

Chapter 4.12

Mitochondrial Genetic Data Indicate a Recent Range Expansion of Gray Mouse Lemurs (*Microcebus murinus*) in the Littoral Forests of Southeastern Madagascar

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Abstract

The gray mouse lemur, *Microcebus murinus*, is a small, nocturnal lemur with a wide distribution in western and southern Madagascar. While most of its habitats are comprised of dry deciduous forest, spiny bush, and gallery forest, a small portion of its distribution extends into evergreen littoral forests of the Tolagnaro region in southeastern Madagascar. In this study, we analyzed spatial patterns of genetic diversity for *M. murinus* in these littoral forests with a mitochondrial genetic marker, the hypervariable region I. We sampled and genotyped 256 individuals at 10 sites in Petriky, Mandena, and Lokaro. These localities represent all known sites for *M. murinus* in the littoral forests of southeastern Madagascar. On a regional scale, we observed two clusters of closely related haplotypes with spatially exclusive ranges. Distances among different haplotypes and the number of haplotypes per population displayed a trend of decreasing diversity in a west-east direction. Forest size decreased, and the degree of isolation of blocks of forest increased in the same direction. On a smaller scale, in the littoral forests of Mandena and Lokaro, an unexpected pattern emerged with one dominant haplotype in all forest blocks. Fragmentation of one formerly large forest at Mandena with subsequent genetic erosion is unlikely to explain this pattern. The observed distribution of genetic diversity could be explained by female philopatry, male-biased dispersal, and recent range expansion of gray mouse lemurs.

Résumé

Des données génétiques mitochondriales indiquent une expansion récente de l'aire de distribution des Petits Microcèbes (*Microcebus murinus*) dans les forêts littorales du sud-est de Madagascar. Le Petit Microcèbe, *Microcebus murinus*,

est un lémurien nocturne avec une aire de distribution étendue sur l'ouest et le sud de Madagascar. Si le principal de son habitat comprend des forêts sèches caducifoliées, des fourrés épineux et des forêts riveraines, une petite partie de son aire de distribution se prolonge dans les forêts littorales sempervirentes de la région de Tolagnaro, dans le sud-est de Madagascar. Dans cette étude, nous procédons à l'analyse des schémas spatiaux de la diversité génétique de *M. murinus* dans ces forêts littorales avec un marqueur génétique mitochondrial, la région hypervariable I. Nous avons observé, échantillonné et produit les génotypes de 256 individus dans 10 stations de Petriky, Mandena et Lokaro. Ces stations représentent toutes les localités desquelles *M. murinus* est connu dans les forêts littorales du sud-est de Madagascar. À une échelle régionale, nous avons observé deux groupes d'haplotypes affines avec des configurations spatiales exclusives. Les distances entre les différents haplotypes et le nombre d'haplotypes par population montraient une tendance vers une baisse de la diversité dans une direction d'ouest en est. Les superficies des forêts diminuaient et le degré d'isolement des blocs de forêts augmentait dans la même direction. À une plus petite échelle, dans les forêts littorales de Mandena et de Lokaro,

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Figure 1. Distribution of *Microcebus murinus* in Madagascar (left; according to Garbutt 1999). Distribution of *M. murinus* and *M. rufus* in the region of Tolagnaro (right). The drawing is based on topographic maps (Foiben Taosarintanin'i Madagasikara, F.T.M.). The arrow symbolizes the probable colonization of the coastal region of Tolagnaro from the southern spiny bush and transitional forest by *M. murinus*. Mm: *M. murinus*; Mr: *M. rufus*. Displayed forest types: Anosyenne and Vohimena Mountains - humid forest; Lavaso Range - mixed forest types; Petriky, Mandena, Lokaro, and Sainte Luce - littoral forest. Transitional and spiny forests west of the Anosyenne Mountains and Lavaso are not shown.

un schéma imprévisible a émergé avec un haplotype dominant dans tous les blocs de forêts. La fragmentation d'une ancienne grande forêt de Mandena avec une érosion génétique consécutive peut difficilement expliquer ce schéma. La distribution observée de la diversité génétique a pu trouver une explication dans une philopatrie femelle, une dispersion biaisée de mâles et une récente expansion des Petits Microcèbes.

Introduction

Gray mouse lemurs, *Microcebus murinus*, are among the world's smallest living primates. They are solitary, nocturnal foragers in a variety of forest types on Madagascar, and have remarkable adaptations to extreme environmental conditions, such as being capa-

ble of torpor (e.g., Ortmann *et al.* 1997). In comparison to other species of the genus *Microcebus*, *M. murinus* has a large distributional range in Madagascar (Fig. 1), comprising the western, dry deciduous forests, the southern xerophytic domain, and the littoral forests (Mittermeier *et al.* 2006).

The region of Tolagnaro (Fig. 1) in southeastern Madagascar is characterized by a steep climatic gradient from the southern xerophytic domain to the eastern humid domain. This gradient is caused by the Vohimena and Anosyenne mountain ranges, which form a climatic barrier and divide the region into western dry, and eastern humid parts (Donque 1972, Ratsivalaka-Randriamanga 1985). South and east of these mountains is a series of littoral forests, which form a distinct, phytogeographic unit (Ratsivalaka-Randriamanga 1987, Lowry and Faber-Langendoen

1991, see Goodman and Ramanamanjato Chapter 2.3). The littoral forests are separated from each other by savannas, wetlands, and watercourses.

Three species of mouse lemur occur in the region of Tolagnaro (Fig. 1). *Microcebus rufus* is found in the humid forests of the Vohimena and Anosyenne Mountains, and in the humid littoral forests of Sainte Luce (Martin 1972). This species also occurs in a humid forest patch of the Lavasoa Range. In this area, its regional distribution coincides with that of *M. murinus*, which occurs in the dryer parts of the Lavasoa (Hapke, unpubl. data). To the west of the Anosyenne Mountains, there is an abrupt vegetational shift from humid to transitional and then to spiny forest, and *M. griseorufus* and *M. murinus* occur locally (Yoder *et al.* 2002). The distribution of *M. murinus* extends to the humid part of southeastern Madagascar near the coast, which includes the Lavasoa Range and the littoral forests of Petriky, Mandena, and Lokaro. The known southeastern limit of its distribution is the embouchure of Lake Mananivo at Lokaro.

Natal dispersal in *M. murinus* is male-biased. Most females remain in their original social group or near the place where they were born, while most males emigrate before reproduction (Radespiel *et al.* 2001, 2003, Wimmer *et al.* 2002, Fredsted *et al.* 2004). Female philopatry and male-biased dispersal are predominant in mammal species (Greenwood 1980). In species with female philopatry, mitochondrial genetic markers typically show less variation within than between local populations because they are only transmitted by females to their offspring (e.g., Melnick and Hoelzer 1992, Hoelzer *et al.* 1994, Perwitasari-Farajallah *et al.* 1999).

In this study, we investigated geographic patterns of genetic diversity in *M. murinus* of the littoral forests of southeastern Madagascar. To this aim, we applied a mitochondrial genetic marker, the hypervariable region I of the D-loop.

Methods

We trapped *Microcebus murinus* with banana-baited Sherman traps set 1 to 1.5 m above ground level. After anesthetization of the animal, we took a tissue sample from each individual by ear-biopsy. We released each animal in the late afternoon at the place of capture. For the preservation of the tissue samples, we used 70% ethanol or a solution with 6 M Urea, 10 mM Tris/pH 8, 10 mM EDTA, 125 mM NaCl, and 1% SDS.

We sampled 256 individuals of *M. murinus* at 10 littoral forest sampling sites between 1998 and 2002 (Figs. 1 and 2; Table 1). The sampling comprised the large littoral forest of Petriky (786 ha), a group of smaller forest blocks up to approximately 150 ha each at Mandena, and one small, isolated forest at Lokaro. The group of forest blocks at Mandena can be further divided into a group of western blocks (Fig. 2: M13, M15/M16, and M20), which are connected by different types of forest habitat, and a group of eastern blocks, which are separated from each other by open heathland of *Erica* sp. The forest block M3 south of these groups could not be investigated since it had been destroyed by fire. We added one additional sample of *M. griseorufus* from Berenty (25.017°S, 46.307°E) for phylogenetic comparisons.

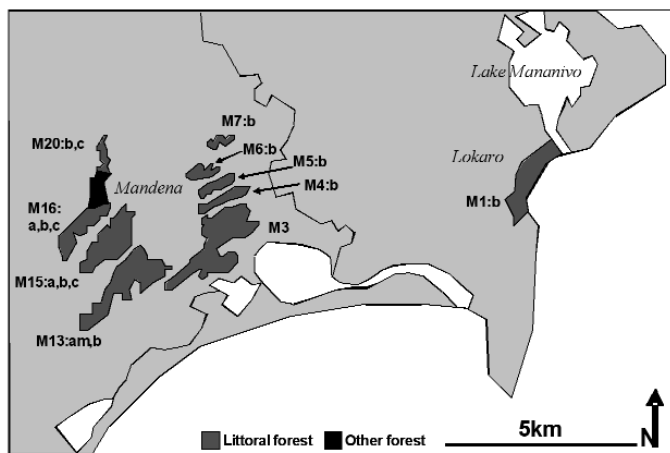


Figure 2. Geographic distribution of mitochondrial haplotypes in Mandena and Lokaro. Schematic drawing is based on a topographic map (Foiben Taosarintanin'i Madagasikara, F.T.M.). M1-M20: numbers of forest blocks; a, b, c, and am: mitochondrial haplotypes. The forest block M3 could not be sampled because it had been destroyed by fire.

Table 1. Observed mitochondrial haplotypes of *Microcebus murinus* at Lokaro (M1), Mandena, and Petriky. Haplotype: displayed is the number of individuals observed with a certain haplotype at each site and over all sites. M1: Lokaro; M4-20: forest blocks of Mandena (Fig. 2); Pe: Petriky; n Ind.: number of individuals analyzed; n. Hap.: number of different haplotypes observed at a site; All. rich.: allelic richness index based on a sample size of eight individuals.

	M1	M4	M5	M6	M7	M13	M15	M16	M20	Pe	Sum
Haplotype											
b	8	11	16	14	9	20	12	52	12		154
am						2					2
a							12	9			21
c							1	18	4		23
ag										1	1
d										10	10
e										38	38
f										3	3
g										4	4
n Ind.	8	11	16	14	9	22	25	79	16	56	256
n Hap.	1	1	1	1	1	2	3	3	2	5	
All. rich.	1.000	1.000	1.000	1.000	1.000	1.849	2.542	2.857	1.999	3.567	

Table 2. Pairwise genetic distances between haplotypes of *Microcebus murinus* in the Tolagnaro region. Below the diagonal: uncorrected p-distances; above the diagonal: number of nucleotide differences.

Clade	Haplotype	A				B				
		a	b	c	am	d	e	f	g	ag
A	a		1	2	2	27	28	27	28	27
	b	0.002		1	1	28	29	28	29	28
	c	0.005	0.002		2	29	30	29	30	29
	am	0.005	0.002	0.005		27	28	27	28	27
B	d	0.065	0.067	0.070	0.065		1	14	9	8
	e	0.067	0.070	0.072	0.067	0.002		15	10	9
	f	0.065	0.067	0.070	0.065	0.034	0.036		11	10
	g	0.067	0.070	0.072	0.067	0.022	0.024	0.026		1
	ag	0.065	0.067	0.070	0.065	0.019	0.022	0.024	0.002	

For the isolation of DNA from the tissue samples, we used the QIAamp DNA Mini Kit (Qiagen) according to the recommendations of the supplier. We used the hypervariable region I of the mitochondrial D-loop as a genetic marker. To this aim, we amplified a section of mitochondrial DNA comprising the Thr- and Pro-tRNA genes and the hypervariable region I with primers mih1coau (5'-GTTATAGTTTCAGGTTAGTCA-3') and mih1cbau (5'-GATCTACTTATCCTTACATGA-3') in *M. murinus*, and with primers mih1coau and MCybfm (5'-CTAGTAGAATGRATCTGAGG-3') for *M. griseorufus*. We performed wax-mediated hot start PCR on a thermal cycler (PTC-200; MJ Research) in 30 µl reactions with 0.5 U AmpliTaq polymerase (Perkin Elmer). We sequenced all PCR products on both strands using nested primers (*M. murinus*: mih1coi

(5'-GAGCGAGAAGAGGGGCA-3') and mih1cbi (5'-ATTATGCCAACCGTAAGCC-3'); *M. griseorufus*: mih1coi and mih1cbi2 (5'-TTATACCWACYGTAAGYCTT-3')), the Thermo Sequenase Fluorescent Primer Cycle Sequencing Kit (Amersham), and a Li-COR two color dnasequencer long readir 4200.

We aligned the obtained sequences using the BioEdit sequence alignment editor (Hall 1999) and the Clustal-W module implemented therein. We calculated average transition/transversion rates and pairwise uncorrected p-distances among non-identical haplotypes using the Mega 3.0 software (Kumar *et al.* 2004). For these calculations, we analyzed the segment of the alignment containing sequences of the mitochondrial D-loop. In order to evaluate a potential influence of differing sample

sizes on the detection of haplotypes, we used the FSTAT version 2.9.3 software (Goudet 2002) to calculate the allelic richness index for the number of haplotypes in each sample based on the smallest sample size of eight individuals.

In order to investigate the phylogenetic relationships among the mitochondrial haplotypes, we performed a phylogenetic tree reconstruction using the maximum likelihood quartet puzzling algorithm implemented in Tree-Puzzle 5.0 (Strimmer and von Haeseler 1996). We applied the HKY model of sequence evolution (Hasegawa *et al.* 1985) with uniform substitution rate and 10,000 puzzling steps. We used the alignment of sequences of the hypervariable region I with *M. griseorufus* as the outgroup for the tree reconstruction.

Results

Within the 256 individuals of *Microcebus murinus*, we observed nine different mitochondrial haplotypes (Table 1). We deposited all sequences in the NCBI GenBank (accession numbers: DQ865140-DQ865149). The sequences of the Thr- and Pro-tRNA genes represented functional tRNA structures in all haplotypes. There was only one variable position within the tRNA genes of the nine haplotypes, and it was situated in a loop of the Thr-tRNA.

The alignment of the analyzed section of the mitochondrial D-loop comprised 419 bp (417 bp and two indel positions in the ingroup). The average transition/transversion ratio of all pairwise comparisons of non-identical *M. murinus* haplotypes was 18.4. The phylogenetic tree reconstruction (Fig. 3) revealed two distinct and statistically well supported clusters of haplotypes. We refer to them herein as clade A and B. Clade A comprised the haplotypes a, b, c, and am, and was confined to the littoral forests of Mandena and Lokaro. Clade B comprised the haplotypes d, e, f, g, and ag, which exclusively occurred in the littoral forest of Petriky. Table 2 displays pairwise genetic distances between all *M. murinus* haplotypes. Distances within the Mandena/Lokaro cluster, clade A, ranged from 0.002 to 0.005, and were considerably smaller than those within the Petriky cluster, clade B (0.002 - 0.036). The two most divergent haplotypes at Petriky were approximately 7 times more distant than those at Mandena. At Lokaro, there was only one haplotype, which was identical to the most frequent one at Mandena. Distances between clades A and B ranged from

0.065 to 0.072.

Most nucleotide differences were transitional substitutions, with up to 14 transitions between haplotypes of clade B, and up to 29 transitions between haplotypes of the two clades. The maximum number of observed, transversal substitutions between haplotypes of the two groups was two.

Table 1 and Figure 2 display the geographic distribution of haplotypes of clade A at Mandena and Lokaro. The most conspicuous aspect of this distribution is the remarkable dominance of haplotype b, which occurred in 154 of the 200 individuals sampled, and in all the littoral forest blocks of Mandena and Lokaro. Additional haplotypes occurred only in the four western blocks of Mandena, M13, M15/M16, and M20, whereas in all forests further east, only haplotype b was observed.

In most cases, sample sizes of the five forests where more than one haplotype was observed were larger than those of forests where only one haplotype was observed (Table 1). The values of the allelic richness index based on the smallest sample size of eight individuals for the samples where more than one haplotype was observed ranged from 1.849 to 3.567 (Table 1).

Discussion

A potential pitfall in population genetic analysis using mitochondrial genetic markers is the amplification of Numts, or nuclear mitochondrial pseudogenes, which are nonfunctional nuclear copies of mitochondrial loci instead of true mitochondrial DNA (e.g., Zischler *et al.* 1995). Our results yield strong evidence that the observed sequences are true mitochondrial haplotypes. The observed strong transition/transversion bias is typical for the evolution of the mitochondrial genome. The observed variation among the nine haplotypes is almost exclusively restricted to the hypervariable region I, whereas the tRNA genes are preserved. This finding indicates a high level of variation in the non-coding, hypervariable region I, and functional constraints in the tRNA genes, which would not be present in nuclear pseudogenes. This is further underscored by the fact that the single variable site within the tRNA genes is a transition in a loop-region.

When regarding the geographic distribution of genetic variation in *Microcebus murinus*, the most conspicuous pattern in our results is the existence of two genetically distinct groups of mitochondrial

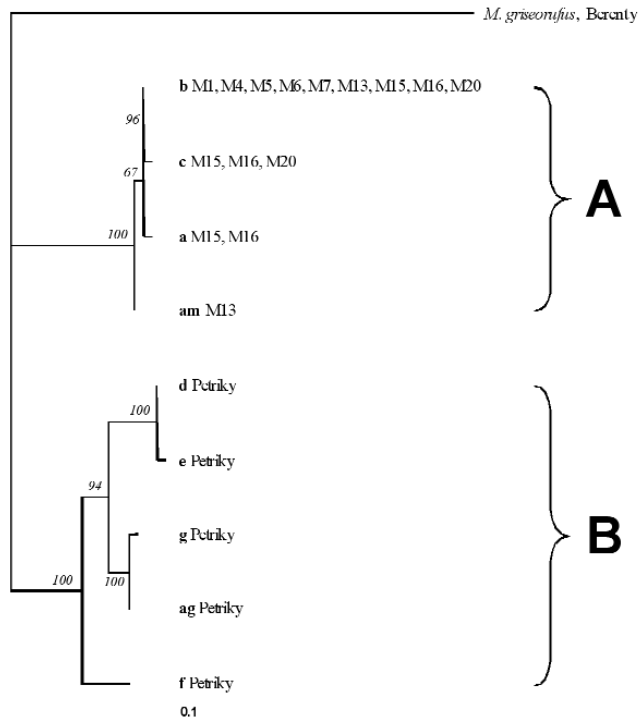


Figure 3. Phylogenetic tree of *Microcebus murinus* populations in the Tolagnaro region. Quartet puzzling maximum-likelihood tree reconstruction based on sequences of the hypervariable region I. Outgroup: *M. griseorufus*. Haplotype identifiers: lower case characters that denominate the individual haplotypes are followed by a list of sampling sites, where these were observed (see also Table 1). The capital letters "A" and "B" denominate the two major clades. Numbers in italics are maximum-likelihood support values, which indicate the statistical support of internal nodes. The length of the bar indicates 0.1 substitutions per position.

haplotypes with spatially exclusive distributions. Such geographic clusters of related mitochondrial haplotypes are typical for female philopatry and male-biased dispersal as exemplified by, for example, several macaque species (Melnick and Hoelzer 1992, Hoelzer *et al.* 1994, Perwitasari-Farajallah *et al.* 1999). Female philopatry and male-biased dispersal have been demonstrated for *M. murinus* in northern (Radespiel *et al.* 2001, 2003) and western (Wimmer *et al.* 2002, Fredsted *et al.* 2004) dry, deciduous forests.

On a smaller geographic scale, the presence of one dominant haplotype in all littoral forests of Mandena and Lokaro is unexpected under female philopatry. Moreover, a trend of decreasing genetic diversity in west-east direction emerges with five different haplotypes in Petriky, two to three haplotypes in the western forest blocks of Mandena, and only one haplotype in the eastern forests blocks of Mandena and at Lokaro. This trend could be related to increasing effects of fragmentation and isolation. The four eastern blocks of forest at Mandena seem to be more strongly isolated from each other by open land than the four western blocks, which are connected by different types of forest habitats such

as an exotic tree plantation, secondary, and riverine forest. One could thus compare one big littoral forest at Petriky to a group of small, connected blocks in the western part of Mandena, a group of small, even more isolated blocks in the eastern part of Mandena, and one small, isolated block at Lokaro.

In this context, it is important to evaluate the potential influence of sample size on the number of observed haplotypes. Indeed, at Lokaro and eastern Mandena, with samples of 8 to 16 individuals all of which each represent one observed haplotype, and western Mandena (M20) with only two haplotypes. The allelic richness index, however, clearly indicates that more than one haplotype would be expected in all samples with several observed haplotypes, even with the sample size of eight individuals. As well as numbers of observed haplotypes, the values of the allelic richness index, which are independent of sample sizes, display a trend of decreasing diversity in a west-east direction. The trend of decreasing genetic diversity is also reflected on the level of sequence divergence - haplotypes in the western clade B at Petriky are much more divergent than those in the eastern clade A at Mandena and Lokaro.

One can construct and compare different scenarios in order to explain the geographic distribution of genetic diversity in *M. murinus* in southeastern Madagascar. Fragmentation and genetic erosion could be to blame. The blocks of forest in Mandena and Lokaro are remnants of one, formerly large forest. Genetic diversity has been decreased by fragmentation to isolated blocks and subsequent genetic erosion. Another possibility is that a bottleneck effect has occurred at Mandena and Lokaro in the course of a recent colonization or a population breakdown.

Among these scenarios, the first is less likely than the second. Under a fragmentation scenario, different, randomly selected matrilineages would be expected to survive in increasingly isolated fragments. This scenario is thus ruled out by the observation of one dominant haplotype, which is present in all forest fragments of Mandena and Lokaro. Under the second scenario, a bottleneck effect could as easily have been caused by recent colonization as by a population breakdown. Neither possibility can be confirmed with the existing mitochondrial data. However, the scenario of a recent colonization of Mandena and Lokaro appears most plausible with respect to the distribution of *M. murinus* in Madagascar. The species is distributed in a large area of western dry deciduous forests and occurs within the southern xerophytic domain. At the southeastern edge, the distribution turns east into a transition zone and extends towards the humid eastern domain, where a small stretch is represented by the littoral forests of Petriky, Mandena, and Lokaro. With respect to the steep climatic gradient within the transition zone of Tolagnaro, a minute climatic change could have led to a gradual vegetation change and a shift to the east of the zone of suitable habitat for *M. murinus*.

When regarding the maximum genetic distances among the two groups of haplotypes, it becomes clear that the last common ancestor of the matrilineages of Mandena and Lokaro has lived seven times more recently than that of the Petriky group. While the radiation of Mandena and Lokaro is thus much younger than that of Petriky, a direct colonization of Mandena from Petriky does not appear very likely. Rivers form strong geographic barriers to the direct migration of lemurs, following the coast to the east and north, to the east of Petriky, and to the east of the easternmost population at Lokaro. In addition, the large genetic distance between the two haplotype groups indicates a long and independent history. It appears more likely that Mandena has been colonized by a population from the west, which originated even further inland.

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