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Molecular Evidence of Reproductive Isolation in Sympatric Sibling Species of Mouse Lemurs

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Abstract

Recent morphological and molecular phylogenetic studies of mouse lemurs (*Microcebus*) occurring in the western and southern regions of Madagascar have shown that species diversity had been considerably underestimated. In large part, this underestimate was due to the lack of sufficient specimen material from given localities to properly assess the level of phenotypic variation within and between populations. The accurate delineation of species boundaries has no doubt been confounded by the diminutive size, nocturnal habits, and subtle morphological variation characteristic of mouse lemurs, which can make field identification of individuals problematic. In this study, we illustrate the use of molecular phylogenetic analysis for revealing reproductive isolation in two sympatrically occurring mouse lemur species, *Microcebus murinus* and *M. griseorufus*. The documentation of these two species from the Berenty Private Reserve in the extreme south of Madagascar verifies the historically-broad distribution of *Microcebus griseorufus*, a species recently resurrected from synonomy.

Key Words

Microcebus; Madagascar; speciation; reproductive isolation; phylogeny

Introduction

Phylogenetic analysis of molecular data is a powerful tool for the examination of organismal diversity. It can offer the investigator a direct method for determining the hierarchical relationships among species and populations, for revealing historical associations among the geographic areas occupied by these species, and for revealing instances of reproductive isolation between closely-related species occupying the same locality (Avise, 2000; Barraclough and Vogler, 2000; Templeton, 2001). Each of these applications of molecular phylogenetic analysis was recently brought to bear on the question of species diversity in mouse lemurs, genus *Microcebus*, from the western and southern regions of Madagascar (Yoder et al., 2000). Morphological analysis of mouse lemur populations, spanning a geographic range from Ankarana in the north to Beza Mahafaly in the south, had revealed the presence of three species new to science and warranted the resurrection of two others from synonymy (Rasoloarison et al., 2000). The molecular phylogenetic study was conducted to test the monophyletic status of individuals ascribed to the various species and to examine their hierarchical relationships. That analysis reinforced the hypothesis of species level status by confirming that each species is reciprocally monophyletic for mtDNA haplotypes. The molecular analysis also suggested that several of the species show extremely limited geographic distributions, thus emphasizing their vulnerability to habitat destruction.

Microcebus murinus is the notable exception, showing a broad western and southern distribution in the Rasoloarison et al. (2000) study, and with known occurrences in the northwestern regions of Madagascar as well (Martin, 1973; Mittermeier et al., 1994; Zimmermann et al., 1998). These observations suggest that the various species of mouse lemur found in the western regions of Madagascar might be quite different from one another ecologically. For example, given that it appears that *Microcebus murinus* is by far the most widely distributed of the currently-described species, with other species having more circumscribed distributions, this supports the idea that *M. murinus* is an

ecological generalist, tolerant of a wide range of vegetational habitats (Ganzhorn and Schmid, 1998; Mittermeier et al., 1994). Also, despite the fact that *Microcebus murinus* was only sampled from a portion of its broad range in the Rasoloarison et al. (2000) study, the results of the phylogenetic analysis suggest a strong pattern of south to north population division during the evolutionary history of that species. For the intraspecific phylogeny, the southernmost localities are progressively more basal (Yoder et al., 2000; Figure 4).

Reproductive isolation in sympatry has long been considered the essential test of species status in mammals and other vertebrates (Mayr, 1942). In the Rasoloarison et al. (2000) study, only one locality, the Kirindy Forest CFPF, yielded more than one species from the same forest block (Figure 1). The molecular phylogeny confirmed that these individuals belonged to separate species by placing them into two independent clades in widely separated parts of the phylogeny. *Microcebus berthae* (formerly *M. myoxinus*) was shown to be deeply nested within one of two primary subclades (designated as the northern clade in the Yoder et al., 2000 study) while *M. murinus* was shown to belong to the other primary subclade (designated as the southern clade). This result implies that there has been secondary contact at the Kirindy Forest between species that have experienced long periods of independent history, with species boundaries being maintained by reproductive isolation.

Prior to the studies described above, numerous reports had suggested the presence of sympatrically occurring species throughout the western regions of Madagascar (Ausilio and Raveloanrinoro, 1998; Mittermeier et al., 1994; Rakotoarison et al., 1993; Randrianambinina, 1997; Thalmann and Rakotoarison, 1994). Careful natural history observation and confirmation has only been provided for two localities, however: Ankarafantiska for *Microcebus murinus* and *M. ravelobensis* (Zimmermann et al., 1998), and the Kirindy Forest (CFPF) for *M. murinus* and *M. berthae* (Schmid and Kappeler, 1994). Notably, both of these studies were conducted in the central and northern portions of the west, with no such studies having been conducted in the southwestern regions of Madagascar. Thus far, only indirect evidence from the analysis of owl pellets has been

presented to suggest sympatric co-occurrence of *Microcebus murinus* with *M. griseorufus* in the south (Rasoloarison et al., 2000). Given that morphological variation among mouse lemur species can be subtle, and that some of the most distinctive variation is only observable in study specimens that require the sacrifice of representative individuals, molecular phylogenetic tests can be extremely valuable for revealing the species identity of newly-discovered populations of cryptic species. The phylogeny yielded by the Yoder et al. (2000) study therefore serves as a framework for assessing both the identity and hierarchical standing of newly-discovered mouse lemur populations and for determining the historical associations among them. In the study presented here, we apply a phylogenetic test for revealing the species identity of a population of mouse lemurs from the Berenty Private Reserve in the extreme south of Madagascar.

Materials and Methods

During a field investigation conducted in March - April, 2000, one of us (F.G.) discovered a population of mouse lemurs in spiny forest habitat within the Berenty Private Reserve. Animals examined from this population are characterized by a long reddish tail, a gray back, a white underside, and reddish markings around the eyes. Two forest plots were investigated, one in a 4.5 ha spiny forest fragment and one in the gallery forest. These two habitats are separated by an abrupt transition along the Mandrare River. Preliminary observations indicated that mouse lemurs were most frequently encountered at heights of 0 - 15 m in the spiny forest and at 2-20 m in the gallery forest. Twenty Sherman live traps (7.7 x 7.7 x 30.5 cm) were set 1-2 m above the ground every 25 m of a regular grid on six nights in the spiny forest, and every 25 m of a transect line on two consecutive nights in the gallery forest. Traps were baited with banana, set at dusk, and checked at dawn. Trapped animals were weighed and measured, and a small (1-2 mm^2) tissue biopsy taken from one or both ears for all captured animals. Ear cuts were used both for identification and for DNA analysis. Ear biopsies were stored at ambient temperature in one ml. of 70% EtOH. Animals were released at dusk at their capture site. A total of 29

individuals were trapped and processed from the spiny forest with only one individual captured from the gallery forest. Of the spiny forest individuals, only three could be positively identified as juveniles. The single female trapped in the gallery forest was of relatively high body mass (67 g) and showed coloration more typical of *Microcebus murinus*, with pale grayish underside and gray head. The single gallery forest individual, and 11 of the 29 spiny forest individuals, were processed for DNA analysis.

Laboratory methods for DNA extraction, PCR amplification, and DNA sequencing exactly followed those reported in Yoder et al. (2000). DNA was amplified and sequenced for the mitochondrial markers D-loop (homologous with the HV1 region in humans), cytochrome oxidase II, and cytochrome *b*, allowing for alignment of homologous sequences from individuals reported in the Yoder et al. (2000) study and the individuals reported in the present study. Sequences unique to the present study have been deposited in GenBank under accession numbers XXXXX - YYYYY. Phylogenetic analysis was performed with PAUP* (Swofford, 1998) version 4.0b10 (Altivec) using minimum evolution. To assess statistical support for hypothesized clades, bootstrap analysis was performed. The laboratory and phylogenetic studies were conducted as blind. The responsible investigators (M.M.B. and A.D.Y., respectively) did not know prior to analysis which one of the 12 samples originated from the gallery forest individual.

Results and Discussion

Natural history observation and morphometric analysis of the Berenty population yielded two hypotheses to be investigated with molecular phylogenetic analysis. First, the marked ecological disjunction between the gallery and spiny forests, along with the distinctions in coloration between animals from the two habitats, indicates that there may be more than one mouse lemur species present at Berenty. Although *Microcebus* has been previously reported to occur in these two habitats, both populations were initially ascribed to *M. murinus* (Hladik et al., 1998; Martin, 1973). Second, morphometric analysis

of individuals from the spiny forest demonstrates that they are quite diminutive when compared with *Microcebus murinus*, but also when compared with *M. griseorufus*, the only other mouse lemur species documented to occur in southern and western Madagascar (Table I). We acknowledge that it is problematic to compare measurements taken in different studies by different investigators. Nonetheless, this apparent size discrepancy led to the initial hypothesis that the spiny forest population might represent yet another undescribed species of mouse lemur.

Phylogenetic analysis confirms the first hypothesis. The individual from the gallery forest population is shown to lie in a portion of the phylogeny distinct from the spiny forest individuals (Figure 2). This same analysis appears to contradict the second hypothesis, however. Whereas the gallery forest individual joins a *Microcebus murinus* clade, the spiny forest individuals are shown to constitute a clade that is the close sistergroup of *M. griseorufus* from Beza Mahafaly. Although the sistergroup relationship of these two clades does not alone confirm their conspecific status, the associated observation of low levels of genetic separation indicates intraspecific variation. The branch lengths separating the Beza Mahafaly and Berenty spiny forest populations are compatible with intraspecific branch lengths observed in other mouse lemur species (e.g., see internal branch lengths for *Microcebus myoxinus*, *M. ravelobensis*, and *M. murinus* in Figure 2). Uncorrected pairwise distances ("p") corroborate branch length estimates. Whereas the average "p" of *Microcebus griseorufus* compared to *M. murinus* in this study is 0.10, that for the comparison of *M. griseorufus* with the spiny forest population is 0.01, a ten-fold difference. By contrast, the comparison of the single gallery forest individual with individuals from the spiny forest shows a "p" of 0.11.

Rather than identify a new species of mouse lemur, the molecular phylogenetic analysis presented here confirms that the Berenty spiny forest population belongs to *Microcebus griseorufus*. This observation therefore confirms original reports of this species' broad western and southern distribution (Kollman, 1910). Also, by placing the gallery forest individual in a basal position

within the *Microcebus murinus* clade, this study supports the previous inference of a progressive pattern of south to north population division during the course of this species' evolutionary history. It remains for further investigation, especially of *Microcebus murinus* from north of the Tsiribihina River, to confirm the latter pattern. The results presented here are most notable, however, for their direct illustration of reproductive isolation between two species of mouse lemurs occurring sympatrically. Thus, Berenty can be included with Kirindy (CFPF) and Ankarafantsika as localities for which sympatric reproductive isolation has been established, and in this case, without the necessity of long-term behavioral study. The study is also significant in that it confirms sympatric reproductive isolation between what are apparently sibling species. This is quite distinct from the case illustrated for Kirindy locality in the Yoder et al. (2000) study. In that example, the reproductive isolation illustrated for *Microcebus murinus* and *M. berthae* seems obviously to be the outcome of secondary contact between two species that have long been separated. In the present case, however, reproductive isolation appears to have evolved relatively recently. This then suggests that there may have been strong selection for character displacement of mate recognition signals that has reinforced the evolutionary boundary between these two species, such as olfactory or auditory clues, in addition to the ecological distinctions of the gallery and spiny forest habitats. Such mechanisms have been shown to be powerful forces driving the speciation process in a variety of organisms (e.g., (Johannesson, 2001; Proulx, 2001; Rundle and Schluter, 1998). It remains for long-term study and increased geographic sampling to illuminate these potential mechanisms in mouse lemurs.

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Table I. Comparison of body mass, tail length, and ear length of *Microcebus griseorufus* from Beza Mahafaly with new population from spiny forest of Berenty.

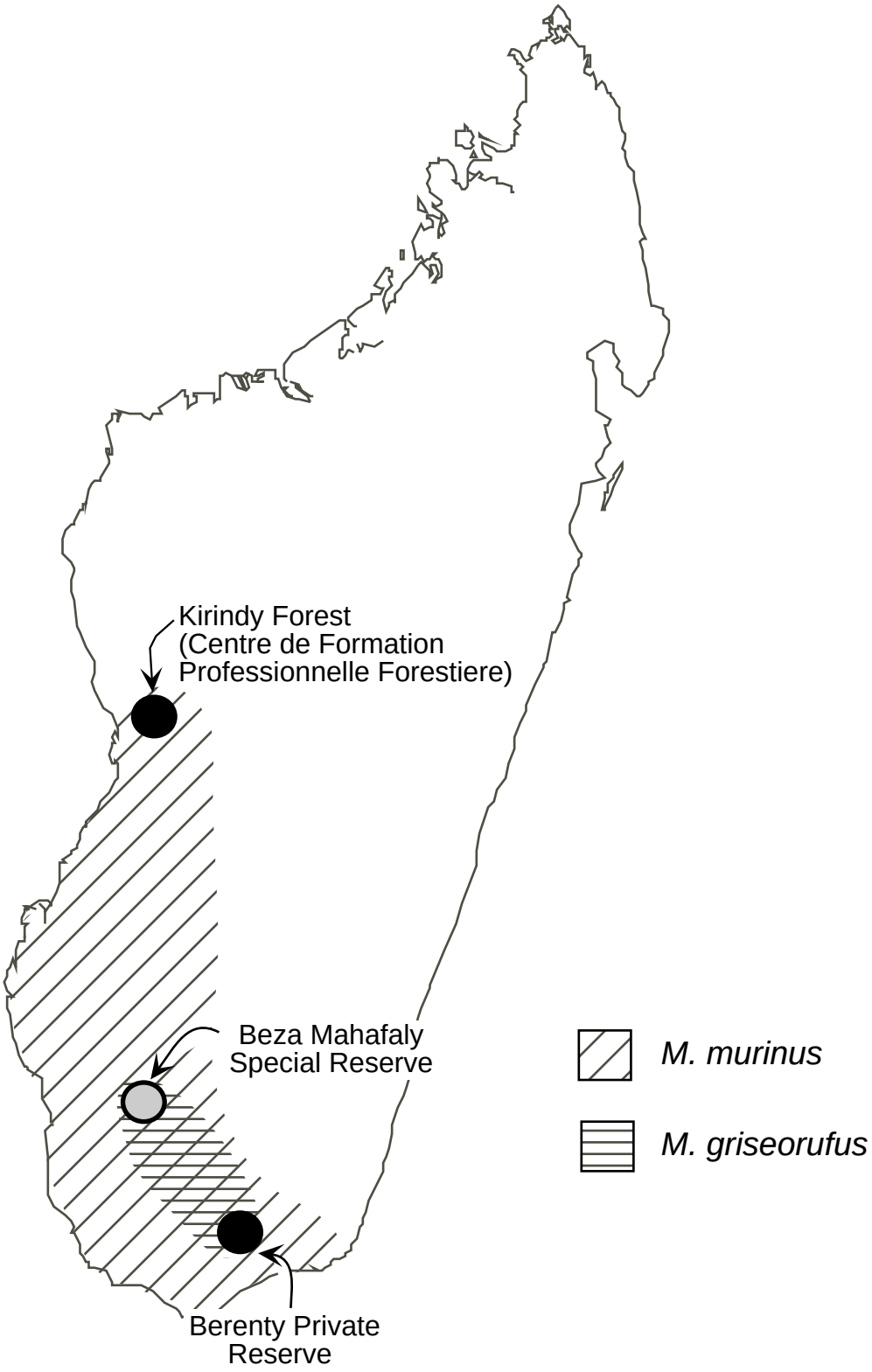
Source	Locality			body mass (g)	tail length (mm)	ear length (mm)
Rasoloarison et al. 2000	Beza Mahafaly		N = 6	63 ± 16 (50-85)	143 ± 6 (136-153)	24 ± 1 (23-25)
Present work	Berenty	Males	N = 5	39 ± 3 (31-49)	140 ± 2 (133-146)	22 ± 1 (20-25)
		Females	N = 11	55 ± 1 (51-67)	151 ± 2 (140-161)	22 ± 0 (21-24)

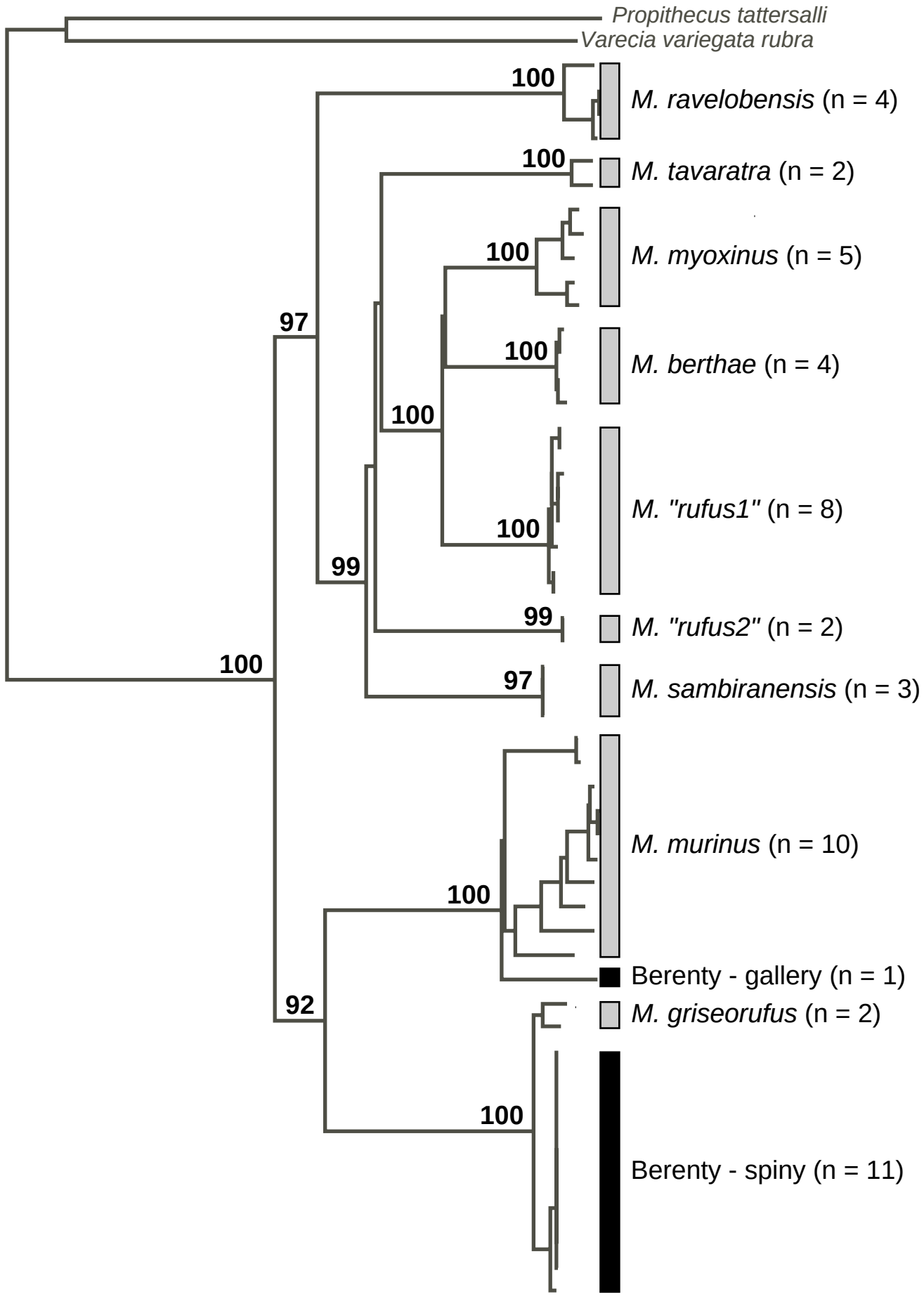
Note: Measurements are given with standard error (± SE) and range (in parentheses).

Figure Legends

Figure 1:
Distribution of *Microcebus murinus* (diagonal hatching) and *M. griseorufus* (horizontal hatching) as confirmed by Rasoloarison et al. (2000) and present study. These distributions are certainly underestimates as *Microcebus murinus* is also known to occur in the northwestern regions of the island and *M. griseorufus* has been observed in additional localities in the southern domain (S. Goodman, pers. com.). Black-filled localities indicate sites where molecular phylogenetic analysis has illustrated sympatric reproductive isolation between *Microcebus murinus* and another mouse lemur species.

Figure 2:
Phylogeny derived from sequence alignment of 2404 bp of combined mitochondrial DNA sequences from the control region homologous with the hypervariable region 1 region in humans, cytochrome *b* and COII. Distance tree was generated in PAUP* using HKY85 correction model and weighted least squares (power = 2) algorithm. Numbers on branches indicate statistical support from 100 bootstrap replicates of the "fast" bootstrap algorithm. Sequences derived from Berenty individuals are unique to this study; others are from the Yoder et al. (2000) study. Branches are drawn to be proportional to estimated probabilities of change along that branch. Black-filled boxes illustrate position of Berenty individuals. Numbers of individuals sampled for each species and / or population are shown in parentheses.


Kirindy Forest
(Centre de Formation
Professionnelle Forestiere)
Beza Mahafaly
Special Reserve
Berenty Private
Reserve
M. murinus
M. griseorufus


Propithecus tattersalli
Varecia variegata rubra
100
M. ravelobensis (n = 4)
100
M. tavaratra (n = 2)
100
M. myoxinus (n = 5)
97
100
M. berthae (n = 4)
100
M. "rufus1" (n = 8)
100
99
99
M. "rufus2" (n = 2)
100
97
M. sambiranensis (n = 3)
M. murinus (n = 10)
100
Berenty - gallery (n = 1)
92
M. griseorufus (n = 2)
100
Berenty - spiny (n = 11)