

**Working Title:** Next-generation *in situ* conservation and capacity building in Madagascar using a mobile genetics lab

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## 1 **Abstract**

2           Madagascar is a biodiversity hotspot that is facing rapid rates of deforestation, habitat  
3           destruction and poverty. Urgent action is required to document the status of biodiversity to  
4           facilitate efficacious conservation plans. Within country, new generations of Malagasy scientists  
5           and conservationists are taking on leadership roles, although many lack access to modern  
6           genetic sequencing and are underrepresented in academic publications, when compared to  
7           international counterparts.

8           With the recent advent of portable and affordable genetic technologies, it is now possible  
9           to tackle logistical considerations. Mobile genetics labs, with the capacity for *in situ* DNA  
10          extraction, amplification and sequencing, can produce scientifically reproducible data under field  
11          conditions, minimizing the time between sample collection and data analysis. Additionally,  
12          mobile labs offer powerful training opportunities for in-country scientists that enable local  
13          students and researchers to actively participate and contribute fully to the research enterprise,  
14          and that further empower these communities to contribute to the conservation dialog.

15          Here, we show “proof of concept” by deploying a miniaturized thermal cyclers alongside  
16          the Oxford Nanopore MinION DNA sequencer in Madagascar, including in the newly  
17          established Anjajavy Protected Area in northwestern Madagascar. We successfully extracted  
18          DNA from tissue samples collected using minimally-invasive techniques, amplified and  
19          sequenced a phylogenetically informative mitochondrial gene (cytochrome-b; *cytb*), and thereby  
20          confirmed the presence of Danfoss’ mouse lemur (*M. danfossi*) within the Anjajavy Reserve.

21          To demonstrate the reproducibility of our methods, we successfully performed our  
22          established molecular and analytical pipeline at two additional locations in Madagascar, where  
23          we also conducted two-day workshops at local higher-education Institutions to demonstrate the  
24          process from tissue samples to DNA sequencing. Ultimately, we show that a mobile genetics  
25          lab can provide reliable and expeditious results, become a powerful educational tool, and allow

26 scientists to conduct genetic analyses, potentially allowing for rapid interventions under  
27 emergency conditions *in situ*.

28

## 29 **Introduction**

30         Madagascar, one of the world’s most threatened biodiversity hotspots, is fighting severe  
31 challenges to the long-term survival of its endemic wildlife due to habitat loss and degradation,  
32 while experiencing one of the fastest population growth rates worldwide (Gardner et al. 2018;  
33 UNFPA 2018). Thus, there is a sense of urgency to accurately report biodiversity to assess  
34 conservation risks and translate these data into policy action. Somewhat paradoxically, thanks  
35 to advances in genetic technologies, our quantification of biodiversity levels continues to rise  
36 despite high deforestation rates. This is largely the result of new biological surveys targeting  
37 poorly known areas, the integration of genetic analyses with more traditional morphological  
38 assessments, and a barcoding approach to species identification. Oftentimes, as new species  
39 are formally described in the literature, they are immediately tagged as “endangered” or  
40 “critically endangered” under International Union for Conservation of Nature (IUCN) regulations,  
41 because their distributions are restricted, and their habitats are highly fragmented.

42         This is particularly true of the lemurs of Madagascar, which are currently considered the  
43 most threatened group of primates on earth (Estrada et al. 2017). Among them, the “cryptic”  
44 nocturnal mouse lemurs (*Microcebus*) have undergone one of the most dramatic taxonomic  
45 expansions, with species numbers increasing from only a few to 24 in the last decades (Hotaling  
46 et al. 2016). Some mouse lemur species are known to live in sympatry and, in certain cases,  
47 there is evidence of hybridization between them, which make species assignment by phenotypic  
48 cues challenging at best (Hapke et al. 2011). Further increasing their appeal as research  
49 models, genomic data are rapidly accruing, including a genome assembly to chromosome-level  
50 for the gray mouse lemur (*Microcebus murinus*) (Larsen et al. 2017).

51           On the flip side of its biodiversity wealth, Madagascar's academic opportunities are rare  
52 and limiting. Species descriptions and updates on the conservation status of lemurs have been  
53 traditionally led by foreign researchers. Although there is an ongoing trend for Malagasy  
54 scientists to take a more active role in project design, data collection and analysis, international  
55 collaborations are still vital and encouraged in Madagascar, both to contribute financially and  
56 technologically, and to facilitate knowledge production and dissemination. Efforts by Malagasy  
57 researchers to encourage these collaborations are especially laudable in the context of national  
58 policy and education. Unfortunately, reliance on international support has led foreign  
59 researchers to take leadership in publishing and securing funding. This is evident by the  
60 underrepresentation of lead-authors affiliated with Malagasy institutions, a trend expected to  
61 contribute to weakening high education development and quality for years to come (Waeber et  
62 al. 2016).

63           Yet, we are now at scientific, academic, and technological crossroads: A new generation  
64 of Malagasy researchers are establishing labs and/or developing research programs in country.  
65 At the same time, new DNA sequencing technologies are revolutionizing the fields of genetics  
66 and genomics, expanding applications worldwide through the creation of miniaturized devices.  
67 These new technological products are relatively affordable and have the potential to sequence  
68 even whole genomes in real time (Tyler et al. 2018). At the forefront of these developments are  
69 devices released from Oxford Nanopore Technologies (ONT) and miniPCR. These companies  
70 have created the MinION (ONT; a portable nanopore-based DNA sequencer platform) and the  
71 miniPCR (a miniaturized thermal cycler). These technologies have already been tested under  
72 field conditions in a variety of projects around the world, including rapid species assessments in  
73 a South American biodiversity hotspot (Pomerantz et al. 2018), disease surveillance to cope  
74 with epidemiological crises in West Africa (Quick et al. 2016) characterization of microbiome  
75 communities under extreme climatic conditions such as Antarctica (Johnson et al. 2017), and  
76 even in outer space (Castro-Wallace et al. 2017). As these technologies are becoming widely

77 available, there is increasing need for operational workflows to simplify analyses and reduce  
78 laboratory costs, while being able to survey remote field sites and produce expedited and  
79 reliable results (Maestri et al. 2019). And far from least, these technologies are proving to be  
80 excellent platforms for engaging in-country students and scientists in the fast-moving area of  
81 field genomics (Watsa et al. 2019).

82 Our interdisciplinary research team has been conducting research in Madagascar for  
83 decades. Recently, we have furnished a mobile genetics lab for the field, both for research and  
84 capacity building. Our objectives were twofold: 1-to test the efficacy of a mobile genetics lab in  
85 Madagascar by sequencing a marker gene from mouse lemurs (i.e., genetic "barcoding") and  
86 providing the first lemur species assessment *in situ*; 2- to build in-country capacity by  
87 conducting workshops at local academic Institutions in Madagascar to gauge the interest and  
88 the potential use of the lab as an educational tool for high education students.

## 89 **Methods**

### 90 *Lab implementation in Madagascar*

91 Our mobile genetics lab included equipment, reagents and supplies needed to process  
92 samples from DNA extraction to sequencing (Table 1). We tested all lab components at Duke  
93 University, North Carolina, USA. The lab was fitted in two Pelican cases and shipped from the  
94 USA to Madagascar in May 2018 (Sup. Inf.). In Sambava, NE Madagascar, we conducted our  
95 first lab test in country, checking reagents and flow cells, testing equipment and troubleshooting  
96 lab protocols to local conditions. This comprehensive testing included DNA extractions, PCRs,  
97 library preparations and sequencing, using tissue samples stored at the office from previous  
98 research missions. Moreover, we trained one of our Malagasy collaborators in the basics of lab  
99 operation. With his assistance, we organized a two-day workshop conducted at the Centre  
100 Universitaire Régional de la SAVA (CURSA) a local branch of the University of Antsiranana in  
101 northeastern Madagascar.

102 In early July, we moved the lab from Sambava to our field site, the Anjajavy Lodge and  
103 Reserve. This site was ideal because it has electricity, an unusually high density of mouse  
104 lemurs and, importantly, because the species of mouse lemur at this site has/have yet to be  
105 genetically confirmed. Anjajavy (S14.99025 E47.22958) encompasses ~1,200 ha of private  
106 Reserve, and ~8,000 ha of recently protected area, predominantly dry deciduous forest. We  
107 conducted our field mission between July 9 and 29, 2018. During the first 10 days of our  
108 mission, we captured and obtained tissue samples from 12 mouse lemurs. During the last 10  
109 days in the field, we extracted all tissue samples and chose a high-quality extraction to  
110 sequence. The process from DNA extraction until sequencing took ~ 8 hours and was  
111 partitioned over two days (Sup. Inf.).

112 After Anjajavy, we traveled to the capital Antananarivo to set the lab at the Vahatra  
113 Association office, the leading Malagasy NGO promoting capacity building and scientific  
114 research in Madagascar. With assistance from Vahatra staff, we conducted our second  
115 workshop at the Vahatra Association's office, for university students and researchers interested  
116 in genetics.

#### 117 *Sample processing and analysis*

118 Genomic DNA was extracted from tissue samples using DNeasy Blood & Tissue Kit  
119 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. We incorporated  
120 mechanical lysis (i.e., bead beating) as an additional step after chemical lysis. We selected to  
121 sequence the entirety of the mitochondrial gene, *cytb*, (~1100bp) which has proven a reliable  
122 phylogenetic marker for mouse lemurs (Hotaling et al. 2016). We amplified *cytb* using the  
123 miniPCR thermal cycler. We used a PCR cleanup protocol with AMPure beads before  
124 proceeding to sequencing. DNA library preparation and flow cell loading were carried out  
125 according to the protocols by ONT. Flow cell pore availability ranged between 85 and >1000  
126 active pores (Sup. Inf.).

127           We allowed each library to run for 1 hour, and we used MinKNOW software to base call  
128 for 3-8% of bases. This low percentage was enough to accomplish great depth in sequencing  
129 coverage with more than 10,000X of the *cytb* amplicon per sample. Sequenced data were  
130 stored as FASTq files and retrieved using MinKNOW software. Raw reads were first filtered by  
131 read length between 1000 and 1400 bases (approximating that of the *cytb* gene) and then  
132 mapped to a *M. murinus cytb* reference sequence (GenBank accession number: U53572)  
133 before generating a consensus sequence. Consensus sequences were aligned to the reference  
134 sequence using Geneious software, and then blasted to our database comprising ~ 270  
135 published (NCBI GenBank) and unpublished mouse lemur sequences from the Yoder lab.  
136 Finally, we created a phylogenetic tree using Neighbor-Joining (NJ) analysis with uncorrected p-  
137 distances as implemented in PAUP version 4a165. One thousand bootstrap trees were also  
138 estimated by NJ with PAUP and we used RAxML version 8.2.12 to draw bootstrap support onto  
139 the nodes from the original data set. The phylogenetic tree was edited in FigTree version 1.4.4.

140           All research protocols complied with Institutional Animal Care and Use of Animals at  
141 Duke University (IACUC# A263-17-12), and field research was approved by the Ministry of  
142 Environment, Ecology and Forests of Madagascar (Permit#  
143 035/18/MEEF/SG/DGF/DSAP/SCB.Re).

144

## 145 **Results**

146           In total, we sequenced DNA from four mouse lemurs using the mobile genetics lab in  
147 Madagascar. Two mouse lemur samples were sequenced in Sambava, one sample was  
148 sequenced *in situ* at the Anjajavy field site, and one sample was sequenced in Antananarivo  
149 (Fig. 1). All mouse lemur samples grouped with those of *Microcebus danfossi*, thus we  
150 genetically confirmed the presence of this species at Anjajavy (Fig. 2). Consensus sequences  
151 generated in this study were stored in GenBank under accession numbers XXX-XXX.

152



Fig.1 Locations for the use of the genetics lab. 1.Sambava: (a) Loading gel in the DLC/SAVA office, (b) Loading MinION prior to sequencing; 2. Anjajavy: (a) deciduous forest, (b) lab setting near forest, (c) Danfoss' mouse lemur; 3. Antananarivo: lab setting at Vahatra office.

153

154 We conducted two workshops to target college students, at the Centre Universitaire  
155 Régional de la SAVA (CURSA) in the town of Antalaha, and at the Vahatra Association office, in  
156 the capital Antananarivo (Fig. 3). During both workshops, we described and demonstrated all  
157 procedures from tissue extraction to sequencing to species assignment. A total of 66 students  
158 attended the workshop at CURSA conducted in Malagasy, where some students actively  
159 participated in hands-on molecular techniques such as pipetting, loading samples in the  
160 miniPCR thermal cycler, setting the agarose gel and loading samples using the Bluegel DNA



161 electrophoresis kit. At Vahatra Association, a total of 25 students (the maximum room capacity)  
162 attended the workshop, which was conducted in English. These participants had more  
163 comprehensive backgrounds in genetics, so workshop participants engaged in all lab activities  
164 as well as discussions about potential implementation of this technology in a variety of research  
165 topics. During the workshop at Vahatra Association, we sequenced a mouse lemur sample from  
166 our recent field mission at Anjajavy.

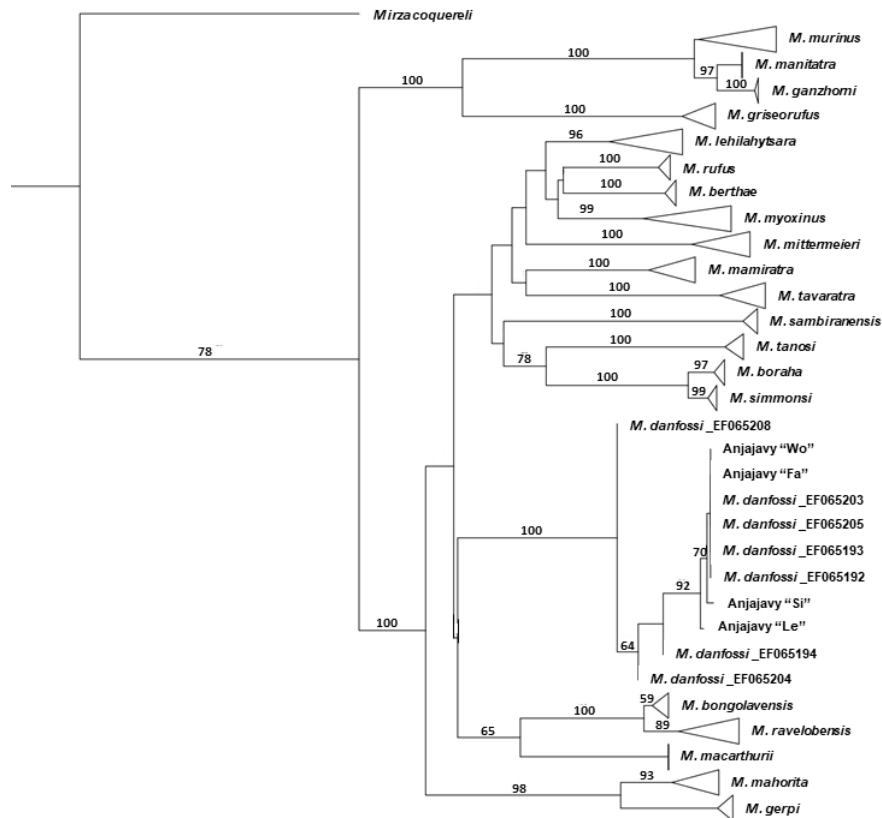


Fig. 2 *Microcebus* phylogeny based on *cytb* sequences. Branches were collapsed for practical purposes, except for the *M. danfossi* lineage, to highlight consensus sequences from Anjajavy; node support above 50 % is shown. GenBank accession numbers for Anjajavy samples are pending, those for published sequences of Danfoss' mouse lemurs are listed next to the species name.

167



Fig. 3 Workshops in Madagascar: a,b at CURSA in Antalaha; c,d at Vahatra office in Antananarivo

168

## 169 Discussion

170 Portable and affordable technologies in the field of genetics have made it feasible to  
171 produce scientifically robust data under field conditions, minimizing the time between sample  
172 collection and data analysis. The capacity for *in situ* genetics also provides training opportunities  
173 to enable active participation of local students in the whole process of scientific research.

174 To our knowledge, we were the first researchers to perform *in situ* sequencing of wild  
175 lemurs in Madagascar. We were able to confirm species assignment for one of the lemurs at  
176 Anjajavy within a week, from lemur capture to tissue collection, to the generation of a  
177 phylogenetic tree. Our results are consistent with the known distribution of *M. danfossi*, between  
178 the Sofia and Maevarano rivers, NW Madagascar (Olivieri et al. 2007). Anjajavy's mouse lemurs  
179 had been tentatively assigned as *M. danfossi* on the basis of morphological assessments

180 (Randrianambinina et al. 2010), but we provide the first genetic confirmation of Danfoss' mouse  
181 lemurs at this site.

182 We would like to emphasize that mobile genetics technology not only minimizes time  
183 between sample collection and analysis, but has the potential to address urgent conservation  
184 concerns in *real time*. Conservation crises can span from needing rapid biodiversity  
185 assessments in threatened habitats, to dealing with wildlife die-off situations or disease-related  
186 cases that require immediate intervention (e.g., Carver 2018).

187 We showed “proof of concept” that this technology can be deployed in remote sites, that  
188 results can be obtained in a speedy manner and that training sessions can prepare advanced  
189 students with the skills and means to conduct genetic analysis *in situ*. As a testament to the  
190 latter point, both MBB and LKG who deployed the lab in Madagascar, had some experience  
191 with molecular genetics and analysis, but are not geneticists themselves. Week-long training in  
192 the USA provided the necessary skills, tools and information to run the lab on the ground in  
193 Madagascar and train others in its use.

194 One unexpected corollary of the workshops was the general interest from participants to  
195 apply these technologies to a large range of research topics. We also learned that there are  
196 facilities and Institutions in country already using MinION technology such the Mahaliana Lab  
197 (<https://www.mahaliana.org/>) and the Pasteur Institute (<http://www.pasteur.mg/>). Connectivity  
198 among the scientific community, both national and international, will be beneficial for  
199 researchers interested in funding, contracting services or collaborating with them. Finally, we  
200 showed that the mobile genetics lab can be a powerful educational tool, for teaching basic  
201 concepts to introductory students, or exemplifying complex procedures for advanced college  
202 students with background in biological sciences. Thus, the potential for this technology to  
203 immediately produce data, coupled with the power of capacity building to engage local  
204 researchers significantly outweighs the logistical challenges to obtain, transport and maintain  
205 lab supplies and reagents in remote settings.

206 In sum, miniaturized and more affordable technologies have the potential not only to  
207 speed up production of knowledge and solve biological and environmental crises in efficacious  
208 manners at remote settings, but also to shape the professional careers of passionate scientists  
209 in less advantageous academic settings and to level the scientific playing field.

210

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225

## 226 **Supporting Information**

227 Travel itinerary, Laboratory protocols, and Sampling methods. The authors are solely  
228 responsible for the content and functionality of these materials. Queries (other than absence of  
229 the material) should be directed to the corresponding author.

230

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283

**Table 1** Mobile genetics lab components

<b>Application</b>	<b>Component</b>	<b>Company</b>
<b>DNA extraction</b>	TerraLyzer (i.e., Craftsman automatic hammer)	Zymo Research
<b>Sample preparation</b>	Mini centrifuge	USA scientific
<b>Sample preparation</b>	Heatblock	BenchMark Scientific
<b>Sample preparation</b>	Pipettes	Gilson
<b>DNA amplification</b>	Thermal cycler	miniPCR
<b>Electrophoresis</b>	Bluegel DNA electrophoresis	miniPCR
<b>DNA quantification</b>	Qubit fluorometer	Invitrogen
<b>DNA sequencing</b>	MinION sequencer	Oxford Nanopore Technologies
<b>DNA sequencing</b>	SpotON flow cells (R9.5)	Oxford Nanopore Technologies
<b>Data collection/analysis</b>	Laptop computer	Lenovo

Note. Agarose gels were prepared by melting ingredients together over a candle.

## Supporting Information

### *Travel itinerary*

Our genetics lab was shipped in two Pelican cases from Washington Dulles Airport, USA to Ivato Airport Antananarivo, Madagascar in early May. The trip included two flights and was ~ 27 hours total. The following morning, the lab was shipped to the town of Sambava (NE Madagascar) via air freight, and less than 24 hours later, it was brought by car to the Duke Lemur Center/SAVA Conservation office for storage with an available fridge/freezer unit. In between international and domestic flights, sensitive reagents and flow cells were kept in coolers and refrigerated with ice packs. In early July, the lab was shipped back from Sambava to Antananarivo via air freight and, less than 48 hours later, we rented a car and drove to the town of Mahajanga, (NW Madagascar) for 13 hours. Less than 24 hours later, we rode a boat from Mahajanga to the field site, the Anjajavy Lodge and Reserve, a trip that lasted 4.5 hours. In between transportation routes, we placed reagents and flow cells under refrigeration and replaced ice packs. In late July, we reverted the original route, taking the genetics lab from Anjajavy back to Antananarivo.

### *Lab protocols*

Primers and PCR: Primers to amplify *cytb* gene were L14724: 5'–CGA AGC TTG ATA TGA AAA ACC ATC GTT G–3', and H15915: 5'–AAC TGC AGT CAT CTC CGG TTT ACA AGA C–3' as previously described in Irwin et al. (1991). Each Polymerase chain reaction (PCR) contained approximately 1.5  $\mu$ L of PCR product, 12.5  $\mu$ L LongAmp Taq DNA Polymerase (New England Bio Labs), 1.25  $\mu$ L of each primer, and 8.5  $\mu$ L water for a 25  $\mu$ L total volume. Samples for the PCR run followed the following settings: initial denaturation 95°C for 2 minutes, 32 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 480 seconds. PCR products were then cleanup using AMPure beads and washed in 70% EtOH before resuspending in water.



Library preparation: DNA products (45  $\mu$ L amplicon) were prepared with 7  $\mu$ L Ultra II End-prep reaction buffer and 3  $\mu$ L Ultra II End-prep enzyme and 5  $\mu$ L water. Samples were washed using AMPure beads and 70% EtOH and resuspended in 30  $\mu$ L water. Adapter ligation and tethering was then carried out with 20  $\mu$ L of Adapter Mix (ONT) and 50  $\mu$ L of NEB Blunt/TA ligation Master Mix (New England Biolabs). The adapter ligated DNA library was then purified with AMPure beads and ABB buffer. Samples were resuspended in 13  $\mu$ L water and placed in a LoBind tube ready for sequencing.

Sequencing: We prepared pre sequence-library following ONT protocols, by mixing 12  $\mu$ L amplicon, 2.5  $\mu$ L water, 25.5  $\mu$ L LBB and 35  $\mu$ L RBF. We inserted flow cell in the MinION frame, and loaded the flow cell's priming port with 800  $\mu$ L of priming mix (576  $\mu$ L RBF and 624  $\mu$ L water) with SpotON cover closed. We additionally loaded 200  $\mu$ L priming mix in priming port with SpotON cover open. The sample was then added to SpotOn por via dropwise fashion. Finally, we covered SpotOn and priming ports, close the MinION lid and open the MinKNOW GUI software to proceed to sequence. Note: We were authorized by ONT to use MinKNOW 18.5.1.0 version to base call offline, because we were off grid at Anjajavy.

Flow cells: We used a total of three flow cells (R 9.4), one at each location: Sambava, Anjajavy and Antananarivo respectively. Flow cell number one, used in Sambava, was transferred with the mobile genetics lab in May, and contained more than 1000 active pores at the time of use, well over the 800 threshold recommended by company. Flow cell number two was brought by a researcher to Anjajavy in July, and contained 85 active pores. Despite the low count, likely the result of disrupted transportation conditions, results obtained from this unit were reliable. In fact, our ability to reliably sequence *cytb* gene speaks to the great redundancy buffer provided by flow cells, when relatively short and single genes are the source of sequencing. Flow cell

number three was brought by another researcher in September to Antananarivo, and used for our last sequencing event. This flow cell contained over 1000 active pores at the time of sequencing.

Note: When we tested the lab at Duke University, we sequenced a sample from a grey mouse lemur (*M. murinus*) from the Duke Lemur Center using the MinION sequencer, and generated a consensus sequence that was 100% identical to the reference sequence that had been generated by Sanger sequencing.

### *Sampling methods*

Mouse lemurs were captured with Sherman traps (3 x 3.5 x 9") baited with small pieces of banana and set along trails at 1.5m height. Traps were set in the afternoon and checked in the mornings for 6 days. All captured lemurs were brought back to the campsite for processing. At the campsite, individuals were weighed, measured, and microchipped for identification (Trovan®). Tissue samples (ear biopsies, 2mm) were taken from anesthetized individuals (Ketamine, 10mg/kg) and stored in 90% alcohol for further analysis. Individuals were released at trapping sites later the same day.

### **References**

Irwin DM, Kocher TD, Wilson AC. 1991. Evolution of the cytochrome *b* gene of mammals. *Journal of Molecular Evolution* **32**: 128-144.