MORPHOLOGY, BEHAVIOUR AND MOLECULAR EVOLUTION OF GIANT MOUSE LEMURS (*MIRZA* SPP.) GRAY, 1870, WITH DESCRIPTION OF A NEW SPECIES.

Kappeler PM, Rasoloarison RM, Razafimanantsoa L, Walter L and Roos C

Key Words: *Mirza*, morphometrics, biogeography, phylogeny, mitochondrial cytochrome *b*, *Microcebus*, new species

Abstract

Giant mouse lemurs (genus *Mirza)* are small nocturnal primates endemic to Madagascar, of which a single species (*M. coquereli*) is currently recognized. It is distributed along Madagascar's west coast, with a gap of several hundred kilometres between two presumed subpopulations. Previous studies in the field and in captivity indicated substantial differences in several aspects of the biology of these two subpopulations. We therefore collected morphometric, genetic and behavioural data from populations representing the southern and northern end of their range to examine these differences in more detail. We obtained standard morphometric field measurements and DNA samples from a total of 74 adult males and females at Kirindy (central western Madagascar) and Ambato (northwestern Madagascar) and compared their social organisation. We also studied a total of 9 *Mirza* specimens housed at the Rijksmuseum van Natuurlijke Historie Leiden (The Netherlands). Our morphometric analyses revealed that the two *Mirza* populations differed significantly in 12 out of 13 measures, with the northern *Mirza* sporting smaller values in all traits except testes volume. Northern *Mirza* spent the day in nests with 2-8 (mean 4.1) individuals, whereas *Mirza* in the south virtually always slept alone. Moreover, reproduction in the northern population occurred several months earlier than in the south. We also sequenced the complete mitochondrial cytochrome *b* (cyt *b*) gene from several specimens and found that (1) the two populations differed by 3.33-3.51 %, which is similar to genetic distances observed among several closely related species of mouse lemurs (*Microcebus*), (2) DNA extracted from tissue on skulls collected in 1868/1870 yielded partial cyt *b* sequences that aligned perfectly with the northern and southern population samples, respectively, and (3) *Microcebus* from Andasibe clearly differed genetically from all other known mouse lemur species, indicating a separate species status for this population. Based on the combination of morphological, behavioural and genetic differences between *Mirza* from Kirindy and Ambato we conclude that they should be separated at the species level. Because *M. coquereli* was described based on a specimen from the southern population, we describe the northern *Mirza* as a species new to science.

Introduction

Madagascar is a global hot spot for biodiversity and conservation (GOODMAN and BENSTEAD, 2003; MITTERMEIER et al., 1998; MYERS et al., 2000; SECH-

REST et al., 2002; YODER et al., 2005). Because of the number of endemic taxa and their phylogenetic history and position, the primates of Madagascar (Lemuriformes), in particular, represent one of the top global primate conservation priorities (GANZHORN et al., 1997a,b; JERNWALL and WRIGHT, 1998). Information about the taxonomic status, geographical distribution and abundance of individual taxa constitute the necessary scientific basis for the development of effective conservation action plans and their legal implementation. In the case of lemurs, these basic data are still far from complete because many new taxa or new localities for known taxa continue to be described, even in the new millennium (GROVES, 2000; LOUIS et al., 2005; MAYOR et al., 2004; RASOLOARISON et al., 2000; THALMANN and GEISSMANN, 2002) and their phylogenetic relationships are far from being resolved (FAUSSER et al., 2004; NIEVERGELT et al., 2002; PASTORINI et al., 2001a,b, 2002a,b, 2003; ROOS et al., 2004; YODER et al., 2000). Ongoing field studies, genetic analyses and museum work all indicate that the full taxonomic diversity of lemurs is still incompletely described and that conservation priorities need to be updated constantly.

The mouse and dwarf lemurs (Cheirogaleidae) represent the largest lemur family. All members of the five currently recognized genera (*Allocebus*, *Cheirogaleus, Microcebus*, *Mirza* and *Phaner*) are relatively small (< 500 g) and nocturnal (MARTIN, 1972). Several field studies of cheirogaleids initiated in the 1990s used extensive trapping, detailed morphometrics and various genetic tools to address questions about their behavioural ecology. These new kinds of data also revealed the existence of new species (SCHMID and KAPPELER, 1994; ZIMMERMANN et al., 1998) and prompted systematic taxonomic work on this group, using a combination of osteological, morphometrical and genetic data (GROVES, 2000; PASTORINI et al., 2001b; RASOLOARISON et al., 2000; RUMPLER et al., 1998; YODER et al., 1996, 2000, 2002). Despite difficulties arising from many synonyms, missing and damaged holotypes or those with vague collection localities and descriptions based on lectotypes or neotypes, the previously single recognized species of west coast mouse lemur (*Microcebus murinus*) could be differentiated into seven different species, three of which were new to science (RASOLOARISON et al., 2000). A similar taxonomic revision of the genus *Cheirogaleus*, albeit based on analyses of museum specimens alone, has indicated the existence of several species of dwarf lemurs along Madagascar's east coast, where traditionally only one species (*C. major*) had been recognized (GROVES, 2000). The overdue re-evaluations of the taxonomic status and phylogenetic position of *Phaner* and *Allocebus* still await further field data (GROVES and TATTERSALL, 1991).

The genus *Mirza* GRAY**,** 1870 is currently represented by a single recognized species: Coquerel's dwarf lemur, *M. coquereli* (GRANDIDIER**,** 1867). While mainly considered as a mouse lemur (e.g. PETTER and PETTER-ROUSSEAUX, 1979), it was resurrected as separate genus by TATTERSALL (1982) because of its larger size and various behavioural and morphological differences with *Microcebus*. The genus *Mirza* is restricted to western lowland forests, where it appears to have a disjunct distribution, with a gap between subpopulations spanning several hundred kilometres (Fig. 1). However, neither the exact limits of remaining population centres, nor the actual and historical presence or absence of *Mirza* in the intermediate regions are known (MITTERMEIER et al., 1994; PETTER et al., 1977; TATTERSALL, 1982).

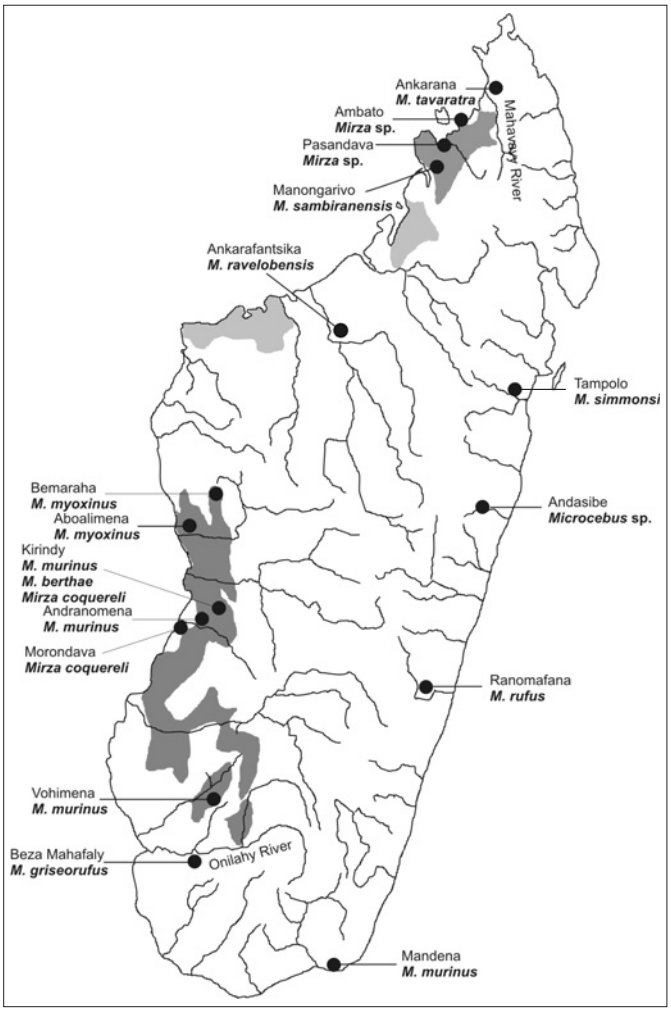

Fig. 1: Distribution of the genus *Mirza* (black: confirmed, grey: inferred). Dots indicate origin of analysed *Mirza* and *Microcebus* samples (see also Table 1).

Today, *Mirza* is found in the Sambirano region, with the northern Mahavavy river as a possible northern boundary of this species' range, whereas in the south, it is known to occur between the Parc National de Bemaraha and the Parc National de Zombitse-Vohibasia, with a possible southern limit of their distribution somewhere north of the Onilahy river (KAPPELER, 2003). Most recently, *Mirza* has also been sighted in the Réserve Naturelle Intégrale Tsingy de Namoroka (S. GOODMAN, pers. comm.), which is between these two distribution nuclei.

The behavioural ecology of *Mirza* has been studied both near the southern and northern ends of its geographical distribution. The first detailed description of the natural history of *Mirza* was provided by PETTER et al. (1971), who collected opportunistic observations near Beroboka, north of Morondava. ANDRIANARIVO (1981) conducted the first systematic study of ranging and social behaviour in the northern population on the Ampasindava Peninsula near Ampasikely, northwest of Ambanja. A study by PAGES (1978, 1980) in Marosalaza forest north of Morondava focused on social organisation and feeding ecology. Finally, an ongoing study in Kirindy Forest (CFPF) north of Morondava has provided data on the social and genetic structure of another southern population (KAPPELER, 1997a; KAPPELER et al., 2002). The largest captive population of *Mirza*, which is housed at Duke University Primate Centre, was the subject of several behavioural and physiological studies (STANGER, 1995; STANGER et al., 1995). This colony was established in 1982 with animals from the Ambanja region captured near Antamboro between the villages of Ampasimbary and Ampasindava (D. HARING, pers. comm.).

These previous studies have indicated the possible existence of behavioural and morphological differences between the northern and southern populations, e.g. in sexual dimorphism, relative testes size and seasonality of reproduction (KAPPELER, 1997a,b, 2003; but see ALBRECHT et al., 1990), but a direct comparison has not been attempted. To investigate possible taxonomic differentiation that may underlie these differences, we initiated a comparative field study of the northern and southern population and examined museum specimens from both regions. We conclude that the observed behavioural, morphological and genetic differences merit separation of the two populations at the species level and describe the northern population of *Mirza* as a new species.

Materials and Methods

Fieldwork

Field studies of *Mirza* were conducted in Kirindy Forest (20°40'S, 44°39'E), where the German Primate Centre maintains a permanent research station, and on the Ambato Peninsula, situated between the town of Ambanja and the island of Nosy Faly, where we found *Mirza* at the same site on Ermitage beach (13°25'S, 48°29'E) where ANDRIANARIVO (1981) conducted his study. At Kirindy, *Mirza* has been studied continuously since 1993, whereas we studied the Ambato population during three separate trips in March 1999, April 2000 and October 2000. The study area in the dry deciduous forest at Kirindy has been described in great detail elsewhere (KAPPELER, 1997a; SORG et al., 2003). At Ambato, *Mirza* was found in a highly degraded 4 ha patch of forest at the tip of a small peninsula next to the Hotel Ermitage Plage. This small piece of forest, vegetationally referable to the Sambirano Domain (HUMBERT, 1955) also contained several prominent mango trees and was accessible through a small set of foot trails.

At both field sites, we set Sherman and Tomahawk live traps and baited them with pieces of banana, mango or pineapple to capture *Mirza*. In addition, several individuals at Ambato were captured by hand from their daytime nests by local assistants. Captured animals were briefly anesthetised with 0.1 ml "Ketanest 100" and subjected to standard morphometric measurements, including body mass, head length and width, head-body length, tail length and hind foot length (following RASOLOARISON et al., 2000; SCHMID and KAPPELER, 1994). Not all measurements were taken from all animals in order to avoid stressing of awakening individuals. In addition, a small piece of ear skin (2x2 mm) was removed and stored in 70 % ethanol for later DNA-extraction and genetic analyses. Some animals at both sites were fitted with a small radio-tag (Biotrack, UK) to facilitate subsequent location and behavioural observations, including nesting habits.

Museum work

The National Museum of Natural History (Rijksmuseum van Natuurlijke Historie, RMNH), Leiden, The Netherlands, houses several *Mirza* specimens (RMNH 39375-39390), which were collected in the late 19th century at the "Bords du Mouroundava" [=Morondava] and the "Baie de Pasandava" [=Ampasindava]. We measured and compared several cranial landmarks on their skulls, using measurements

employed by RASOLOARISON et al. (2000), and examined mounted specimens externally. Small pieces of dried tissue could be obtained from the base of the skull of some specimens, and we successfully isolated and amplified DNA from these samples for comparison with samples obtained during our field studies.

Statistical analyses

Quantitative analyses of morphometric data were limited to adult individuals, i.e. those weighing more than 250 grams (KAPPELER, 1997a). Cranial measurements obtained at the RMNH could not be compared statistically because the skull of one of three northern specimens was heavily damaged and one was a subadult. We used t-tests to compare mean measurements from males and females or from combined samples of individuals from Kirindy and Ambato, respectively. We tested for possible interactions between sex and origin by using a 2-way ANOVA. Because of the large number of tests based on this data set, alpha was set at 0.01 for all tests to guard against Type I errors.

Molecular genetics

Genetic analyses were based on two different sample types. Ear clips from eight wild-caught *Mirza*, representing populations from Kirindy and Ambato, were collected during field surveys and stored in 70 % ethanol before further processing. Tissue material from six museum specimens of *Mirza* from Morondava and Pasandava was obtained from the RMNH. DNA from the tissue materials was extracted with the QIAamp DNA Mini Kit as recommended by the supplier and stored at -20° C. The complete mitochondrial cytochrome *b* (cyt *b*) gene was PCR amplified (SAIKI et al., 1988) with the oligonucleotide primers CYT-L: 5'-AAT GAT ATG AAA AAC CAT CGT TGT A-3' and CYT-H: 5'-AAC TGC AGT CAT CTC CGG TTT ACA AGA C-3'. Standard, wax mediated hot-start PCRs were carried out for 40 cycles, each with a denaturation step at 94° C for 60 sec., annealing at 60° C for 60 sec. and extension at 72° C for 90 sec., followed by a final extension step for 5 min.. Because of the expected difficulties to amplify longer fragments from museum material, we amplified only a 192 bp long fragment of the cyt *b* gene (position 525 to 716 of the complete gene), using primers 5'-ACA CGA TTC TTT GCA TTC CAC-3' and 5'-AGT AGA AGT AGG AGA AAG AGG-3' with PCR conditions as described above with the exception that the extension time was reduced to 30 sec.. The results of the PCR amplifications were checked by running an aliquot on a 1 % agarose gel, stained with ethidium bromide. Subsequently, PCR products were cleaned with the Qiagen PCR Purification Kit and sequenced on an ABI 3100-Avant sequencer using the BigDye Terminator Cycle Sequencing Kit from Applied Biosystems and the primers as indicated above. The respective sequences were deposited in GenBank and are available under the accession numbers DQ093169-DQ093182, DQ095782 and DQ095783.

Sequences were easily aligned by eye due to the lack of insertions or deletions and checked for their potential to be correctly transcribed in order to eliminate data set contaminations with pseudogenes. For a comprehensive evaluation of the sequence data, we expanded our data set with self-generated sequences from two specimens of *Microcebus rufus* from Andasibe and with orthologous sequences already deposited at GenBank from most currently recognized *Microcebus* species (YODER et al., 2000), one individual of *Mirza coquereli* (YODER et al., 1996) and one individual of

Allocebus trichotis (ROOS et al., 2004), which was used as outgroup for phylogenetic tree reconstructions. The final alignment comprised 24 sequences with 1140 bp in length. Further details about individuals and sequences are summarized in Fig. 1 and Table 1.

Table 1: Origin, sample type and GenBank accession number of analysed species for genetic studies.

species	**abbre-viation**	**origin**	**sample type**	**GenBank**
Allocebus trichotis	-	-	sequence	AY441461
Microcebus murinus	-	Mandena	sequence	AF285565
M. murinus	-	Vohimena	sequence	AF285564
M. murinus	-	Kirindy	sequence	AF285561
M. murinus	-	Andranomena	sequence	AF285559
M. griseorufus	-	Beza Mahafaly	sequence	AF285568
M. ravelobensis	-	Ankarafantsika	sequence	AF285532
M. tavaratra	-	Ankarana	sequence	AF285534
M. berthae	-	Kirindy	sequence	AF285543
M. myoxinus	-	Aboalimena	sequence	AF285539
M. myoxinus	-	Bemaraha	sequence	AF285535
M. sambiranensis	-	Manongarivo	sequence	AF285556
M. rufus	-	Ranomafana	sequence	AF285551
M. simmonsi	-	Tampolo	sequence	AF285553
Microcebus sp.	-	Andasibe*	feces	DQ095782
Microcebus sp.	-	Andasibe*	feces	DQ095783
Mirza sp.	-	Antanboro, close to Pasandava	sequence	U53571
Mirza sp.	Ambato 1	Ambato	tissue	DQ093169
Mirza sp.	Ambato 2	Ambato	tissue	DQ093170
Mirza sp.	Ambato 3	Ambato	tissue	DQ093171
Mirza sp.	Ambato 4	Ambato	tissue	DQ093172
Mirza sp.	Pasandava 10	Pasandava	tissue,RMNH 39375	DQ093173
Mirza sp.	Pasandava 11	Pasandava	tissue,RNMH 39376	DQ093174
Mirza coquereli	Kirindy 1	Kirindy	tissue	DQ093175
M. coquereli	Kirindy 2	Kirindy	tissue	DQ093176
M. coquereli	Kirindy 3	Kirindy	tissue	DQ093177
M. coquereli	Kirindy 4	Kirindy	tissue	DQ093178
M. coquereli	Morondava 12	Morondava	tissue,RNMH 39385	DQ093179
M. coquereli	Morondava 13	Morondava	tissue,RNMH 39381	DQ093180
M. coquereli	Morondava 14	Morondava	tissue,RNMH 39380	DQ093181
M. coquereli	Morondava 15	Morondava	tissue,RNMH 39382	DQ093182
* individual kept at the Zürich Zoo, Zürich, Switzerland				

Because of the upcoming description of three new *Microcebus* species from the east coast of Madagascar (LOUIS et al., 2005), a BLAST search was performed to identify the species affinity of the "*M. rufus*" individuals in our data set. Absolute pairwise differences within and between species and genera were calculated with PAUP 4.0b10 (SWOFFORD, 1999) and DnaSP 3.52 (ROZAS and ROZAS, 1998).

Phylogenetic tree reconstructions based on complete cyt *b* sequences were carried out with the maximum-parsimony (MP), neighbor-joining (NJ) and maximum-likelihood (ML) algorithms as implemented in PAUP or TREEPUZZLE 5.0 (STRIMMER and VON HAESELER, 1996). For MP analyses, all characters were treated as unordered and equally weighted throughout. NJ and ML trees were constructed with the TrN + I + Γ model of sequence evolution, because it was selected as best fitting model with MODELTEST 3.06 (POSADA and CRANDALL, 1998). Relative support of internal nodes was performed by bootstrap analyses (MP and NJ) with 1,000 replications or by the quartet puzzling support values on the basis of 1,000 puzzling steps (ML).

To examine the existence of significantly different lineage-specific evolutionary rates observable in the data set, we performed a relative rate test with the RRTree program (ROBINSON et al., 1998) for all possible pairwise comparisons and using the *Allocebus trichotis* sequence as outgroup.

Divergence dates were estimated with the r8s program, version 1.7 (SANDERSON, 2003) on the basis of estimated branch lengths as deduced from the NJ reconstruction. Age calculation was conducted with the nonparametric method and Powell's optimisation, with all other settings set by default. As calibration points we used the proposed 24.2 million years ago (mya) for the divergence between *Mirza* and *Microcebus* (YODER and YANG, 2004).

Results

Morphology

Two sets of morphometric data were available for the present analyses: external measurements form individuals captured at Kirindy (26 adult females and 30 adult males) and Ambato (8 adult females and 10 adult males), as well as cranial measurements from museum specimens at RMNH (7 from Morondava and 2 from Pasandava). Descriptive statistics for measurements taken at Kirindy and Ambato are summarized in Table 2. *Mirza* from Kirindy were on average heavier than animals from Ambato (Fig. 2; 2-way ANOVA: Origin $F_{1;70} = 4.38$, $p = 0.04$; Sex $F_{1;70} = 0.23$, NS; Origin*Sex $F_{1;70} = 4.56$, $p = 0.03$); despite the fact that several Ambato females were pregnant. Because there was no significant sex effect on body mass, only results of comparisons of combined-sex samples are presented below. The two populations did not differ significantly in body length ($t = 1.58$, $df = 24$, $p = 0.12$), but in tail length ($t = 11.0$, $df = 48$, $p < 0.001$), head length ($t = 3.29$, $df = 51$, $p < 0.001$), head width ($t = 5.97$, $df = 51$, $p < 0.001$), canine height ($t = 10.3$, $df = 55$, $p < 0.001$), ear length ($t = 11.0$, $df = 40$, $p < 0.001$), hind foot length ($t = 5.42$, $df = 49$, $p < 0.001$), as well as the length of their femur ($t = 2.07$, $df = 35$, $p < 0.05$), humerus ($t = 3.03$, $df = 36$, $p < 0.001$), radius ($t = 6.62$, $df = 35$, $p < 0.001$) and tibia ($t = 5.21$, $df = 35$, $p < 0.001$). In all cases, *Mirza*

from Ambato had the smaller means (Table 2). Northern *Mirza* only had marginally larger testes (t = 1.91, df = 38, p = 0.06).

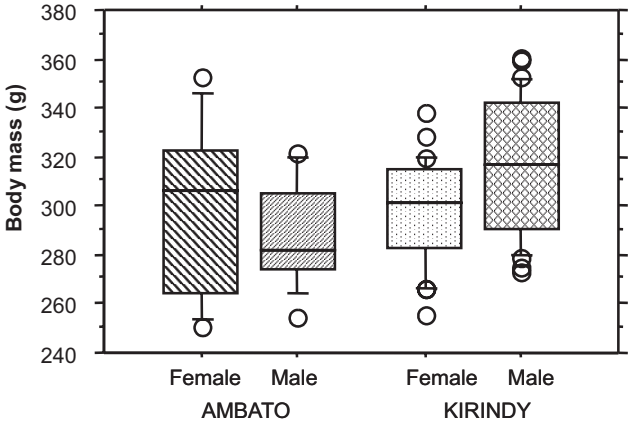

Fig. 2: Box plots depicting variation in body mass among male and female *Mirza* from Ambato and Kirindy.

Proportional differences between members of the two populations were most pronounced with respect to canine height, tail length, ear length and testes volume (Fig. 3). When we used head length to control for size effects, most variables scaled allometrically with a clear separation between northern and southern populations with little to no overlap (Fig. 4). The northern *Mirza* clearly is a scaled-down version of the southern *Mirza*.

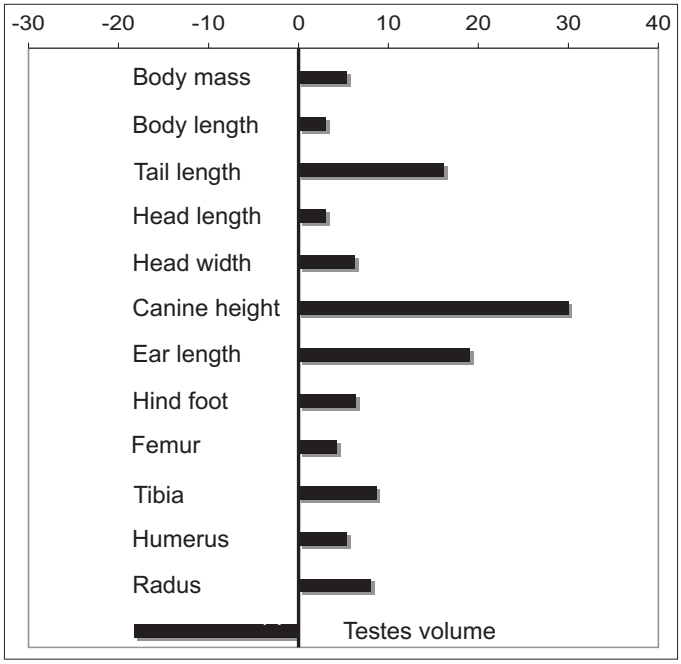

Fig. 3: Proportional differences between northern and southern *Mirza* in morphometric variables. The northern population (Ambato) served as a reference for the Kirindy population.

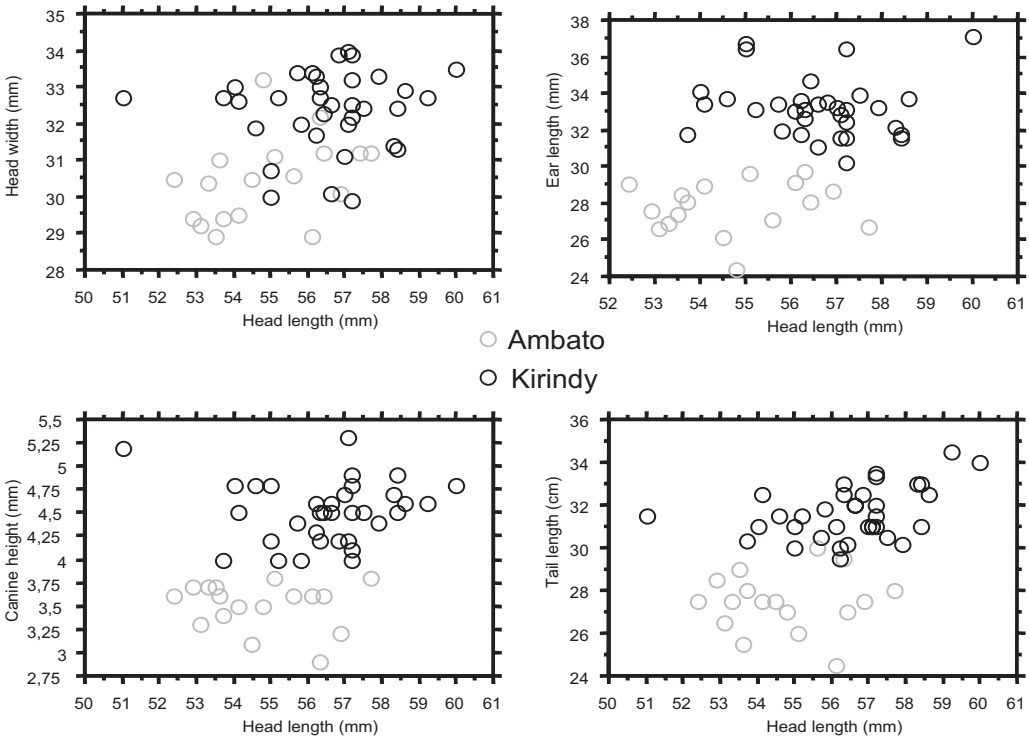

Fig. 4: Allometry of the two *Mirza* populations. Head width and the three most deviant variables (ear length, tail length and canine height; see Fig 3) are scaled against head length for northern (grey) and southern (black) *Mirza*.

Behaviour

Adult *Mirza* at Kirindy occupied home ranges of about 4 ha that overlapped to various extents with those of neighbours (KAPPELER, 1997a). They spent the day in self-constructed nests, most of which were built 1-2 m below the top of *Securinega* (Family Euphorbiaceae) trees (SARIKAYA and KAPPELER, 1997). Radio-collared adult males and females were never found to share a nest during the day (KAPPELER, 1997a). At Ambato, in stark contrast, we always found *Mirza* sleeping in groups of 2-8 (mean 4.1; N = 18 nests) individuals. Because many of these nests contained radio-collared or marked individuals, who were observed at dawn while leaving a nest, or because nests were emptied by hand during the day, we could identify the age/sex class of most individuals. We found that nests at Ambato contained on average 0.77 adult females, 1.06 adult males, 0.44 juveniles and 1.89 unidentified individuals. This form of gregariousness had already been noted by ANDRIANARIVO (1981) and is not due to a lack of nests in this disturbed habitat because i) we also captured 5 animals from a single nest in a much larger forest at the foothills of Ambato Massif several kilometres to the east, and ii) each radio-tagged individual used between 2-5 different nests on the 3-7 days they could be located. The social organization of the *Mirza* populations at Kirindy and Ambato are therefore fundamentally different in this respect.

Reproductive activity in the Morondava region is restricted to a few weeks in November (PAGES, 1978, 1980; KAPPELER, 1997a). At Ambato, we captured 4 pregnant females with clearly palpable foetuses (3 singletons; 1 pair of twins) during late September and early October in 2000. Mating at Ambato therefore presumably takes place in July and August. Members of the captive population at the Duke University Primate Centre reproduced year-round (STANGER et al., 1995).

Molecular genetics

Complete mitochondrial cytochrome *b* gene sequences were generated from eight wild caught giant lemurs and two *Microcebus* individuals from Andasibe, as well as a 142 bp long fragment from six museum specimens representing the two *Mirza* populations. The short fragment displayed five diagnostic mutations to distinguish between the populations from Morondava-Kirindy and Pasandava-Ambato (Fig. 5).

To obtain a comprehensive overview of *Microcebus* and *Mirza* evolution, we expanded our data set with orthologous sequences from all currently recognized *Microcebus* spp. and one *Mirza* individual, which were already deposited at Gen Bank (YODER et al., 1996, 2000). Mouse lemurs distributed along the east coast, traditionally comprised within *M. rufus*, were available from Ranomafana, Anda-

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                                                                      71
U53571          TACCTTTTATCATCACAGCCCTAGTAATAGTTCACCTCCTTTTCCTTCACGAAACAGGATCCAATAACCCA
Ambato1         ............................................................................
Ambato2         ............................................................................
Ambato3         ............................................................................
Ambato4         ............................................................................
Pasandava10     ............................................................................
Pasandava11     ............................................................................
Kirindy1        ....C.........G..........G.C.....................................................
Kirindy2        ....C.........G..........G.C.....................................................
Kirindy3        ....C.........G..........G.C.....................................................
Kirindy4        ....C.........G..........G.C.....................................................
Morondava12     ....C.........G............C.....................................................
Morondava13     ....C.........G..........G.C.....................................................
Morondava14     ....C.........G..........G.C.....................................................
Morondava15     ....C.........G..........G.C.....................................................


                                                                     142
U53571          CTAGGTACCACATCAGACTCAGACAAAATCCCATTCCACCCATATTACACAATCAAAGACCTGCTAGGACT
Ambato1         ............................................................................
Ambato2         ............................................................................
Ambato3         ............................................................................
Ambato4         ............................................................................
Pasandava10     ............................................................................
Pasandava11     ............................................................................
Kirindy1        .....C.....................................................T..................
Kirindy2        .....C.....................................................T..................
Kirindy3        .....C.....................................................T..................
Kirindy4        .....C.....................................................T.........C........
Morondava12     .....C.....................................................T..................
Morondava13     .....C.....................................................T.........C........
Morondava14     .....C.....................................................T..................
Morondava15     .....C.....................................................T..................
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Fig. 5: 142 bp long alignment of the partial cyt *b* sequences including data obtained from museum material. Region represents position 554 to 695 of the complete cyt *b* gene with dots indicating identity with the reference sequence.

Table 2: Descriptive statistics (mean ± SD) for 13 morphometric variables of adult male (M) and female (F) *Mirza* from Ambato and Kirindy.

	Ambato						**Kirindy**					
	F		**M**		**sexes combined**		**F**		**M**		**sexes combined**	
	mean	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**	**mean**	**sd**
body mass	299.00	36.10	287.00	21.60	292.50	28.60	299.00	20.90	316.80	27.10	308.30	25.90
body length	24.10	0.96	24.50	0.41	24.28	0.75	24.90	1.92	25.03	1.11	25.01	1.27
tail length	27.90	1.30	27.10	1.41	27.27	1.38	31.30	1.08	31.81	1.29	31.69	1.23
head length	55.20	1.59	54.50	1.71	54.80	1.64	56.50	1.30	56.40	2.00	56.50	1.73
head width	30.90	1.25	30.10	0.97	30.40	1.15	32.10	0.91	32.50	1.15	32.30	1.07
canine height	3.32	0.28	3.63	0.12	3.50	0.25	4.47	0.37	4.60	0.38	4.55	0.38
ear length	27.10	1.72	28.30	0.91	27.80	1.41	32.70	1.32	33.60	1.72	33.10	1.63
hind foot	54.10	2.09	52.80	1.37	53.30	1.78	56.10	1.73	56.90	2.40	56.70	2.22
femur	58.10	2.45	59.60	1.16	58.70	2.03	60.40	3.33	61.70	3.22	61.20	3.32
tibia	67.20	3.14	69.60	1.71	68.20	2.74	73.10	2.66	74.90	3.06	74.12	2.98
humerus	44.40	2.67	44.70	0.90	44.50	1.98	46.20	2.44	47.40	1.82	46.90	2.12
radius	46.90	2.05	47.30	1.52	47.10	1.73	50.40	1.30	51.60	1.61	51.20	1.58
testes			21.60	5.29					17.65	5.79		

Table 3: Minimum and maximum pairwise genetic distances (in %) within and among analysed species.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1 *Mirza* sp. (Ambato)	0.18-0.79											
2 *M. coquereli* (Kirindy)	3.33-3.51	0.44-0.53										
3 *Microcebus berthae*	16.40-16.67	17.11-17.37	-									
4 *M. myoxinus*	16.67-17.54	16.75-17.37	4.30-4.65	1.32								
5 *M. ravelobensis*	17.54-17.81	17.46-17.72	10.18	10.26-10.61	-							
6 *M. tavaratra*	16.14-16.40	17.28-17.54	7.54	8.16-8.51	10.70	-						
7 *M. sambiranensis*	16.40-16.58	16.93-17.19	6.75	7.72-7.90	11.29	8.33	-					
8 *M. simmonsi*	16.32-16.58	17.02-17.28	6.49	7.81-7.98	11.49	8.33	7.02	-				
9 *M. rufus*	15.97-16.14	16.29-16.49	3.86	4.30-4.83	9.91	7.72	7.28	6.82	-			
10 *M.* sp. (Andasibe)	16.14-16.40	16.49-16.75	4.30	4.91-5.26	10.26	7.63	7.02	6.67	3.77	0.00		
11 *M. griseorufus*	16.84-17.28	16.14-16.40	12.19	13.07	13.07	13.60	12.02	12.63	12.72	12.19	-	
12 *M. murinus*	17.63-18.42	17.19-17.90	13.42-13.77	14.12-14.74	12.90-13.25	14.47-14.56	13.33-13.60	13.16-13.42	13.77-14.04	12.98-13.42	9.83-10.18	0.61-2.46

sibe and Tampolo. The type location of *M. rufus* is "*A few miles north of Fianarantsoa*, *central Betsileo*", which is close to Ranomafana and hence, the individual analysed herein most likely represents *M. rufus*. BLAST search with sequences from the Tampolo individual revealed that this specimen corresponds to *M. simmonsi* (LOUIS et al., 2005) from Betampona and Zahamena. The individuals from Andasibe are closely related to specimens from the Parc National de Mantadia, but not referable to any known species and are therefore described as a new species below.

We analysed individuals of *M. berthae* and *M. murinus* from different locations to compare their within-species variation with that observed between the *Mirza* populations. The average observed genetic differences among all sequences was 15.29 %, with the greatest differences being detected between genera (16.14-18.42 %). Among *Microcebus* spp., distances ranged from 3.77-14.74 %. The two *Mirza* populations differed in 3.33-3.51 %, which is similar to distances observed among several closely related *Microcebus* spp.. The average variations within the two *Mirza* populations from Ambato (n=4) and Kirindy (n=4) were 0.46 and 0.48 %, respectively (Table 3).

Phylogenetic analyses were conducted with the maximum-parsimony, neighbor-joining and maximum-likelihood methods. All obtained trees showed the same topology and differed only in their support values for certain branches (Fig. 6). The monophyly of each of the two genera *Mirza* and *Mircocebus* was highly supported

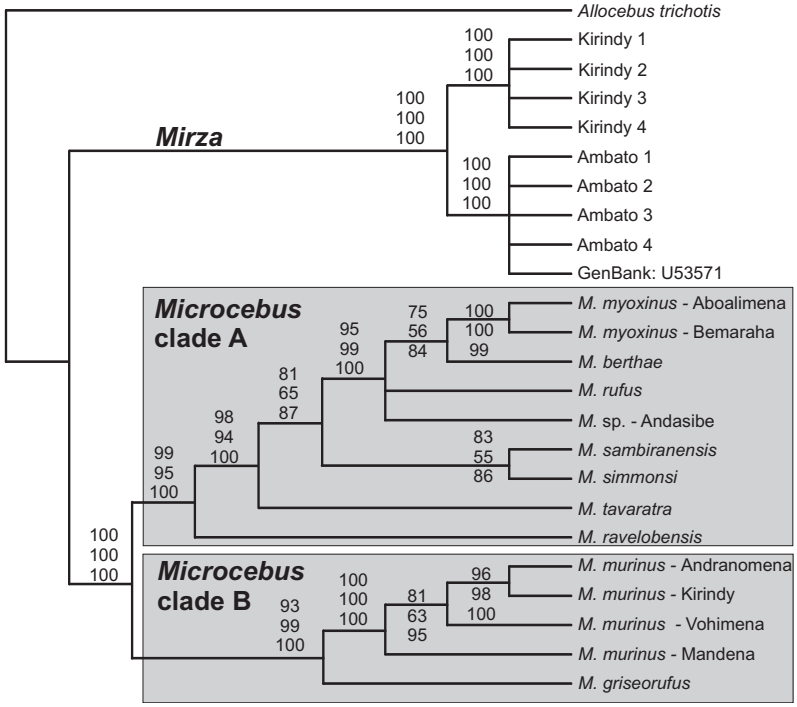

Fig. 6: Phylogenetic relationships as obtained from complete mitochondrial cyt *b* gene sequence data. Numbers on nodes indicate support values for internal branches (top: MP, middle: NJ, bottom: ML). *Microcebus* is divided into two main clades (A and B).

(100 %). Within *Mirza*, the two populations from Kirindy and Ambato are clearly separated into two significantly supported clades. The genus *Microcebus* is also divided into two major groups with one comprising the larger bodied species, *M. murinus* and *M. griseorufus* (designated as clade B) and the other, with all the remaining, smaller bodied species (clade A). Within clade B, a major split occurred between *M. griseorufus* and *M. murinus*. In clade A, *M. ravelobensis* was the first to split off, followed by *M. tavaratra*. The remaining species were further separated into a clade consisting of *M. sambiranensis* and *M. simmonsi*, and a clade containing *M. myoxinus*, *M. berthae*, *M. rufus* and *M.* sp. nov.. Relationships within *Microcebus* were mainly resolved and statistically highly supported.

Because significant rate differences were detected among lineages (data not shown), the calculation of splitting events was hampered by the absence of a molecular clock-like sequence evolution. To deal with these difficulties, time estimations were carried out using nonparametric methods that relax the stringency of the molecular clock assumption. Calculations were performed on the basis of branch lengths obtained from the NJ reconstruction in PAUP under the assumption of the TrN + I + Γ model of sequence evolution and applying the proposed 24.2 mya for the divergence between *Mirza* and *Microcebus* (YODER and YANG, 2004) as calibration point (Fig. 7). Accordingly, the initial split within the genus *Microcebus* occurred about 12.5 mya, which is in agreement with an earlier estimate of 12.0 mya (YODER

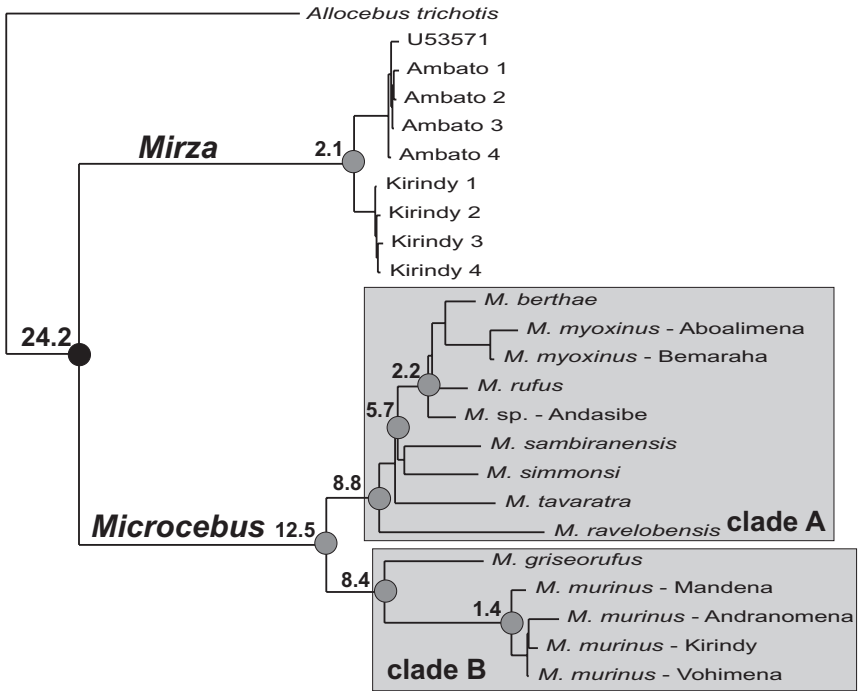

Fig. 7: Estimation of the most recent common ancestors based on complete mitochondrial cyt *b* sequence data. The black circle indicates the divergence between *Mirza* and *Microcebus* 24.2 mya, which was used as calibration point. Grey circles and respective numbers refer to calculated divergence ages in mya.

and YANG, 2004). In clade A, *M. ravelobensis* split off from other species about 8.8 mya. Later on, two radiation-like separations among the remaining species occurred about 5.7 and 2.2 mya, respectively. In clade B, *M. griseorufus* diverged from *M. murinus* about 8.4 mya. The two *Mirza* populations from Kirindy and Ambato were separated about 2.1 mya which is comparable with the most recent splitting event between species of the *Microcebus* clade A (2.2 mya) and much earlier than the separation of *M. murinus* populations (1.4 mya).

Discussion and Conclusions

In this study, we analysed and compared the external morphology, behaviour, reproductive activity and genetic variability at a mitochondrial DNA locus of samples from the two presumed main populations of *Mirza*. Our analyses revealed several pronounced differences between these two populations. First, northern *Mirza* were significantly smaller in all but one external measurement (body length). Because our sample from Ambato included several pregnant females, future comparisons of non-pregnant females should therefore reveal even more pronounced differences in mean body mass. These differences in external morphology were most pronounced in ear length, tail length and canine size, which were all about 20-30 % shorter in animals from Ambato. Comparisons of all external measurements that controlled for differences in body size indicated northern *Mirza* to be scaled-down versions of the animals from the south. Pelage colour was not systematically recorded (cf. RASOLOARISON et al., 2000), but variation within populations appeared to be as great as variation between populations. There may be a tendency for the pelage of northern animals to be less grey and slightly more reddish; their tails also appeared less dark towards the tip and their ventral parts were brighter. The mounted specimens at the RMNH were too faded to permit a meaningful direct comparison, however. The difference in ear size was the most pronounced and easily recognizable difference in external appearance between the two populations; also in mounted specimens.

Second, as in the captive population at Duke University Primate Centre, northern *Mirza* males had larger testes than the animals at Kirindy (see KAPPELER, 1997a). Because our field measurements of testes size were taken months before or after the presumed mating season of northern *Mirza*, the present data presumably represent an underestimate of maximal testes size. As in virtually all other seasonally breeding lemurs, testes size of *Mirza* at Kirindy increased several-fold in the few weeks before the brief annual mating season (KAPPELER, 1997a). Because northern *Mirza* are smaller and because they have absolutely larger testes outside the mating season than *Mirza* at Kirindy, their maximal testes size may be the largest one in relation to body size among strepsirrhines (KAPPELER, 1997b). The differences in weaponry (canine size) and testes size between the two populations nicely correspond to each other and match theoretical expectations for primates with different levels of mate monopolisation (SETCHELL and KAPPELER, 2003). These data suggest that the northern *Mirza* is more promiscuous than the southern individuals, and they match the differences in nesting behaviour described above.

Northern *Mirza* also reproduced months before the population at Kirindy. Because of our limited sampling, we do not know whether reproduction in Ambato is

strictly seasonal, as in Kirindy, or aseasonal, as in captivity. Reproduction in lemurs is generally controlled photoperiodically and occurs earlier in taxa at higher latitudes (RASMUSSEN, 1985). *Microcebus murinus*, for example, which are found along much of the west coast reproduce several weeks earlier in Ampijoroa, compared to Kirindy about 500 km to the south (EBERLE and KAPPELER, 2004; RADESPIEL et al., 2002). Future studies therefore need to determine whether reproduction in northern *Mirza* is only advanced or aseasonal, compared to the southern population.

Third, behavioural differences in daytime nesting were striking between northern and southern *Mirza*. Whereas the animals in Kirindy virtually always spent the day alone in their nests (apart from mothers and their offspring), we found on average more than 4 individuals at Ambato sharing a nest. We find it noteworthy that these nests tended to include several adult males with fully developed testes. In *M. murinus*, where the most detailed data on sleeping group composition exist, adult females form sleeping groups, whereas males typically sleep alone (RADESPIEL et al., 1999; WIMMER et al., 2002). It will therefore be interesting to study the social organisation of northern *Mirza* in more detail. Among other things, it will be interesting to determine whether co-sleeping females are also closely related (cf. KAPPELER et al., 2002).

At Ambato, *Mirza* density based on census walks was calculated to be much higher (1086 individuals/km^2) than at Kirindy (120 individuals/km^2; KAPPELER, 1997a), and also higher than the 385 individuals/km^2 estimated by ANDRIANARIVO in 1981. This high density may be due to the isolated status of this forest fragment, the presence of mango and other introduced food trees, and the fact that, except for a few groups of *Eulemur macaco macaco*, no other lemurs were present. To what extent *Mirza* competes with introduced *Rattus*, which was found in several *Mirza* nests, remains unknown. Thus, *Mirza* at Ambato occurs at relatively high density for unknown reasons, but so far they have only been studied at this one locality.

Finally, variation in cyt *b* sequences within the two *Mirza* populations was of similar magnitude as variation within several *Microcebus* samples. Differences between the two *Mirza* populations were within the lower end of the range of pairwise differences determined for several other pairs of widely recognized *Microcebus* species. DNA obtained from the museum specimens at the RMNH fell squarely within the clusters of the respective field samples. We therefore conclude that the average genetic differences at this locus are comparable to differences observed between other pairs of closely related species. In addition, we found that the cyt *b* sequences of one *Microcebus* taxon differed with similar magnitude from those of its closest neighbours. We therefore conclude that these mouse lemurs need to be considered as a separate species.

In conclusion, the two populations of *Mirza* compared in this study differ consistently and significantly in most external morphometric measures, they display radically different social organisations and reproductive patterns, and they exhibit genetic differentiation indicative of typical species differences. We therefore conclude that the northern and southern populations of *Mirza* deserve to be separated at the species level. Because the type locality of *Mirza coquereli* (GRANDIDIER, 1867) is Morondava, the northern population needs to be named. We are unaware of any ex-

isting synonym for members of this genus (GROVES, 2001; HILL, 1953; SCHWARZ, 1931). We therefore describe the *Mirza* from northern Madagascar as a new species:

Mirza zaza sp. nov. KAPPELER and ROOS

Holotype: RMNH 39377, skull catalogued as "d" by JENTINK (1887) in his Catalogue ostéologique, skin as "c" by JENTINK (1892) in his Catalogue systématique of the Leiden mammal collections. An adult male, collected on 25th September 1865 at Congoni, Ampasindava Peninsula, by F.P.L. Pollen & D.C. van Dam. The skin is mounted. The skull is generally in good condition, except for a broken palate bone. No postcranial skeleton is available. Measurements (in mm; see definitions in RASOLOARISON et al., 2000): greatest skull length: 52.1; basal skull length: 43.3; greatest orbital diameter: 30.4; occipital width: 24.4; zygomatic breadth: 30.5; skull height: 20.4; maxillary canine height: 5.0; maxillary tooth row (P1-M3): 14.9.

Paratypes: RMNH 39376, skull Cat. ost. "c", skin Cat. syst. "b", a subadult female, collected in 1868 in "Baie de Pasandava" (Ampasindava) by Pollen & van Dam. The skin is mounted. The skull is in good condition.

RMNH 39375, skull Cat. ost. "b", skin Cat. syst. "a", an adult female, also collected in 1868 in "Baie de Pasandava" by Pollen & Van Dam. The skin is mounted, but the skull is greatly damaged.

DNA from both specimens is stored at the Gene Bank of Primates, German Primate Centre, Germany (GBP 1024 and 1025).

Type locality: Madagascar: Province d'Ansiranana, "Baie de Pasandava" [= Ampasindava], Congoni (13°40'S, 48°15'E)

Description: Northern dwarf lemurs are covered with short greyish-brown fur that turns distinctly more grey ventrally. Hindlimbs are slightly longer than forelimbs and locomotion is quadrupedal. The tail is long, bushy and darker towards the tip. Ears are relatively short and rounded.

Diagnosis: Distinguished from *Mirza coquereli* by being generally smaller and by having relatively shorter ears, tails and canines (Fig. 8). Differs from *M. coquereli* in 3.33-3.51 % in the complete mitochondrial cytochrome *b* gene.

Etymology: The name *zaza* is the Malagasy word for children. We chose this name for two reasons. First, it refers to the fact that the northern population is the more diminutive of the two species. Second, we wish to emphasize the responsibility of the current generation of Malagasy children for the conservation of this and other members of their fauna for future generations. Malagasy name: Tanta; English name: Northern giant mouse lemur; German name: Nördlicher Riesenmausmaki; French name: Microcèbe géaut du nord

Distribution: As with many other newly described lemur species, the known range of *M. zaza* is essentially limited to the collection sites of Ambato and Pasandava. Intensive surveys are now required to obtain additional information about this species' distribution and abundance, so that potential study sites and conservation measures can be identified.

Fig. 8: *Mirza zaza* (top, Photo: D. Haring) and *M. coquereli* (bottom, Photo: M. Eberle). Ear length is the most prominent external difference between the two species, with about 20 % shorter ears in *M. zaza*.

Within the genus *Microcebus*, a large number of different species are recognized on the basis of morphological and molecular genetic data (LOUIS et al., 2005; PASTORINI et al., 2001b; RASOLOARISON et al., 2000, YODER et al., 2000). Mouse lemurs from Andasibe, however, are not referable to any known species. Since large genetic distances were detected between this population and other *Microcebus* spp. and no synonym is available for this population, we name this taxon as a new species:

Microcebus lehilahytsara sp. nov. ROOS and KAPPELER

Type Series: 9 alive specimens (6 males, 3 females) housed at the Zürich zoo, Switzerland. Specimens were caught at the type location by Samuel Furrer and Robert Zingg in March 2005. DNA from all individuals is stored at the Gene Bank of Primates, German Primate Centre, Germany (GBP 1033-1042).

Type Locality: Madagascar: Province Toamasina, Andasibe (18°55'S, 48°25'E).

Measurements: External head length: males (n=3): 33.5 (33.0-34.0) mm; females (n=1): 35.0 mm. Head-body length: males (n=3): 91.0 (90.0-92.0) mm; females (n=1): 90.0 mm. Body mass: males (n=6): 48 (38-64)g; females (n=3): 45 (30-54) g. Body mass data were collected in May 2005.

Diagnosis and Description: *M. lehilahytsara* is one of the smaller-bodied mouse lemurs. Head-body length is similar to that in the smallest primate species *M. berthae*. The fur is dense and short, bright maroon with an orange tinge on the back, head and tail, turning creamy-white on the ventral side. A distinct white

Fig. 9: Male *Microcebus lehilahytsara* from the type locality Andasibe (Photo: R. Zingg).

stripe, extending from the upper end of the rhinarium to the lower forehead, is present. Ears are short and round. The tail is uniformly colored and used for storing fat. The scrotum is furred and testes are noticeably large (Fig. 9). The species differs from other *Microcebus* spp. in at least 3.77 % in the complete mitochondrial cytochrome *b* gene.

Etymology: The species name "*lehilahytsara*" is a combination of the Malagasy words "lehilahy" and "tsara" which mean "man" and "good", respectively. We name this species in honour of Steven M. Goodman, who conducted numerous field surveys in Madagascar and provided important information about its biodiversity. By choosing this name, we express our appreciation for his crucial contributions to the description and preservation of Madagascar's lemurs and other animals. English name: Goodman's mouse lemur, German name: Goodman's Mausmaki; French name: Microcèbe de Goodman

Distribution: Currently, the species is only known from Andasibe and the Parc National de Mantadia. Further surveys are required to confirm the species' occurrence in other areas.

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This paper is dedicated to Mme. Berthe Rakotosamimanana on the occasion of her retirement from the Université d'Antananarivo to acknowledge her crucial role in the study and conservation of Madagascar's fascinating lemurs over many decades, and to the memory of the late Jean-Jacques Petter, inspiring pioneer of modern fieldwork on many lemurs, including *Mirza*.

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Appendix

Mirza specimens housed at the RMNH, Leiden.

RMNH	Skin	Skull	Sex	Collection place	Collection date	Collecter
39375	a	b	female	Pasandava Bay	1868	FPL Pollen & DC van Dam
39376	b	c	female	Pasandava Bay	1868	FPL Pollen & DC van Dam
39377	c	d	male	Congoni	25.09.1865	FPL Pollen & DC van Dam
39378	d	e	male	Mouroundava River	1870	DC van Dam
39379	e	f	male	Mouroundava River	1870	DC van Dam
39380	f	g	male	Mouroundava River	1870	DC van Dam
39381	g	h	male	Mouroundava River	1870	DC van Dam
39382	h	i	female	Mouroundava River	1870	DC van Dam
39383	i	j	female	Mouroundava River	1870	DC van Dam
39384	j	k	female	Mouroundava River	1870	DC van Dam
39385	k	l	female	Mouroundava River	1870	DC van Dam
39386	-	a*	female	Mouroundava [River]	[1870]	DC van Dam
39387	l**	-	-	-	-	DC van Dam
39388	m**	-	-	-	-	DC van Dam
39390	n**	-	-	-	-	DC van Dam
* complete skeleton; ** preserved in alcohol						

Author´s address:

Peter M. Kappeler: Department of Behavioral Ecology & Sociobiology, German Primate Centre (DPZ), Kellnerweg 4, 37077 Göttingen and Department Sociobiology/Anthropology, University of Göttingen, Berliner Str. 28, 37073 Göttingen, Germany.

Rodin M. Rasoloarison, Léonard Razafimanantsoa: Department of Behavioral Ecology & Sociobiology, German Primate Centre (DPZ), Kellnerweg 4, 37077 Göttingen and Université d'Antananarivo, Antananarivo, Madagascar.

Lutz Walter: Primate Genetics, German Primate Centre, Kellnerweg 4, 37077 Göttingen, Germany.

Christian Roos: Primate Genetics, German Primate Centre, Kellnerweg 4, 37077 Göttingen, Germany and Gene Bank of Primates.

Correspondence to: pkappel@gwdg.de