sampling and some wide regions only had one or two individuals sampled. This was the case of the Daraina region, where two mouse lemur individuals were sampled in the forest fragments of Bekaraoka and Bobankora (Figure 1). The two specimens were identified as belonging to the species *M. tavaratra,* together with four other individuals sampled north and northwest of Daraina in the national parks of Ankarana, Ankavana and Analabe (Olivieri *et al.,* 2007). However, sympatric mouse lemur species are known to inhabit several regions of Madagascar and it is unclear whether Daraina only harbours one species particularly as the regions south of Daraina are unsampled. Moreover, a recent study by Markolf *et al.* (2011) has suggested that for mouse lemurs, the sample sizes used to identify species through mtDNA, as in the Weisrock *et al.* (2010) study may be too small leading to an upward bias in the number of recognised species.
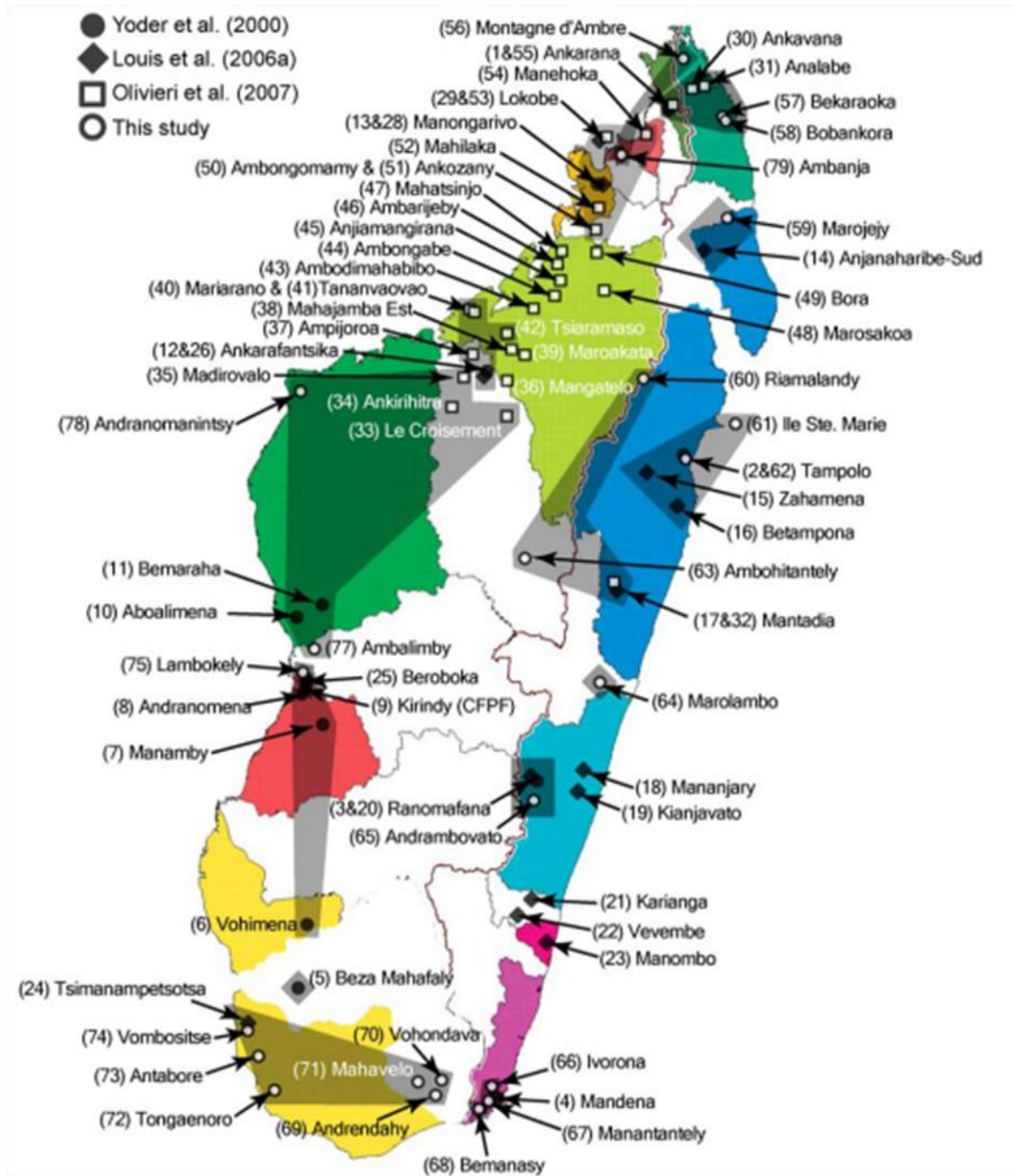
Figure 1: The geographic distribution of *Microcebus* species across Madagascar.

This map shows the most up to date distribution of mouse lemurs obtained from the analysis of mitochondrial and nuclear loci. (Source: *in* Weisrock *et al.*,2010).

1.3. The Study site: Daraina region

Despite being relatively small (2,580 km^2) the Daraina region has a surprisingly diverse habitat (Quéméré *et al.,* 2010[b]) within the area geographically limited by the Loky and the Manambato rivers. The forested area corresponds to 17% of the total area (440 km²) and is composed of gallery forests, littoral forests and highly fragmented humid or dry deciduous forest patches undergoing gold mining and wood exploitation (Goodman *et al.,* 2006). Forests are surrounded by human-altered degraded grasslands used for zebu cattle grazing, dry scrub and agricultural land (Meyers, 1993; Randrianarisoa *et al.,* 1999).

All samples were obtained from three forest fragments,Bekaraoka, Binara and Solaniampilana,withn the Daraina or Loki/Manambato region in the north of Madagascar (Figure 2). The three fragments, though geographically close, are ecologically very different. Solaniampilana and Bekaraoka are classified as western dry forest, while Binara is an evergreen humid forest (Moat and Smith, 2007). The fragments are separated from each other by a minimum of 10km and a maximum of 18km in a straight trajectory. The total surface area of the Bekaraoka, Binara and Solaniampilana forest fragments were estimated using the ArcGis® 9.3 program and the Madagascar Vegetation Mapping Project, from this last, forest classification data was used (source: http://www.kew.org/gis/projects/mad_veg/datasets.html). The estimated surface areas were, respectively, 54.24 km², 45.38km² and 12.86 km². It is also important to stress that the Bekaraoka and Binara forests are located in the "Station Forestière à Usages Multiples", (S.F.U.M. "Multiple Usage Forest Station"), a 570 km² Protected Area managed by the Fanamby NGO in collaboration with the Ministry of Environment and Forests (Quéméré *et al.,* 2010[b]), while the Solaniampilana forest is currently situated outside this protected area.

Based on known home range for the genus *Microcebus* (Mittermeier *et al.,* 2010), and distance between the sampled sites, it is plausible to assume that direct dispersal would be very unlikely to occur. To our knowledge the species of *Microcebus* present in this region are poorly known and

only two individuals have been sequenced so far, one from Bekaraoka and one from Bobankora fragments (Weisrock *et al.,* 2010).
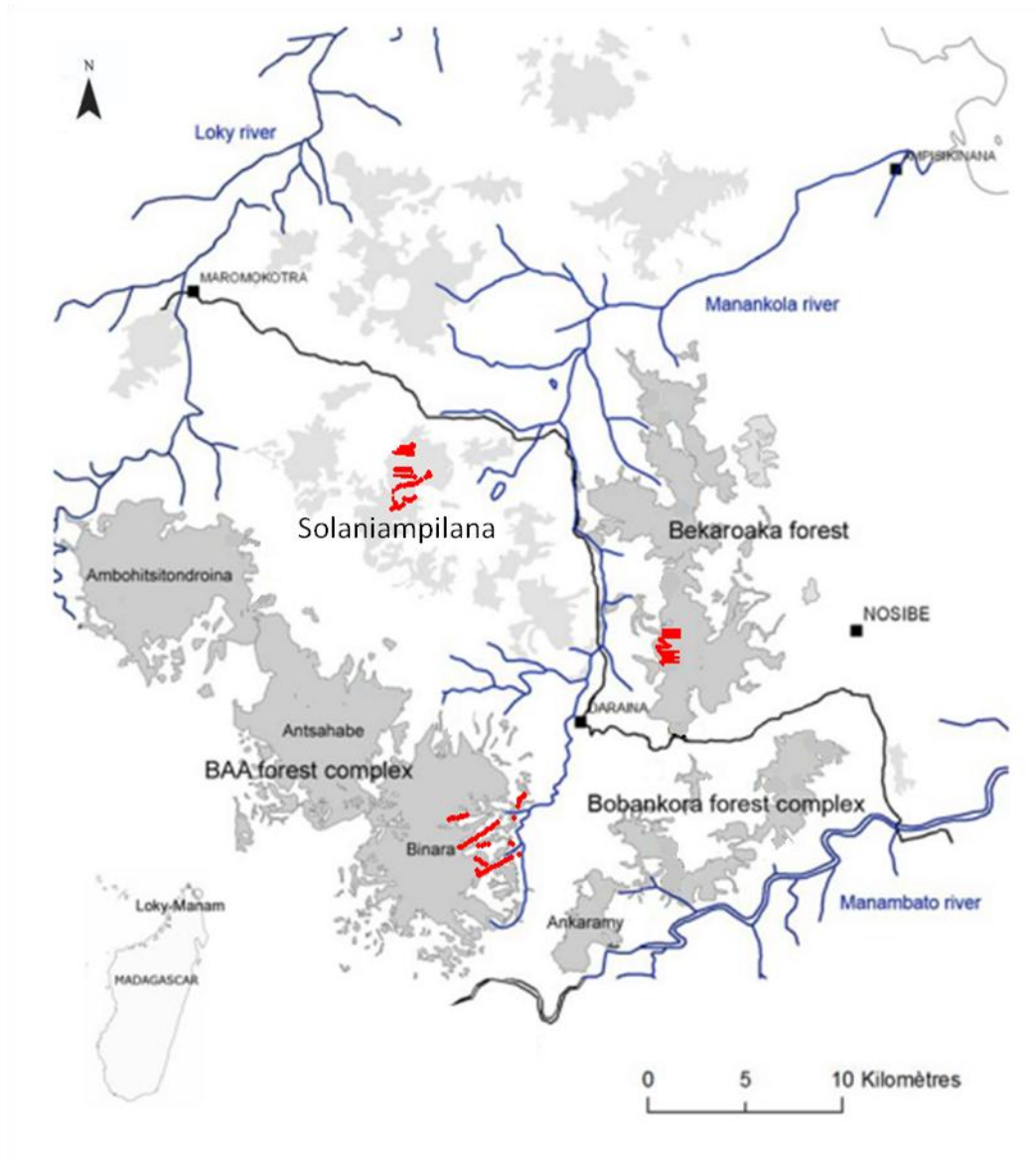
Figure 2: Trapping locations for the individuals sampled in this study. The map above shows the area of Daraina and the different forest fragments sampled; the red dots are the geographic locations where individuals were captured.

2. Materials and Methods

2.1. The *Microcebus* samples collection and DNA extraction

The *Microcebus* samples were collected in July and August of 2010 using Sherman traps (H.B. Sherman Traps®). For each animal captured, morphometric measurements were recorded and tissue, faecal and hair samples were collected. A total of 209 individuals were captured. However, only the tissue samples (ear biopsies) of a maximum of 72 individuals were used and sequenced in this study (see below for details and annex table 1 for sample information). The biopsies were stored in Queens Lysis Buffer (Seutin *et al.,* 1991; Dawson *et al.,* 1998) for preservation purposes. Once in the laboratory, the samples were kept at 4˚C until laboratory analysis. All animals were marked with 1-3 systematic ear biopsies (ca. 2mm²) for future identification in the field following Rakotondravony *et al.,* (2009), thus some storage tubes contained more than one biopsy. All field handling and sampling procedures adhered to the legal requirements of Madagascar and were approved by the Ministry of Water and Forests.

Total genomic DNA was extracted from 72 ear biopsies. Each biopsy was incubated overnight at 37˚C in 300µl digestion buffer (100mM EDTA, 100mM NaCl, 50mM Tris pH8 and 1% SDS) and 30µl of Proteinase K at 10mg/ml (Promega #V3021). A standard mammalian DNA isolation protocol (adapted from Laird *et al.,* 1991) was used as it was found to be the cheapest and quickest of all other procedures considered (see Annex – table 2). The extractions were performed in a DNA free Hood and each set of samples included a negative control to ensure that no cross-contamination occurred and a positive control to validate the genotypes.

2.1.1. Mitochondrial DNA (mtDNA) amplification and sequencing

Three different mtDNA regions/loci were amplified and sequenced for individuals from the three forest fragments. The mtDNA regions were selected on the basis of previously published molecular studies on *Microcebus* species. Two conserved regions of the mtDNA adapted for cross

species comparisons, Cytochrome *b* (*cyt b* -1110bp fragment) and Cytochrome oxidase II (COII -684bp fragment), were amplified with the flanking tRNA primers L14724: 5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' and H15915: 5´-AACTGCAGTCATCTCCGGTTTACAAGA-3´ (Irwin *et al.,* 1991), and L7553: 5´-AACCATTTCATAACTTTGTCAA-3´ and H8320: 5´-CTCTTTAATCTTTAACTTAAAAG-3´ (Adkins *et al.,* 1994), respectively. Another 514bp fragment from the hypervariable d-loop region of the mtDNA was also amplified using the universal mammalian control region primers L15997: 5´- CACCATTAGCACCCAAAGCT-3´and H16498: 5´-CCTGAAGTAGGAACCAGATG -3´ (Gerloff *et al.,* 1999). PCR amplifications were carried out in a 10µl reaction with final concentrations of 1.0µM for each primer, 1.5mM of $MgCl_2$, 0.2mM of each dNTP, 1 x NH4-reaction buffer, 0.05 units of GoTaq® Flexi DNA polymerase (Promega #M8305), and 10–100ng of DNA template. Standard PCRs were run with an initial denaturing temperature of 94˚C for 2min, followed by 30 cycles of 1min at 94˚C, 1min at 45-55˚C and 1min and 15secs at 72˚C, and a final extension of 10 min at 72˚C. Both PCR conditions and cycles followed the protocols from Guschanski *et al.*(2007) and Horvarth *et al. (*2008). For details on the annealing temperatures see table 1. An aliquot of 3 µl PCR products, including the positive and negative controls, were loaded on a 2% agarose gel to verify successful amplification and possible contamination. The percentage of samples that were discarded from the data analysis due to none or poor amplification are also represented in table 3 (sequencing success≈74%). PCR products were directly sequenced using both forward and reverse primers and BigDyeH Terminator v3.1 (Applied Biosystems, Foster City, CA). Cycle sequencing was performed in a total volume of 10 µL including 0.5mM primer, 2µl Big Dye, 2µl buffer, 50-100ng of PCR product and water up to 10 µl. Cycle sequencing conditions were carried out with an initial denaturing temperature of 96ºC for 1min, followed by 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec, 60˚C for 1min15sec, and a final hold at 4˚C. The sequences were analyzed in an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA). Individual sequences were checked by eye and edited using *MEGA*© version 5 (Tamura *et al.,* 2011), with the final alignment of all sequences for each loci being performed in BIOEDIT© version 7.0.9.0 (Hall, 1999). The single loci were then

concatenated into larger sequences to have all regions of the mtDNA to be analysed as a single loci. It is important to note that no information regarding sampling location was associated to the sequences in order to ensure blind analysis of the DNA (Heckman *et al.,* 2006), although for each individual was identified with a number.

2.1.2. Microsatellite amplification and genotyping

Genetic markers developed for another species, the *M. murinus* species, were used in this study. This was because there were no microsatellite markers designed for *M. tavaratra* and as markers for *M. Murinus* have been shown to provide good results when tested across different mouse lemur species (Radespiel *et al.,* 2001; Hapke *et al.,* 2003). Five polymorphic dinucleotide microsatellite loci were used to genotype 11 individuals from each fragment (table 1). The microsatellite loci were amplified in a 10µl reaction containing 0.15mM of each primer, 5 µl of My Taq HS Mix (Bioline-25045) and 1µl of template DNA. An initial denaturation of 15 s at 94°C was followed by 36 cycles of 30 s at 94°C, 1 min at 55-58°C, 1 min at 72°C, and a final extension at 72°C for 10 min. Negative controls were used in every PCR reaction in order to check for contamination. Primers were labelled with a fluorescent dye (either Hex or FAM) and amplification products were genotyped using ABI 3130 DNA equipment.

Table 1: Mitochondrial and nuclear DNA loci genotyped in this study.

Locus	No. Indiv	Lenght (bp)	Primer	Sequence	Repeat motif	Anealing temp (ºC)	Reference
cyt b [a]	33	1110	L14724 (F)	CGAAGCTTGATATGAAAAACCATCGTTG		50	Irwin *et al.,* 1991
			H15915 (R)	AACTGCAGTCATCTCCGGTTTACAAGA			
COII [a]	59	684	L7553 (F)	AACCATTTCATAACTTTGTCAA		45	Adkins *et al.,* 1994
			H8320 (R)	CTCTTTAATCTTTAACTTAAAAG			
d-loop [a]	68	514	L15997 (F)	CACCATTAGCACCCAAAGCT		55	Gerloff *et al.,* 1999
			H16498 (R)	CCTGAAGTAGGAACCAGATG			
Mm03 [b]	33	93-149	Mm03 (F)	AGCCTCACTGTTTCAGTTGTGT	(GA)18	55	Radespiel et al., 2001
			Mm03 (r)	GGCAGGAAATGTCATCTGG			
Mm08 [b]	33	130-198	Mm08(F)	CAGTTGGTGAATGGGCTAGG	(TC)18	55	Radespiel et al., 2003
			Mm08(R)	GAGACCATAATGCTGCAAGTAACC			
Mm22 [b]	33	204-240	Mm22 (F)	GATATTTGCAGTGACGTCAAA	(CA)16	58	Hapke et al., 2003
			Mm22 (R)	AACTTCGACCCTTCCCAGTA			
Mm30 [b]	33	213-234	Mm30 (F)	GATGCTGAACCTCTGTCTG	(CA)11	58	Hapke et al., 2003
			Mm30 (R)	GGCATTTGCGCAAGGTCG			
Mm40 [b]	33	142-167	Mm40 (F)	GAGAACAAGGATAGAATGTAAA	(CA)17 (CAAAA)10	55	Hapke et al., 2003
			Mm40 (R)	TTTCCATTAACCTCTTACAACT			

[a] Mitochondrial DNA regions/loci
[b] Nuclear markers (microsatellites)

3. Lemur taxonomy and genetic diversity

3.1. Phylogenetic trees

The geographic distribution and taxonomy of lemurs is still under debate, and this is true for mouse lemurs (Weisrock *et al.,* 2010; Markolf *et al.,* 2011). Moreover, several regions of Madagascar are known to harbour more than one mouse lemur species. As noted above only two individuals from the Daraina region have been sequenced to date and both have been assigned to the *M. tavaratra* species together with individuals from surrounding regions (Weisrock *et al.,* 2010). In order to determine whether our samples belonged to the same clade or species, phylogenetic reconstructions were carried out. Four mtDNA sequences belonging to four mouse lemur species (*M. tavaratra, M. mittermeieri, M. sambiranensis* and *M. simmonsi*) downloaded from GenBank (NCBI) were used as the reference sequences. These four species were specifically selected based on the fact that they are geographically distributed around and close to the Daraina region (Mittermeier *et al.,* 2010; Weisrock *et al.,* 2010). Loci *cyt b* and COII were concatenated for all the sampled individuals and for the GenBank sequences. (see Annex, table 3 for details on GenBank sequences)Thehylogenetic tree was drawn with the aim to find out to which group our samples fit best. Phylogenetic trees per loci were also performed in order to avoid lost of information due to short sequences or missing sites (see Annex – figures 1-3).

According to the results obtained in the first phylogenetic tree constructed, we then performed the same analysis including the d-loop region but only using *M. tavaratra* sequences (1 sequence from Weisrock *et al.* (2010), 1 from Yoder *et al.,* (2000) and 4 from Olivieri *et al.,* (2007). This tree allowed us to detect if a possible population structure was present in our data set. The evolutionary model that best fits the data for each phylogenetic tree was calculated in jModelTest[©] 0.1.1 (Posada, 2005). In this case, the test used 40 candidate models to determine which one had less information lost, according to Akaike Information Criterion (AIC). All model types (equal/unequal base frequencies (+F), with/without proportion of invariable sites (+I) and with/without rate variation among sites (+G)) were included in the calculations. The phylogenetic

trees were then constructed using MEGA$^{©}$ version 5 (Tamura *et al.,* 2011) under the best fitting model using a maximum likelihood (ML) method and 1000 bootstrap replicates. Note that different evolutionary models were used for the different mtDNA regions (table 2).

Table 2: Evolutionary models for phylogeny inference.

These were selected in jModelTest to be used later in the construction of phylogenetic trees in MEGA v.5.						
Locus	Lenght (bp)	Variable sites	No. of Haplotypes	Favoured Model[b]	Mean Bayesian -lnL (full data)	Gamma shape
cyt b [a]	1108	218	18	GTR + G	3011.6	0.2430
COII[a]	529	63	16	TPM + G	1497.5	0.0100
d-loop[a]	393	300	31	GTR + G	3417.3	0.8540
cyt b + COII[a]	1788	303	20	GTR + I + G	4565.38	0.3810
cyt b + COII + d-loop[a]	2308	62	10	TrN + I + G	3586.02	0.0100

[a]GenBank sequences were used for these calculations
[b]Model selected according to the Akaike Information Criterion in the program jModelTest

3.2. Genetic diversity indexes

The aim here was to characterize the population structure using both, mtDNA and microsatellite data. Analysis were performed using the commonly used statistics to study population structure such as F_{ST} statistics, analysis of molecular variance (AMOVA) and clustering methods in STRUCTURE.

3.2.1. mtDNA

Haplotype diversity (Hd), defined as the probability that two randomly chosen haplotypes are different, and nucleotide diversity (π), the average proportion of nucleotide differences between all possible pairs of sequences in the sample (Nei, 1989), were calculated using the mtDNAin the program DnaSP v5.0 (Librado and Rozas, 2009). An exploratory analysis was performed for the

genetic diversity indices. These were calculated (i) independently for the each mtDNA locus (COII, *cyt b* and d-loop) using 33 individuals, (ii) for the concatenated sequences using the same 33 individuals, and (iii) using all the individuals sequenced at the COII and d-loop regions(59 and 68 respectively). This genetic diversity results have shown to not vary with the number of samples used and with the number of sequences. For this reason most of the results and discussions are focused on the 33 individuals and in the largest data set for each mtDNA region (*cyt b* 33 indiv., COII 59 indiv., d-loop 69 indiv.).

The relationships among the haplotypes were also displayed using a median-joining network (Bandelt *et al.,* 1999), constructed in NETWORK[©] v4.6.0.0 (available at www.fluxus-engineering.com). A network was built for each mitochondrial loci.

Genetic diversity for each forest was calculated in Genetix v4.05.2 (Belkhir *et al.,* 1996/1997) and evaluated using the observed and expected heterozygosities (Ho and He) and the common measure F_{IS} which detects if populations depart from the HWE.Pairwise F_{ST} were performed using ARLEQUIN v3.11 (Excoffier, 2006) in order to look for genetic deifferentiation between populations. 10,000 permutations were performed in order to assess the significance of the estimated values.

A hierarchical analysis of molecular variance (AMOVA) was also performed using the conventional F-statistics, where the frequencies of haplotypes are used to test the significance of the variance components associated with various hierarchical levels of genetic structure (Excoffier *et al.,* 1992). In this case, variation among and between populations was determined by means of a non-parametric permutation approach.

3.2.2. Nuclear DNA: microsatellites

Microsatellite data were obtained for the five loci from 33 individuals. Expected and observed heterozygosity (H_e and H_o, respectively), as well as the *inbreeding* coefficient (F_{IS}, measuring

departures from HW proportions) were calculated per locus per populationusing Genetix v4.05.2 (Belkhir *et al.,* 1996/1997). The significance of the deviations from HW equilibrium were determined by running 1,000 permutations per locus. Pairwise F_{ST} and AMOVA analyses (overall, and locus by locus) were run in ARLEQUIN v3.11 with the significance of values being estimated from 10,000 permutations.

Further analysis based on genotypic information was performed using the program STRUCTURE 2.3.3 (2010). This program aims at identifying genetic clusters and requires the user to provide a minimum and a maximum number of genetic clusters (K). Runs were carried out with the "admixture model" and the "correlated allele frequency" model, without any prior information and with an initial alpha value of 1.0. The principle of this program is to group the data into k groups that are at HW and linkage equilibrium. Data are analysed for each given K value using a number of independent replicates (usually twenty) and a likelihood value is estimated under each K. We used as a minimum value K=1 and as maximum K=6, this choice was based on the Evanno *et al..* (2005) study, which suggests to use a value larger than the number of samples: more precisely they suggest to add three to that number but this is admittedly an arbitrary number. In our case, since there were three forest fragments, K=6 seems more than reasonable. Altogether twenty independent runs of 60,000 iterations were performed for each K, with a burn in period of 20,000 generations.

4. Results

Altogether, DNA from all individuals could be easily extracted and amplified, but the sequencing success proved to be different across loci. Out of the 72 individuals 68, 59 and 33 individuals could be sequenced for the d-loop, COII and *cyt b*, respectively, giving an overall success rate of 74% (table 3). All individuals sequenced for the *cyt b* were also sequenced for the other two loci, allowing us to have concatenated data for 33 individuals.

Table 3: Rate of success for mtDNA sequencing of *M. tavaratra* tissue samples.

Population	N (total)	Sequenced *cyt b*	Sequenced *COII*	Sequenced *d-loop*	Average	Sequencing success
BEK	21	11	19	20	17	79%
SOL	26	12	23	26	20	78%
BIN	25	10	17	22	16	65%
total	72	33	59	68	53	74%

4.1. Phylogenetic analysis: the Daraina mouse lemurs belong to the *M. tavaratra* clade

The results of the jModelTest[©] 0.1.1 (Posada, 2005) analysis allowed us to identify the GTR $+\Gamma$ $+I$ (General Time Reversible model plus Gamma distribution and Invariable sites) as the best evolutionary model for our data (i.e. the concatenated sequences of the 2 loci cytb and COII). but information for each evolutionary model can be found in table 2.

When we analysed our sequences together with the previously published *M. tavaratra* sequences and four other mouse lemur species (*M. mittermeieri, M. sambiranensis, M. simmonsi and M. rufus*), we found that the ML trees constructed for the two conserved mtDNA regions, grouped our samples together with the *M. tavaratra* individuals in one well-supported clade (Figure 3). This result was also found when the two genes were analysed independently (Annex Figures 1 & 2). The fact that these mtDNA genes exhibited the same pattern (grouping all M.tavaratra sequences) with high bootstrap values, strongly suggests that all our samples can be assigned to the

M. tavaratra species. Such defined clustering was not found in the d-loop region. In this tree it is possible to find *M. sambiranensis* in the same clade as our samples and the GenBank sequences of *M. tavaratra* and *M. mittermeieri* represented in the two main clades (see Annex – figure 3). This hypervariable region therefore exhibits similar haplotypes across species.
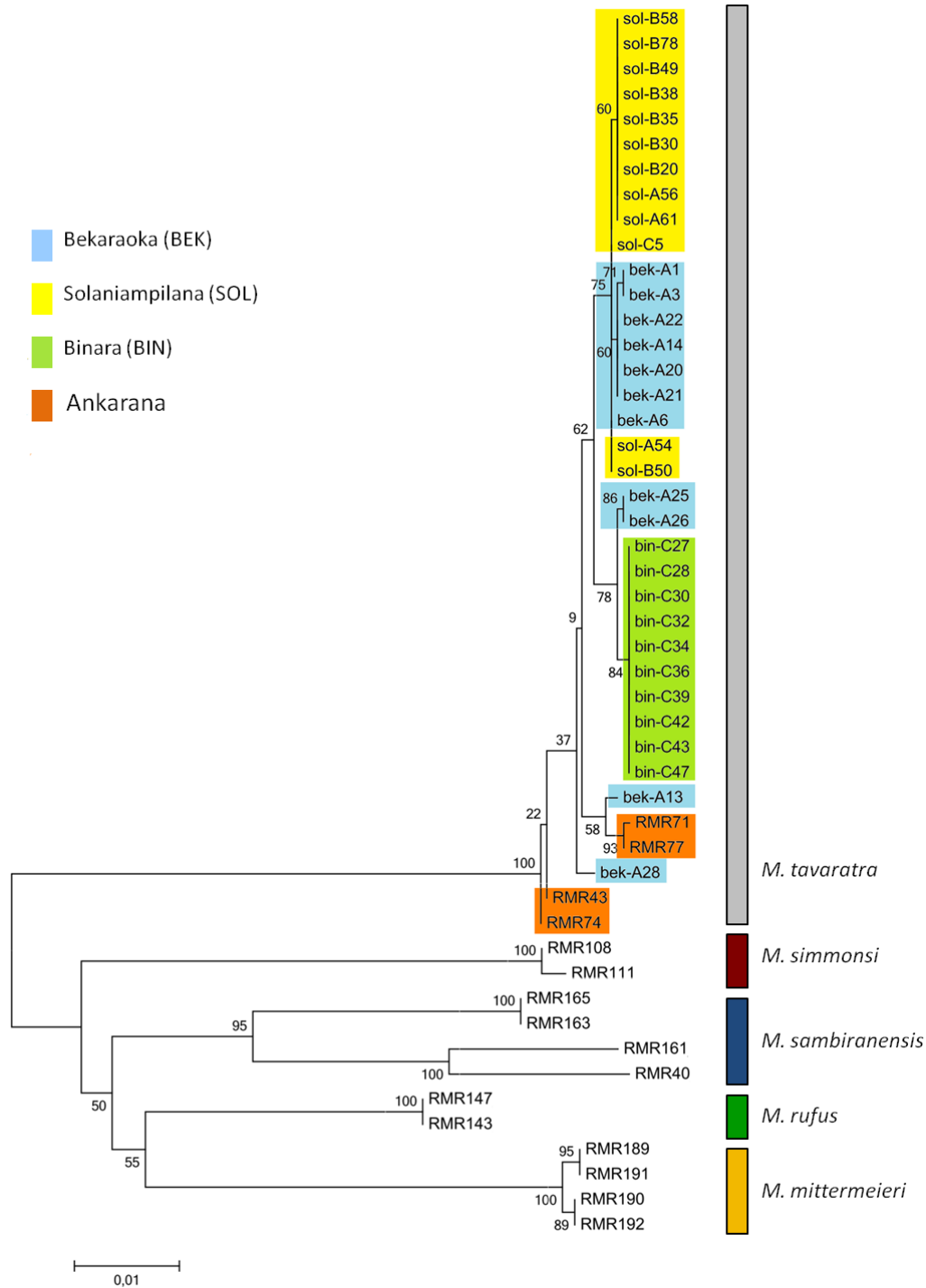
Figure 3: Phylogenetic tree for the mtDNA (*cyt b* and COII) of four *Microcebus* species. In this tree we have the concatenated sequences of our individuals and also of the reference sequences retrieved from GenBank.

Figure 4 represents the phylogenetic tree obtained from the concatenated sequences for 33 individuals for which the three loci were sequenced. This figure shows no simple grouping of the data, with the exception of the Binara samples which are monophyletic, due to the fact that only one haplotype was observed across the three loci (i.e. there was no mtDNA polymorphism in Binara, see below). The Solaniampilana samples exhibit some clustering but this is also probably due to a lower level of genetic diversity since several individuals from Solaniampilana also grouped with individuals from Bekaraoka. This last forest fragment exhibited the most divergent haplotypes, and had individuals in all the "sub-clades" of the tree with the exception of the sub-clade comprising the Binara individuals.

4.2. Mitochondrial DNA diversity and differentiation within *M. tavaratra*

Sequence comparisons of 1,110 bp of mtDNA *cyt b* gene revealed 16 polymorphic sites for a total of 33 individuals from the 3 forests, of which 9 sites were parsimony informative. These polymorphisms defined 7 distinct haplotypes with a haplotype diversity of Hd=0.805 ($\pm$ 0.036) and nucleotide diversity of π=0.0031 ($\pm$ 0.0004). Since the sample size for the cytb gene was smaller than that of the other two mtDNA loci (59 and 68 individuals for the COII gene and the d-loop, respectively) one could argue that the lower cytb diversity is likely to be underestimated. We therefore compared the results obtained for the same 33 individuals to the results obtained with the larger sample sizes for the other two mtDNA regions and found that they did not significantly differ from the smaller data set (see Annex − table 4). This suggests that the diversity calculated for the cytb is probably not strongly underestimated.

Six haplotypes were identified for the COII gene (684 bp), with 9 sites being polymorphic (8 were parsimony informative) and Hd=0.645 ($\pm$ 0.042) and π=0.004 ($\pm$ 0.0002). The control region (d-loop, 514 bp) showed high haplotype diversity across all populations (Hd=0.805 $\pm$ 0.001) and a high nucleotide

diversity (π=0.021 ± 0.001). Polymorphism was found for 43 sites, with 36 parsimony informative sites, and these mutations resulted in 13 distinct haplotypes.

As presented in table 4, the three populations showed variable levels of genetic diversity with Binara and Solaniampilana exhibiting either no diversity or very low nucleotide diversity for the mtDNA regions amplified, whereas Bekaraoka exhibited much higher levels, at both the haplotype and nucleotide diversity level. There were clear differences across loci. Whereas Bekaraoka population revealed high haplotype diversity for all loci, Solaniampilana had intermediate diversity for the d-loop and *cyt b* regions, but a lower value for the COII gene. However, the most striking result was the lack of diversity found for the Binara fragment. All the individuals (N=22) showed the same haplotype for all mtDNA region. When the same analyses were performed for the concatenated sequences, a similar pattern was observed for each population (table 4).

Table 4: mtDNA haplotypes and nucleotide diversity. Genetic diversity was calculated for the largest sample size of each forest fragment, for each mtDNA locus. A set of 33 individuals with concatenated sequences for the three loci was also analysed.

| | N = 33 sequenced individuals | | | | | N = 59 sequenced individuals | | | | | N = 68 sequenced individuals | | | | | N = 33 sequenced individuals | | | | |
| | Cytochrome b (1110 bp) | | | | | Cytochrome oxidase II (684 bp) | | | | | D-loop (514 bp) | | | | | concatenated mtDNA (2308 bp) | | | | |
Population	N	h	S	Hd (SD)	$\pi \pm SD$	N	h	S	Hd ± SD	$\pi \pm SD$	N	h	S	Hd ± SD	$\pi \pm SD$	N	h	S	Hd ± SD	$\pi \pm SD$
Bekaraoka	11	5	14	0.709 (0.137)	0.003 (0.002)	19	5	8	0.790 (0.057)	0.0045 (0.0027)	20	9	40	0.858 (0.054)	0.021 (0.011)	11	7	59	0.873 (0.089)	0.0075 (0.004)
Solaniampilana	12	2	1	0.409 (0.133)	0.0004 (0.0004)	23	2	1	0.087 (0.078)	0.0001 (0.0003)	26	5	12	0.508 (0.108)	0.007 (0.004)	12	3	10	0.439 (0.158)	0.0017 (0.001)
Binara	10	1	0	0.000 (0.000)	0.000 (0.000)	17	1	0	0.000 (0.000)	0.000 (0.000)	22	1	0	0.000 (0.000)	0.000 (0.000)	10	1	0	0.000 (0.000)	0.000 (0.000)
All populations	33	7	16	0.805 (0.036)	0.0031 (0.0004)	59	6	9	0.645 (0.042)	0.0036 (0.0002)	68	13	43	0.805 (0.032)	0.0214 (0.001)	33	10	62	0.828 (0.041)	0.007 (0.0006)

Genetic differentiation between the forest fragments showed that all pairwise F_{ST} values were significant at the locus level and for the concatenated sequences (table 5). Overall, the most distinct population was Binara showing F_{ST} values ≥0.516 as expected for a sample having one fixed haplotype.

Table 5: Genetic distances among study populations. Population pairwise F_{ST} are represented below the diagonal line and they are highly significant as p-values (above the diagonal line) are exactly equal to zero.

	cyt b			COII			d-loop			3 loci concatenated		
	BEK	SOL	BIN	BEK	SOL	BIN	BEK	SOL	BIN	BEK	SOL	BIN
BEK		0.000	0.000		0.000	0.000		0.000	0.000		0.000	0.000
SOL	0.432		0.000	0.345		0.000	0.321		0.000	0.339		0.000
BIN	0.632	0.779		0.516	0.950		0.562	0.730		0.549	0.763	

The hierarchical AMOVA based on haplotype frequencies revealed that for the two conserved mtDNA genes (*cyt b* and COII), 61 to 65% of the total variation was within populations and 35 to 39% was among populations (table 6). When this analysis was performed for the d-loop hypervariable region and for the concatenated sequences, the difference between the variations within and among populations were less evident indicating, nevertheless, higher levels of genetic differentiation within populations (45% within populations and 54% among populations).

Table 6: AMOVA results of *Microcebus* species from three forest fragments in Daraina.

Source of variation	d.f.	Sum of squares	Variance components	% of variation	Fixation Index (FST)	p-value[a]
cyt b - 33 indiv. (all locations)						
Among pops	2	7.083	0.30525	61.24	0.612	0.000
Within pops	30	5.795	0.19318	38.76		
COII - 59 indiv. (all locations)						
Among pops	2	10.633	0.26515	64.81	0.648	0.000
Within pops	56	8.062	0.14396	35.19		
d-loop - 68 indiv. (all locations)						
Among pops	2	12.474	0.26695	54.48	0.545	0.000
Within pops	65	14.496	0.22302	45.52		
concat. - 33 indiv. (all locations)						
Among pops	2	6.462	0.27394	54.79	0.548	0.000
Within pops	30	6.780	0.22601	45.21		

[a]Probability of finding a more extreme variance component and F_{ST} index than observed by chance alone after 1000 permutations.

Haplotype networks were drawn for each locus and for the concatenated data (Figure 4). Binara only exhibits one haplotype for each mtDNA region, and this haplotype is shared only with individuals from Bekaraoka population for COII and d-loop regions. The Solaniampilana and Bekaraoka populations always share one haplotype for all the mtDNA regions analysed. As individuals from Bekaraoka were found to have less genetic differences with the other populations, the locations of the traps where the individuals were captured were placed on a map from Daraina (figure 2).

4.3. Microsatellite diversity within *M. tavaratra*

All of the five nuclear loci were amplified for 33 individuals, most of them the same for which the 3 mtDNA loci had been amplified. All loci analysed were polymorphic with a minimum of 3 and a maximum of 11 alleles (table 3). Overall, none of the F_{IS} values were significantly different from zero, so the three populations showed no departure from HWE, despite the fact that the loci were originally developed for another mouse lemur species (*M. murinus*). Hence, all the data was kept in the analysis.

Interestingly, the microsatellite results differed strongly from those obtained with the mtDNA. The population that had previously shown no variation (Binara) was variable for the 5 nuclear loci, exhibiting the highest number of alleles per locus (table 7).

Table 7: Nuclear DNA diversity calculated for each populations when 5 microsatellite loci were analysed.

Locus	Mean No. Alleles	H_O	H_E	F_{IS}
BEK	5.8	0.739	0.727	0.016
SOL	6.8	0.816	0.782	0.043
BIN	7.8	0.796	0.746	0.661

*None of the p-values for the F_{IS} are statistically significance.

The AMOVA showed that around 94% of the variation corresponds to within populations' diversity, hence showing that the populations are very little differentiated from each other at the microsatellite level, as opposed to the result obtained with the mtDNA loci, where higher variation was found within populations.

Table 8: AMOVA results for the microsatellite data obtained in this study.

Source of variation	Sum of squares	Variance components	% of variation	Fixation Index (FST)	p-value[a]
Among pops	9.258	0.121	5.84	0.058	0.000
Within pops	123.62	1.958	94.16		

[a]Probability of finding a more extreme variance component and F_{ST} index than observed by chance alone after 1000 permutations.

Although the AMOVA had shown no variation among populations, a population structure analysis was performed in STRUCTURE 2.3.3 (2010) using different K values (number of possible populations) in order to determine if any differentiations did exist. The results revealed by the Structure program support the low differentiation among populations, classifying all individuals as belonging to the same population, whichever the total number of clusters used (figure 6).
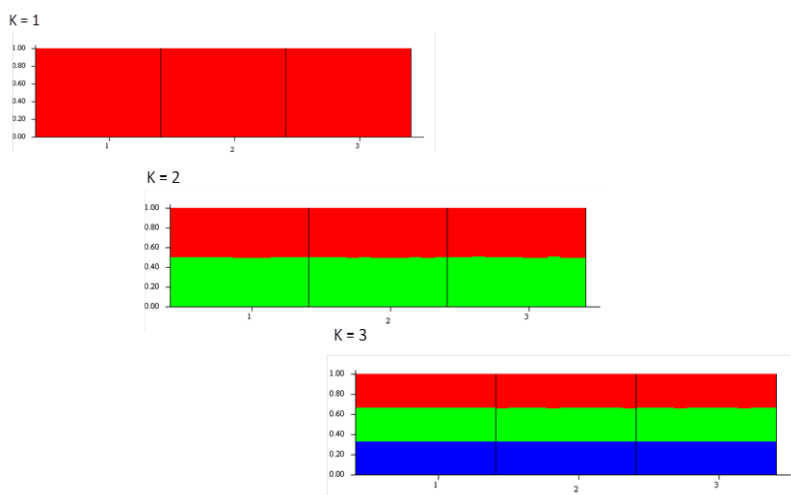
Figure 4: Output from Structure program when k=1, 2 and 3. In the analysis performed in structure, the program did not detect any differentiation between the three population.

5. Discussion

5.1. Samples and laboratory techniques

Testing several extraction protocols, as well as optimizing DNA amplification and sequencing, can be a time demanding task but it is extremely useful for improving results' quality and to ease on future work which use similar methods. Given that I was involved in the project around several lemur species for which hundreds of samples were collected, a crucial issue was to develop efficient methods that would also be cheap. This was the case for the faecal samples of sifakas (more than 1500 faecal samples have been collected for *P. coronatus* and *P. coquereli*) but also for the mouse lemurs for which more than 400 individuals are expected to be collected for the long-term project. Out of all three methods tested (Annex – table 1), the mammalian DNA isolation protocol adapted from Laird *et al.,*(1991) protocol was considered the best for several reasons. It yielded good DNA, used few organic solvents hence reducing health hazard and associated pollution, offered the lowest budget and it could be performed in one single day, after over-night digestion.

This study aimed to use a data set of 20 to 25 individuals per forest fragment, but this was not always the case. The lowest number of samples per fragment for which genetic analysis was performed was ten. While this number is not very high I should stress that for endangered species, the number of individuals genotyped can typically be smaller depending on the ease of access and species. For the longer term I expect to genotype more individuals but for this Masters project this is still a reasonable number of samples in comparison to several previous lemur phylogenetic and population genetics studies (For instance, Schneider *et al.,* (2010) used a large sample of 195 *M. murinus* individuals from 15 locations but only analysed one mtDNA locus and no microsatellite data). In a recent study Markolf *et al.,* (2011) tried to estimate the minimum number of individuals that should be analysed per sampled location to determine whether genetically differentiated populations can be identified as putative species. Their work focused on mouse lemurs from the southwest of Madagascar and used a simple permutation approach using genetic data from the d-

loop mtDNA region. They suggested that ten individuals per location was the minimum required number of individuals sampled per location in order to avoid false positives. In our case this did not seem to be problematic since the only monophyletic group associated to a forest fragment (Binara) had a minimum of 10 individuals sequenced, and in fact it suggests that the number of individuals sequenced here was reasonably high. This was also confirmed by the analysis of larger samples for the different mtDNA loci. Overall, the sequencing success rate for our samples was 74% and our results suggest that the larger the locus to be amplified, the lower the sequencing rate. Because we used good quality DNA (tissue, as opposed to faecal material), only samples which provided bad sequences were repeated two or three times. For the nuclear DNA (microsatellites), most genotypes could be read on the first amplification and only one repetition was performed for those individuals for which the genotype was potentially problematic (no alleles, or possible missing allele).

5.2. Only one *Microcebus* species appears to be present in Daraina

Species delimitation is a complex but crucial issue for understanding the factors driving the diversification of biota (Wilmé *et al.,* 2006) and for defining regions of conservation priority, defined as hotspots (Markolf *et al.,* 2011). In this study, one of the main aims was to determine if all the individuals sampled in the three forest fragments of Daraina belonged to the same species and if these could be assigned to a previously described *Microcebus* species recorded in the same area (*M. tavaratra*) by Weisrock *et al.,* (2010). Our results did not show high genetic divergence among lineages and all our samples grouped with the *M. tavaratra* reference sequences in one monophyletic clade with very high statistical support. We can thus confidently suggest that all our samples belong to the same species. Another result was that the tree depth was much less important for *M. tavaratra* than for some of the other mouse lemur species, hence suggesting that perhaps only part of the species haplotypic diversity has been uncovered for some species, and that more individuals should be analysed in the future to confirm monohyly. Indeed, as discussed above, the identification of mouse lemurs'

species should use several individuals per location to validate monophyly of putative species sampled across sometimes wide areas. Markolf *et al.,* 2011 suggest using at least ten individuals per forest fragment. While it should be clear that this number is, to a large extent, arbitrary and should not be taken at face value, it is worth mentioning that in most previously published genetic studies less than five individuals are usually used to "define species". In Daraina, Weisrock *et al. (*2010) only used two individuals from two forest fragments to define the specimens' species.

We did, however, find that some species did not appear as monophyletic when a phylogenetic tree was constructed for the most variable region of the mtDNA amplified in this study (d-loop locus). Actually, two species showed individuals belonging to the two main clades, namely *M. tavaratra* and *M. mittermeieri* (figure 3 in Annex). The fact that these two species have individuals belonging to different clades is not necessarily surprising as the d-loop sequences exhibited high nucleotide diversity with 300 polymorphic sites out of 389bp sequence length (table 2).

This amount of polymorphic sites across the five species, suggests that homoplasy is very high and therefore that this locus cannot be used to estimate species divergence, for this group of species and is better adapted to monitor within species diversity. The fact that *M. mittermeieri* and *M. tavaratra* had sequences in the two most divergent clades suggests that these two species may have larger effective population sizes than the other species. Another possibility is that it might be related to the geographical distribution of the individuals sampled.

In this study we chose to use phylogenetic trees as these are a useful tool for establishing genealogical relationships among a group of organisms and therefore species identification (Clement *et al.,* 2000), but it would be interesting to perform the same analyses with a program that constructs phylogenetic networks, as more information might be obtained (i.e. hybridization, horizontal gene transfer) (Huson and Scornavacca, 2010).

5.3. Genetic diversity

Genetic indices can provide valuable information on the recent evolutionary history of species and on possible changes of their environment and habitat (Goossens *et al.,* 2006; Fouquet *et al.,* 2007; Rezende *et al.,* 2009). Evolutionary changes, including past or recent expansions or contractions due to human or natural effects, selection events, together with other species characteristics such as the social structure, can all affect patterns of genetic diversity (Gaggioti *et al.,* 2009). Recent years have seen an increase in the number of studies trying to infer populations or environmental changes from the analysis of several genetic markers (Avise *et al.,* 2000). This study is thus important as it provides the first within species diversity data for *M. tavaratra*.

5.3.1. Mitochondrial DNA variation in *M. tavaratra*

One of the most surprising result obtained in this study was the lack of genetic diversity in the Binara forest across all mtDNA loci despite the fact that the other fragments (Bekaraoka and Solaniampilana), appeared to maintain a high level of haplotype diversity. While this seemed very surprising at first we also found that not much variation was found at the nucleotide level for the Solaniampilana fragment, even for the most variable region (d-loop: $\pi=0.007 \pm 0.004$). Different species and social units, as well as sampling areas pose some difficulties for scaling genetic diversity across studies, but the values of genetic diversity obtained in this study do not disagree much with previous studies (i.e. Matocp and Villablanca, 2001; Radespiel *et al.,* 2008), with the exception of Binara.

When we compare the three fragments it is unclear why the levels of genetic diversity would be very different between these. For instance, the vegetation in Bekaraoka and Solaniampilana is classified as dry deciduous whereas Binara is an evegreen forest. We should mention here that originally different habitats were thought to play an important role in the distribution of *Microcebus* species across Madagascar, causing a division between eastern wet-adapted and western dry-adapted mouse lemurs. However, Yoder *et al.,* (2000) have demonstrated that a

primary division into northern and southern clades is more likely to be correct. Indeed, ecological plasticity within clades seems to be typical, even at the intraspecific level (Yoder *et al.,* 2000). Thus, the probability that the habitat ecology is the cause for the observed genetic differentiation in daraina seems unlikely

Similarly, the sizes of the forest fragments do not appear to be correlated to the amount of diversity since Binara is much larger than Solaniampilana (45.3 versus 12.8 km2). One could imagine that the Binara individuals were all genetically related through matrilines but the geographic distribution of the sampled individuals suggests that it is unlikely. Indeed, when the trapping location for each individual was displayed on a map from the Daraina region (figure 2) we observed that this fragment was the one with the most disperse points. The protection status of the forests is also not clearly related to the amount of diversity since both Binara and Bekaraoka are within the S.F.U.M. and Solaniampilana is not.

Altogether, this suggests that the lack of variation in Binara may be due to some sampling bias, which we cannot identify at this stage, and might be related to ecological and behavioural characteristics that would need to be investigated (particularly regarding the behaviour and reproductive success of females). In fact, we noticed that the number of females sampled in Binara was particularly high (84% vs 16%), and for Solaniampilana (64% vs 36%), but for Bekaraoka was higher for males (33% vs 67%). Though, it is unlikely that this alone would explain the results fully.

Interestingly a similar result was found by Guschanski *et al.,* (2007) study on *M. ravelobensis,* where 3 out of the 9 populations sampled had no haplotype diversity. In two cases the sample sizes were small but in one it was comparable to ours (N = 15). The authors suggested that fragment size and geographical isolation were two factors that could explain the lack of genetic diversity for those populations, but as noted above the first factor does not seem reasonable neither does isolation as natural corridors connect Binara to the Antsahabe neighbouring fragment. At this stage, our results suggest that more fragments need to be sampled and Binara further investigated.

When pairwise F_{ST} were calculated for the mtDNA data, the results were congruent with the diversity values obtained before, i.e. high F_{ST} values between Binara and the other two forests. However, these could not support the hypothesis proposed earlier, that forest fragments on opposite banksides of rivers are expected to show higher differentiation than population on the same river side. Many studies have shown that rivers can be a strong barrier for gene flow even for taxa with different biological and/or ecological characteristics (Goossens *et al.,* 2005; Jalil *et al.,* 2008; Eriksson *et al.,* 2004; Louis *et al.,* 2006[b]). This has been repeatedly shown in Madagascar where major rivers are often the limit between species or subspecies of lemurs (Wilmé *et al.,* 2006; Mittermeier *et al.,* 2008). In a recent study carried out by Quéméré *et al.,* in 2010[b] in the Daraina region, they demonstrated that the Manankola river was the main landscape feature generating genetic differentiation between populations of golden crowned sifaka and therefore the main barrier to gene flow in *P. tattersalli*. Given that sifakas are much more mobile species with bigger home ranges and a greater ability to cross open areas than mouse lemurs, we expected that the Bekaraoka samples might be particularly differentiated from the other two populations, but our results do not appear to support this (table 6). In fact, Bekaraoka appears to share haplotypes with both Solaniampilana and Binara populations (figure 5).
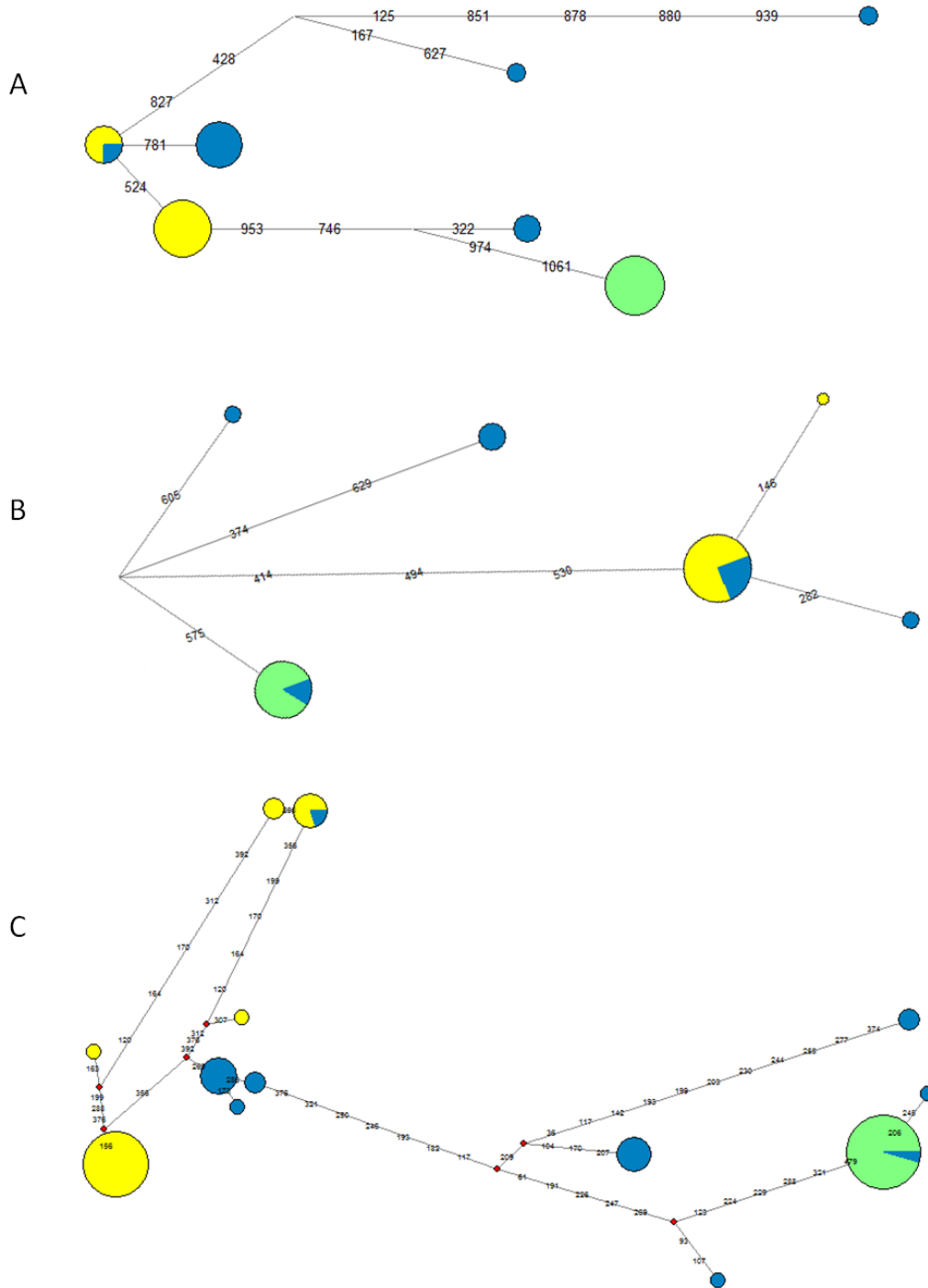
Figure 5: Haplotype networks for each mtDNA loci.

The figure above shows the haplotype network produced for the three loci. Each circle represents one haplotypes and the colours represent the populations. A) *cyt b* network shows that only one haplotypes for Binara; B) in COII network, Bekaraoka has individuals showing all haplotypes with exception of one from Solaniampilana; C) the d-loop haplotype network is the most complex among all as this is it a hypervariable region of the mtDNA.

Although I should stress again that we only had data from three forests, an absence of rivers acting as barriers for gene flow have been observed in other cases such as spider monkeys (Collins and Dubach, 2000), long-tailed macaques (de Ruiter and Geffen 1998), and a number of lemur species (Pastorini *et al.,* 2003; Craul *et al.,* 2008) (in Vigilant and Guschanski, 2009).

5.3.2. Microsatellite markers

The microsatellite data analysed herein provided one important result, which was to show that the Binara samples actually exhibited genetic diversity. This was important as the lack of mtDNA diversity first led us to think that there was a technical issue such as contaminations or geographically more concentrated sampling. As noted above, the sampling of the mouse lemurs from Binara was probably the most geographically widespread and the microsatellite data confirm this as it appeared to be the genetically most variable forest. Several cautionary remarks must be made, though. The markers used were developed for another species (*M. murinus*) and it is expected that markers developed in one species will create downward biases in the estimation of diversity when used in another species (Ellegren, 1997; Chikhi *et al.*, 2008). Second, only five markers were used here which, is typically considered to be a small number to study genetic differentiation and diversity (but see Sousa *et al.,* in press, for the analysis of fish data sets with five microsatellite loci and sophisticated inferential methods). The lack of microsatellite primers designed for specific mouse lemur species is of course not unique to *M. tavaratra* and several published studies have done this before, making comparison across studies possible (i.e. sea snake-*Hydrophiinae* sub-family species, Lukoschek and Avise,2011; Sea otter-*Mustellidae* family Kretschmer *et al.,* 2009). Indeed, these markers have also been amplified in other *Microcebus* species (e.g. *M. rufus, M. ravelobensis*). Altogether, the genetic diversity values obtained for *M. tavaratra* are on the same order than those observed for the same microsatellites used in previous studies (Radespiel *et al.,* 2001; Hapke *et al.,* 2003) (Table 9).

Table 9: Genetic diversity of each locus across *Microcebus* species.

Locus	This study (*M. tavaratra*)				Radespiel et a., 2001 (*M. murinus*)				Hapke et al., 2003 (*M. griseorufus*)			
	No. Indiv.	No. Alleles	H_O	H_E	No. Indiv.	No. Alleles	H_O	H_E	No. Indiv.	No. Alleles	H_O	H_E
Mm03	11	10	0.82	0.88	173	15	0.85	0.84	-	-	-	-
Mm08	11	16	0.36	0.57	172	29	0.93	0.92	-	-	-	-
Mm22	11	16	0.91	0.80	-	-	-	-	16	11	0.857	0.750
Mm30	11	5	0.73	0.82	-	-	-	-	15	3	0.297	0.333
Mm40	11	9	0.91	0.90	-	-	-	-	16	8	0.766	0.688

5.4. Further research and conservation implications

To our knowledge, this is the first study to perform genetic analysis on larger numbers of individuals from the *Microcebus* species present in this area of the north of Madagascar. In addition to the genetic data, morphometric information was also collected and will be analysed and compared with the results obtained in this study in the future.

Altogether, this study suggests that the level of mtDNA diversity is relatively high in Bekaraoka and lower in Solaniampilana and Binara. Nevertheless, a strong genetic differentiation was detected among the three populations despite a rather small distribution area. Further sampling could be performed in Daraina in order to verify if the genetic patterns detected in this study are maintained for larger and more disperse samplings. Multi-species genetic studies, morphometric information and spatial distribution could be combined to draw inferences about population size and subdivision, gene flow and hybridization, and past demographic changes (Guschanski *et al.*, 2007).

Nevertheless, from the information gathered in this study, one could suggest that the genetic diversity detected in these populations can be considered a positive outcome for the conservation of this species. At the same time neighbouring fragments might show unique diversity, a feature that can have implications for species divergence. Such estimates of genetic diversity, population structure and connectivity obtained in studies like this one, are crucial for the effective

conservation of many species in order to develop effective conservation priorities (Quéméré *et al.,* 2009). More and more collaborations are taking place between researchers and conservation managers as both are interested in ecological aspects of species, such as population isolation, and in establishing connectivity between populations to provide gene flow and thus increase the genetic diversity and potential viability of threatened and small populations (Vigilant and Guschanski, 2009).

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ANNEX

Fig 1: Phylogenetic tree for the *cyt b* region of *Microcebus* spp mtDNA

M tava cytb Ankar1
M tava cytb Ankar2
bin-C47
bin-C43
bin-C42
bin-C39
bin-C36
bin-C34
bin-C32
bin-C30
bin-C28
bin-C27
sol-C5
sol-B78
sol-B58
sol-B50
sol-B49
sol-B38
sol-B35
sol-B30
sol-B20
sol-A61
sol-A56
sol-A54
bek-A22
bek-A21
bek-A20
bek-A14
bek-A6
bek-A1
bek-A3
M tava cytb Ankar3
bek-A28
bek-A25
bek-A26
M tava cytb Ana1
M tava cytb Ankav2
bek-A13
M tava cytb Ankav1
M mittermeieri cob Marojejy1
M mittermeieri cob Marojejy3
M mittermeieri cob Marojejy2
M mittermeieri cob Marojejy4
M simmonsi cob Tampolo1
M simmonsi cob Tampolo2
M. rufus   cob Andramb1
M. rufus   cob Andramb2
M sambi cob Ambanja1
M sambi cob Ambanja2
M sambi cob Ambanja3
M sambi cob Manongarivo1
M sambi cob Manongarivo2

64
99
56
64
78
100
19
45
99
100
100
87
100

0.01

Fig 2: Phylogenetic tree for the COII region of *Microcebus* spp mtDNA
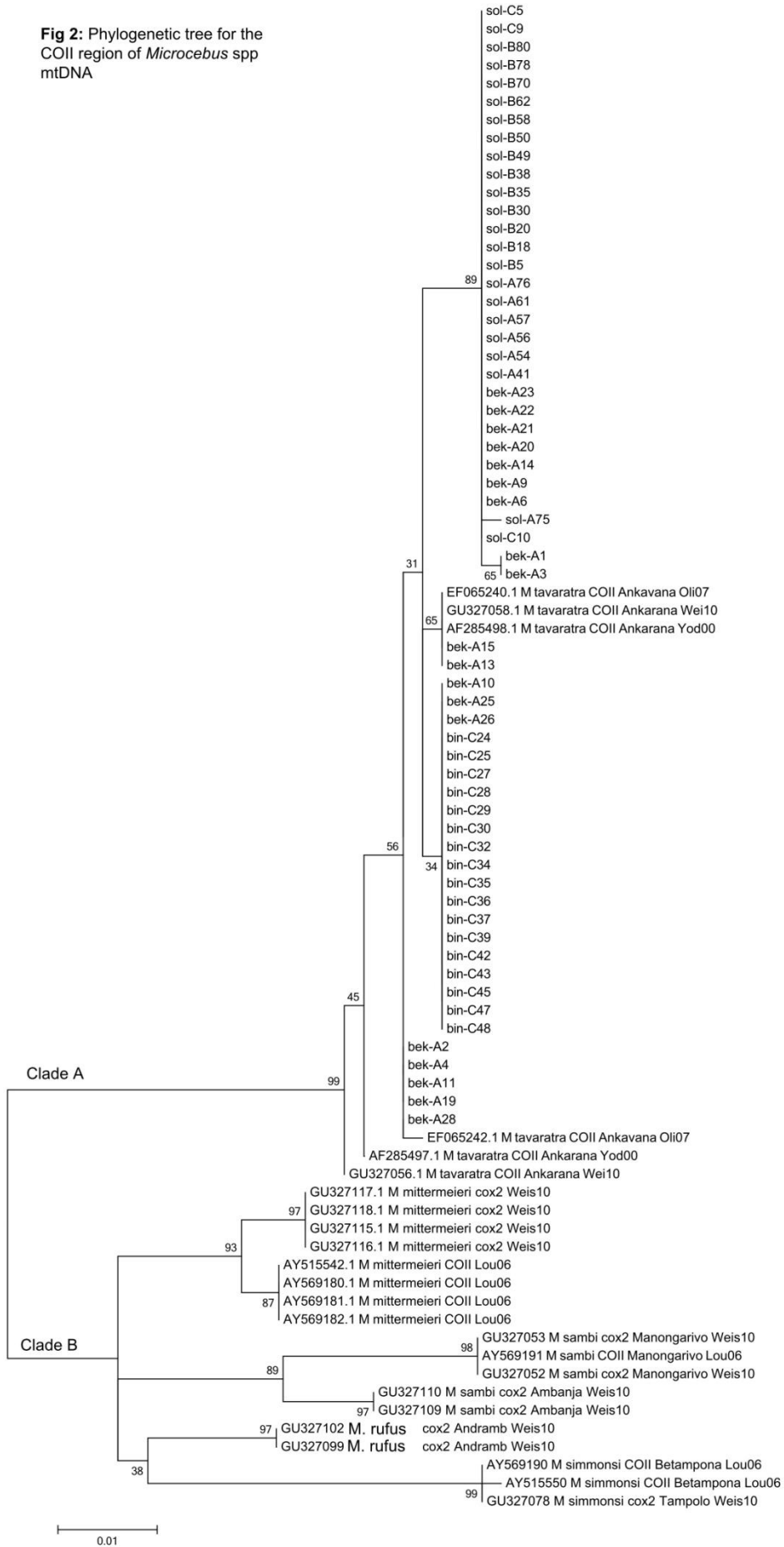
Fig 3: Phylogenetic tree for the
d-loop region of *Microcebus* spp
mtDNA

sol-C10
sol-C16
sol-C9
sol-B80
sol-B78
sol-B70
sol-B62
sol-B58
89 sol-B49
sol-B38
sol-B35
sol-B30
sol-B20
sol-B18
sol-B5
40 sol-A61
sol-A56
sol-A57
62 bek-A1
bek-A3
bek-A8
bek-A9
8 bek-A14
55 bek-A20
bek-A21
bek-A22
bek-A23
sol-A75
48 sol-A31
sol-B50
45 bek-A6
sol-A48
92 sol-A54
sol-A76
17 sol-C5
sol-A41
63 EU810369.1 M mitt D-loop Antsa Rade08
21 EU810366.1 M mitt D-loop Ampari Rade08
99 EU810368.1 M mitt D-loop Antsa Rade08
EU810367.1 M mitt D-loop Anjia Rade08
99 EF065222 M sambira D-loop Mahilaka Oli07
EF065238 M sambira D-loop Ambongo Oli07
99 AF285478 M sambira D-loop Manongarivo Yod00
73 EF065225 M sambira D-loop Mahilaka Oli07
33 89 AF285477 M sambira D-loop Manongarivo Yod00
bek-A19
94 bek-A28
bek-A11
2 bek-A2
29 bek-A4
AF285456.1 M tava D-loop Ankarana Yod00
EF065217.1 M tava D-loop Analabe Oli07
bek-A17
bin-C48
bek-A25
15 bin-C47
bek-A26
bin-C24
bin-C25
49 bin-C26
bin-C27
bin-C28
bin-C29
bin-C30
bin-C32
90 bin-C33
bin-C34
bin-C35
bin-C36
bin-C37
bin-C39
bin-C40
bin-C41
bin-C42
bin-C43
bin-C44
bin-C45
EF065218.1 M tava D-loop Ankavana Oli07
92 EF065216.1 M tava D-loop Ankavana Oli07
bek-A13
91 bek-A15
DQ534959.1 M tava D-loop Ankarana Andr06
DQ534965.1 M tava D-loop Analamera Lou08
99 89 DQ535014.1 M mami D-loop Lokobe Andr06
DQ535013.1 M mami D-loop Lokobe Andr06
71 DQ535015.1 M mami D-loop Lokobe Andr06
88 AY254491.1 M mitt D-loop Anjana Andr06
91 EF175302.1 M mitt D-loop Anjana Andr06
68 EF175289 M simmonsi D-loop Betampona Lou08
EF175294 M simmonsi D-loop Tampolo Lou08
94 EF175297 M simmonsi D-loop Tampolo Lou08
58
91 EF175292 M simmonsi D-loop Tampolo Lou08

0,5

44

Table 1: Information on the 72 samples used in this study.

x: sequencing and/or genotyping performed / - : no sequencing, no genotyping nor sexing performed / F: female / M: male

Forest	Sex (F/M)	Indiv. ID	trap ID (1st capture)	GPS South/North	GPS East/West	[DNA] ng/µl	Cyt-b	COII	D-loop	Genotyping	sexing pcr_1	sexing pcr_2	sexing pcr_3
Bekaraoka	F	bek-A1	J12	-13,1732405	49,7031348	34,25	x	x	x	x	F	F	F
Bekaraoka	M	bek-A2	A11	-13,1704576	49,7028922	21,40	-	x	x	-	M	-	-
Bekaraoka	M	bek-A3	C35	-13,1746721	49,7078386	47,60	x	x	x	x	M	-	-
Bekaraoka	F	bek-A4	F17	-13,159830	49,70462	22,10	-	x	x	-	M	-	-
Bekaraoka	M	bek-A6	F31	-13,159800	49,70724	24,80	x	x	x	x	-	-	-
Bekaraoka	M	bek-A8	D47	-13,1635951	49,7102932	27,80	-	-	x	-	M	-	-
Bekaraoka	M	bek-A9	J23	-13,168586	49,7003726	28,90	-	x	x	-	-	-	-
Bekaraoka	M	bek-A10	B46	-13,1727642	49,709958	32,00	-	x	-	-	-	-	-
Bekaraoka	M	bek-A11	A48	-13,1694483	49,7099222	10,50	-	x	x	-	M	-	-
Bekaraoka	F	bek-A13	D17	-13,1635381	49,7045234	33,10	x	x	x	x	-	-	-
Bekaraoka	M	bek-A14	D11	-13,1635407	49,7034574	20,30	x	x	x	x	-	-	-
Bekaraoka	M	bek-A15	D19	-13,1635457	49,7049016	17,10	-	x	x	-	M	M	M
Bekaraoka	F	bek-A17	B37	-13,1728688	49,7080599	25,20	-	-	x	-	F	-	-
Bekaraoka	F	bek-A19	F11	-13,159770	49,70356	178,20	-	x	x	-	F	F	F
Bekaraoka	M	bek-A20	D11	-13,1635407	49,7034574	48,00	x	x	x	x	M	-	-
Bekaraoka	F	bek-A21	D14	-13,1635343	49,7039821	96,30	x	x	x	x	F	-	-
Bekaraoka	M	bek-A22	D10	-13,1635526	49,7032645	28,17	x	x	x	x	M	-	-
Bekaraoka	F	bek-A23	D44	-13,1635547	49,7097836	18,20	-	x	x	-	F	-	-
Bekaraoka	M	bek-A25	A173	-13,1667237	49,7023731	28,93	x	x	x	x	-	-	-
Bekaraoka	M	bek-A26	A182	-13,1669008	49,7032412	20,23	x	x	x	x	-	-	-
Bekaraoka	M	bek-A28	E23	-13,1616512	49,7057354	9,90	x	x	x	x	M	-	-
Solaniampilana	F	sol-A31	A21	-13,0904707	49,5780733	17,00	-	-	x	-	F	-	-
Solaniampilana	F	sol-A41	C32	-13,0864267	49,5766511	4,40	-	x	x	-	F	F	F
Solaniampilana	M	sol-A48	F62	-13,0935987	49,5852463	286,38	-	-	x	-	M	M	M
Solaniampilana	F	sol-A54	B2	-13,0885412	49,5821374	32,52	x	x	x	x	-	-	-
Solaniampilana	M	sol-A56	B17	-13,088527	49,5794077	12,07	x	x	x	x	-	-	-
Solaniampilana	F	sol-A57	B25	-13,0885483	49,5779306	42,50	-	x	x	-	F	-	-
Solaniampilana	F	sol-A61	A7	-13,0904286	49,5811647	35,79	x	x	x	-	F	-	-
Solaniampilana	M	sol-A75	A350	-13,0945818	49,5827611	70,60	-	x	x	-	M	-	-
Solaniampilana	M	sol-A76	A328	-13,0921161	49,5829521	29,36	-	x	x	-	-	-	-
Solaniampilana	M	sol-B5	D26	-13,0811278	49,5780628	29,36	-	x	x	-	-	-	-
Solaniampilana	M	sol-B18	E22	-13,0791261	49,5786474	77,10	-	x	x	-	M	-	-
Solaniampilana	F	sol-B20	E27	-13,0791142	49,577714	22,20	-	x	x	x	M	-	-
Solaniampilana	F	sol-B30	F16	-13,0770213	49,5798725	12,40	x	x	x	x	F	F	F
Solaniampilana	M	sol-B35	F27	-13,0770237	49,5778653	61,70	x	x	x	x	F	F	?
Solaniampilana	M	sol-B38	F35	-13,0770731	49,5764099	14,50	x	x	x	x	M	-	-
Solaniampilana	F	sol-B49	B21	-13,0885482	49,5786636	19,86	x	x	x	x	M	-	-
Solaniampilana	F	sol-B50	B31	-13,0885322	49,5768253	20,59	x	x	x	x	-	-	-
Solaniampilana	F	sol-B58	A23	-13,0904855	49,5777759	12,10	x	x	x	x	F	-	-
Solaniampilana	F	sol-B62	E17	-13,0791212	49,5795312	27,90	x	x	x	-	F	-	-
Solaniampilana	F	sol-B70	C2	-13,0864786	49,5820878	27,63	-	x	x	-	F	-	-
Solaniampilana	M	sol-B78	D20	-13,0811425	49,5793298	10,13	-	x	x	x	F	-	-
Solaniampilana	F	sol-B80	D25	-13,0811159	49,5783042	76,10	x	x	x	-	M	M	M
Solaniampilana	F	sol-C5	D46	-13,0810386	49,5745138	11,36	-	x	x	x	F	F	F
Solaniampilana	F	sol-C9	F3	-13,0770447	49,5821807	29,60	x	x	x	-	F	F	F
Solaniampilana	M	sol-C10	F19	-13,0770109	49,5793158	34,50	-	x	x	-	-	-	-
Solaniampilana	F	sol-C16	A23	-13,0904855	49,5777759	47,40	-	x	x	-	-	-	-

Figure 1 (cont.)

Forest	Sex (F/M)	Indiv. ID	trap ID (1st capture)	GPS South/North	GPS East/West	[DNA] ng/µl	Cyt-b	COII	D-loop	Genotyping	sexing pcr_1	sexing pcr_2	sexing pcr_3
Binara	F	bin-C24	J49	-13,2562391	49,6146327	21,60	-	-	x	x	F	F	F
Binara	M	bin-C25	J63	-13,2541764	49,619209	72,15	-	x	x	x	F	-	-
Binara	F	bin-C26	A23	-13,2409987	49,6315928	31,40	-	x	x	-	-	-	-
Binara	F	bin-C27	A25	-13,2406297	49,6316001	16,50	-	-	x	x	-	-	-
Binara	F	bin-C28	A31	-13,2394128	49,6322926	28,90	x	x	x	-	-	-	-
Binara	F	bin-C29	G28	-13,2492236	49,6040399	24,60	x	x	x	-	F	-	-
Binara	F	bin-C30	G29	-13,2492799	49,6041985	71,73	-	x	x	x	F	-	-
Binara	F	bin-C31	G46	-13,2489989	49,606321	24,6	-	-	-	-	-	-	-
Binara	F	bin-C32	G47	-13,2489963	49,6064388	33,50	x	x	x	x	-	-	-
Binara	F	bin-C33	G1	-13,2502303	49,600224	383,39	x	x	x	-	M	F	F
Binara	F	bin-C34	G70	-13,2489331	49,6089411	7,75	-	-	x	x	-	-	-
Binara	M	bin-C35	A115	-13,2411731	49,6308148	29,06	x	x	x	-	-	-	-
Binara	F	bin-C36	A107	-13,2400677	49,6308164	22,20	-	x	x	x	M	-	-
Binara	M	bin-C37	A102	-13,2394039	49,6311758	19,20	x	x	x	-	F	-	-
Binara	F	bin-C38	A97	-13,2388806	49,6313388	8,09	-	-	-	-	-	-	-
Binara	M	bin-C39	A78	-13,2376217	49,6323851	16,16	-	x	x	x	-	-	-
Binara	F	bin-C40	A71	-13,2371379	49,6327773	20,80	x	x	x	-	M	-	-
Binara	F	bin-C41	A62	-13,242489	49,6308486	13,80	-	-	x	-	F	-	-
Binara	F	bin-C42	I88	-13,275477	49,6183375	17,77	-	-	x	x	-	-	-
Binara	F	bin-C43	I113	-13,2740836	49,6192682	43,14	x	x	x	x	-	-	-
Binara	F	bin-C44	G51	-13,2490179	49,6067988	28,60	x	x	x	-	-	-	-
Binara	F	bin-C45	G52	-13,2489404	49,6069533	14,74	-	-	x	-	-	-	-
Binara	F	bin-C46	G49	-13,2490231	49,6066996	439,71	-	x	x	-	F	F	F
Binara	F	bin-C47	J76	-13,2573636	49,61258	23,74	x	x	x	x	F	-	-
Binara	F	bin-C48	J88	-13,2587102	49,6104777	42,20	-	x	x	-	-	-	-

Table 2: Tissue extraction protocols and respective budgets.

Kit/Protocol	Reference	price per sample
Quick-gDNA ™	Zymo Research Corporation, Irvine, CA	1,85 €
DNeasy Blood & Tissue	Qiagen, Valencia, CA	3,43 €
Invisorb® Spin Tissue	Invitek GmbH, Berlin, Germany	3,62 €
ISOLATE Genomic DNA	Bioline, Lisboa, Portugal	2,26 €
Phenol Chloroform	Vallet D, Petit EJ, Gatti S, Levréro S, Ménard N (2008) A new 2CTAB/PCI method improves DNA amplification success from faeces of Mediterranean (Barbary macaques) and tropical (lowland gorillas) primates. Conserv Genet 9(3):677–680. doi:10.1007/s10592-007-9361-8	1,02 €
standard mammalian DNA isolation protocol	Laird et al. 1991	**0,55 €**
Silicon Dioxide	Rohland N, Siedel H and Hofreiter M (2009) A rapid column-based ancientDNA extractionmethod for increased sample throughput. Molecular Ecology Resources	0,59 €

Table 3: GenBank accession numbers for the reference sequences used to perform the phylogenetic tree for the two concatenated loci (cyt b + COII)

Microcebus species	Indiv. ID	Location	cyt b	COII	D-loop
M. tavaratra	003y03ana	Analabe	EF065206	EF065242	EF065217
	003y03anka	Ankarana	EF065212	EF065240	EF065216
	004y03anka	Ankarana	EF065199	EF065241	EF065218
	007y03kar	Ankarana	EF065210	EF065239	EF065220
	YLE110	Ankarana	AF285534	AF285498	AF285457
	RMR 43	Ankarana	AF285533	AF285497	AF285456
	RMR71	Ankarana	GU327258	GU327058	-
	RMR74	Ankarana	GU327254	GU327056	-
	RMR76	Ankarana	GU327260	GU327059	-
	RMR77	Ankarana	GU327262	GU327061	-
	RMR78	Ankarana	GU327261	GU327060	-
M. mittermeieri	RMR189	Marojejy	GU327316	GU327115	-
	RMR190	Marojejy	GU327317	GU327116	-
	RMR191	Marojejy	GU327318	GU327117	-
	RMR192	Marojejy	GU327319	GU327118	-
M. sambiranensis	RMR161	Ambanja	GU327309	GU327108	-
	RMR163	Ambanja	GU327310	GU327109	-
	RMR165	Ambanja	GU327311	GU327110	-
	RMR40	Manongarivo	GU327246	GU327051	-
M. simmonsi	RMR108	Tampolo	GU327273	GU327072	-
	RMR111	Tampolo	GU327276	GU327075	-
M. rufus	RMR143	Andrambovato	GU327300	GU327099	-
	RMR147	Andrambovato	GU327303	GU327102	-

Table 4: Results comparison between two sets of data with different number of individuals sequenced

Population	N = 33 sequenced individuals Cytochrome oxidase II (COII)				N = 59 sequenced individuals Cytochrome oxidase II (COII)				N = 33 sequenced individuals D-loop				N = 68 sequenced individuals D-loop			
	N	h	Hd ± SD	π ± SD	N	h	Hd ± SD	π ± SD	N	h	Hd ± SD	π ± SD	N	h	Hd ± SD	π ± SD
Bekaraoka	11	5	0.782 ± 0.107	0.004 ± 0.003	19	5	0.790 ± 0.057	0.005 ± 0.003	11	7	0.873 ± 0.089	0.021 ± 0.012	20	9	0.858 ± 0.054	0.021 ± 0.011
Solanampilana	12	1	0.000 ± 0.000	0.000 ± 0.000	23	2	0.087 ± 0.078	0.0001 ± 0.0003	12	3	0.439 ± 0.158	0.007 ± 0.004	26	5	0.508 ± 0.108	0.007 ± 0.004
Binara	10	1	0.000 ± 0.000	0.000 ± 0.000	17	1	0.000 ± 0.000	0.000 ± 0.000	10	1	0.000 ± 0.000	0.000 ± 0.000	22	1	0.000 ± 0.000	0.000 ± 0.000