A New Primate Species in Madagascar:
Mirza zaza

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

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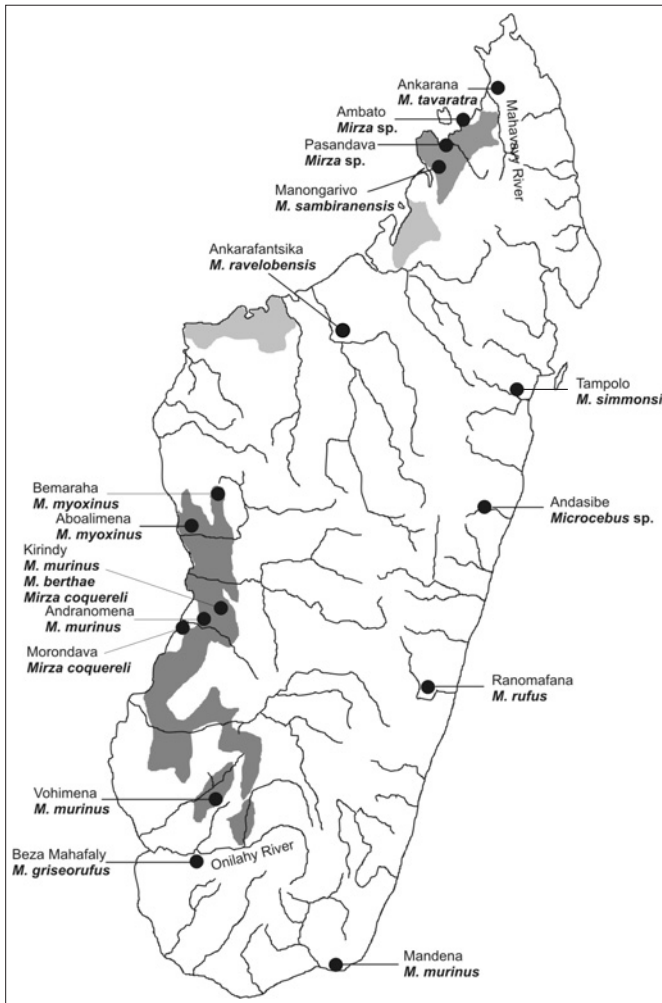
Fig. 1: Distribution of the genus *Mirza* (black: confirmed, grey: inferred). Dots indicate origin of analysed *Mirza* and *Microcebus* samples (see also Table 1).

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 (KAPPE-LER, 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 $TrN + I + \Gamma$ 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).
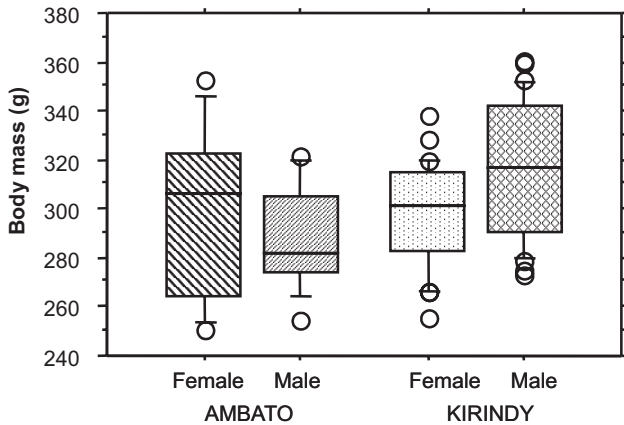
Fig. 2: Box plots depicting variation in body mass among male and female *Mirza* from Ambato and Kirindy.

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

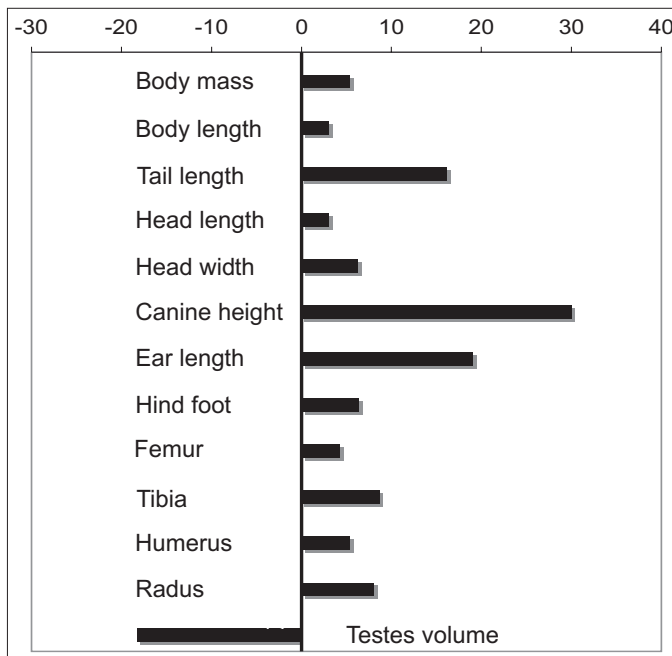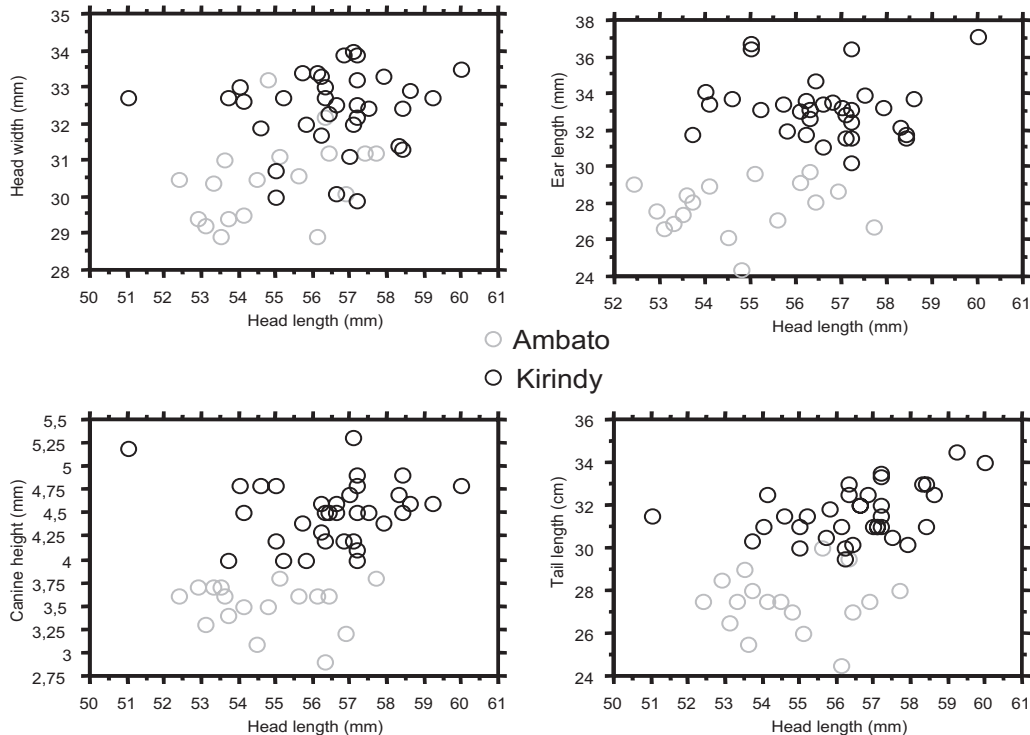Adult *Mirza* at Kirindy occupied home ranges of about 4 ha that overlapped to various extents with those of neighbours (KAPPELER, 1997a). They spent the day in self-constructed nests, most of which were built 1-2 m below the top of *Securinega* (Family Euphorbiaceae) trees (SARIKAYA and KAPPELER, 1997). 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 Fiana-rantsoa, 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
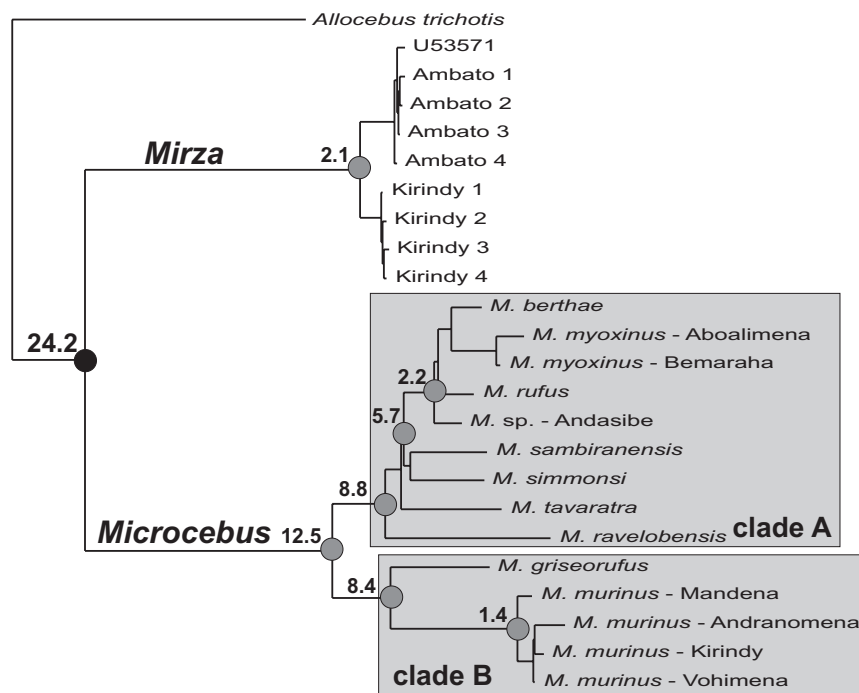
Fig. 7: Estimation of the most recent common ancestors based on complete mitochondrial cyt *b* sequence data. The black circle indicates the divergence between *Mirza* and *Microcebus* 24.2 mya, which was used as calibration point. Grey circles and respective numbers refer to calculated divergence ages 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. RASO-LOARISON 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.

Acknowledgements

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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						

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THE USE OF SEVERAL MICROSATELLITE LOCI APPLIED TO 13 NEOTROPI-CAL PRIMATES REVEALED A STRONG RECENT BOTTLENECK EVENT IN THE WOOLLY MONKEY (*LAGOTHRIX LAGOTRICHA*) IN COLOMBIA.

Ruiz-Garcia M

<u>Abstract</u>

Samples of blood and hairs of 371 free individuals belonging to 13 Neotropical Primate species were analyzed at 10 DNA microsatellites. The main results obtained were as follows: 1- The gene diversity levels obtained indicated that *Alouatta seniculus* and *Ateles fusciceps robustus* presented the highest levels, meanwhile *Cebus capucinus* and *Aotus nancymae* showed the lowest levels. 2- Effective and population sizes were estimated by means of the gene diversity levels found employing different mutation models. The values obtained for the mutation rate per generation of 7×10^{-5} and the N_e/N ratio $= 0.15$ seem to be those more realistic with the history of these species. 3- The unique species clearly showing to cross a recent bottleneck was *Lagothrix lagotricha*. Other species, such as *Aotus nancymae*, *Ateles chamek* and *Ateles geoffroyi*, showed some evidence of recent bottlenecks but considerably more weak than that detected for *Lagothrix lagotricha*.

<u>Introduction</u>

In the last decades, even nowadays, the constant destruction of the Neotropical rain forests as well as the action of the hunters to obtain protein resources have provoked a striking habitat fragmentation and a considerable diminution of the effective (= reproductive) numbers for many different mammal species, including a considerable number of Primates, which could have a well-evidenced incidence on the genetic variability of these species. Several highlighting examples, from an ecological and from a genetic standpoints, are as follows: 1- Perhaps, the most outstanding case is that related with the lion tamarins, *Leontopithecus rosalia* in the Rio de Janeiro state, *L. chrysomelas* in the southern Bahia state, *L. chrysopygus* in the Sao Paolo state and *L. caissara* in coastal Sao Paolo and Paraná states (DIETZ et al., 1994). They were selected as flagship species representing the Brazilian Atlantic rainforest and, fortunately, a considerable amount of ecological and population genetics studies have been carried out on them. Diverse strategies of conservation have been undertaken with the lion tamarins being the most noteworthy those as looking for natural reserves, such as the Uma Biological Reserve (COIMBRA-FILHO et al., 1993), emergency action plans (GUSMAO-CAMARA, 1993), translocation effects on genetic diversity (DIETZ et al., 1999), compatibility among forests and agricultural patches (VALLADARES-PADUA et al., 1999) and genetic variation

within and among populations of several *Leontopithecus* species by means of allozymes (FORMAN et al., 1986) as well as DNA microsatellite loci (GRATIVOL et al., 2001). Several of these studies put forward that the lion tamarins have near-absence of allozyme variation (polymorphism loci percentage around 3 % and heterozygosity levels around 1 %). These results agree quite well with the population number censuses reported for these species. For *L. chrysomelas*, SEAL et al. (1990) reported around 850-3100 animals in wild, 550 in protected areas and 285 in captivity, whereas they reported 450, 290 and 550 animals for *L. rosalia*, 450, 88-450, and 64, for *L. chrysopygus*, respectively. For *L. caissara,* an overall number of 400 individuals has been surveyed. For instance, KIERULFF and RYLANDS (2003) determined that during 1990-1992, 109 groups of *L. rosalia* living in the Rio of Janeiro state summing up a total of 562 individuals. Therefore, the outstanding low levels of gene diversity in *Leontopithecus* seem to be clearly related with their small effective numbers. 2- A second Brazilian species, which has given insights on the relationship among ecological, genetics and conservation parameters, has been *Brachyteles arachnoides.* This is the largest Neotropical primate, the called muriqui. At the current moment, only 650-750 individuals seem to be present in wild. MITTERMEIER et al. (1987) pointed out that only among 15 and 18 isolated forest remnants were inhabited by the muriqui. In agreement with this, COIMBRA-FILHO et al. (1993) also detected 15 widely scattered populations, although the number of individuals that they reported suggested a noticeable higher amount (2000 individuals). Most recently, other 15-20 localities inhabited by the muriqui have been discovered (MARTUSCELLI et al., 1987; AURICCHIO, 1997). Such as it was the case of the lion tamarins, several ecological, conservation and genetics studies have carried out with the muriqui. Several of them are conformed by management plans (MENDES, 1994), determination of demography dynamics (STRIER, 1991, 1993), female dispersion patterns (PINTES and STRIER, 1999), food resources (TALEBI-GOMES, 1999) and determination of gene diversity levels (POPE, 1996). Regardless of its small population size, the allozyme gene diversity for this species was high (28 % of polymorphic loci and 0.11 of heterozygosity level). These results put forward that the genetic diversity of this species was probably very high throughout all its original range. A compelling addition to this inference is that the decline of the muriqui´s populations has been extremely rapid regard to the generation length and, therefore, much of the original genetic variability in the current populations is still retained. 3- A third example, outside from Brazil, is that of diverse *Alouatta* species in Central America. The destruction and fragmentation of the Central American forests, yellow fever epidemics and devastating hurricanes have diminished the population sizes of *Allouatta palliata* and *A. pigra*. The studies of MALGREM (1979), MALGREM and BRUSH (1978) revealed threaten genetic diversity levels at 20 loci for the first species (10 % of polymorphic loci and a expected heterozygosity of 0.01). Adding credence to these findings, JAMES et al. (1997) showed a mean heterozygosity level at allozyme loci of 0.021 and 5.6 % of polymorphic loci as well as a very reduced mtDNA gene diversity for *A. pigra* in Belize.

The development in the last few years of molecular procedures based in the advent of the polymerase chain reaction (PCR), let to the population geneticists to analyze and determine the genetic structure and the levels of genetic variability of a variety of wild species throughout small fragments of tissues, hairs or faeces. Among

the most remarkable molecular markers for these tasks are the STRPs (Short Tandem Repeat Polymorphisms, microsatellites). This kind of markers are composed by short repetitive elements, being the repetition tandems integrated by one to six nucleotide base pairs. These markers are very frequent inside the eukaryotic genomes, are randomly distributed, and are very polymorphic. Additionally, one very determinant property is that the DNA amounts needed to analyze these molecular markers is very few, which let to the researcher to use non-invasive procedures to sample wild animals. Despite of the importance of determining levels of genetic variation in species in threaten or dangerous situations with these markers, however, the major part of the New World Primate species, out from Brazil and Central America, are not studied from a conservation genetic point of view. In the current work, three main aims were analyzed as follows: 1- The levels of genetic diversity in nine Colombian Primate species (*Cebus albifrons*, *C. apella*, *C. capucinus*, *Saimiri sciureus*, *Alouatta seniculus*, *Lagothrix lagotricha*, *Ateles fusciceps robustus*, *A. belzebuth belzebuth* and *A. hybridus*), in one Peruvian primate (*Aotus nancymae*), in one Bolivian-Peruvian species (*Ateles chamek*), in one Costa Rica species (*Alouatta palliata*) and in one Guatemalan species (*Ateles geoffroyi*) were estimated by using hypervariable DNA microsatellite markers. 2- Several tests were used to detect feasible recent bottleneck events in these Primate species and 3- The calculation of theoretical effective (= reproductive) numbers throughout of all the history of each one of the species studied by means of two different mutation model are shown. Thereby, to address these three questions, hairs and blood from 371 Neotropical Primate individuals of 13 species were directly taken from wild.

Materials and Methods

A total number of 371 animals, belonging to 13 Primate species, were surveyed throughout of different Colombian, Peruvian, Bolivian, Guatemalan, Venezuelan and Costa Rican localities (Fig. 1). All the samples obtained were composed by little amounts of hairs with roots and ¼ part of the samples obtained was blood. The hair and blood samples were directly obtained in wild by the author, by using food traps, or by colleagues, which captured animals for biomedical research or for translocation processes. The hairs were quickly kept in absolute ethanol and the blood in disodic EDTA. The specific number of individuals sampled was as follows: I obtained 33 hair samples of *Cebus albifrons versicolor* from the middle Magdalena Valley, (Colombian Departments of Antioquia and Bolivar), 22 samples of *Cebus apella apella* from the Leticia region (Colombian Amazon), ten samples of *Cebus capucinus capucinus* from the Uraba region (northwestern of Antioquia Department), 37 samples surveyed of *Saimiri sciureus macrodon* from the Leticia region (Colombian Amazon), including a few samples of individuals captured in the slopes of eastern Andes from Colombia, belonging to the *S. s. albigena* subspecies, 56 samples of *Alouatta seniculus seniculus* from all the regions where this species lives in Colombia, 30 samples of *Lagothrix lagotricha lagotricha* from the Leticia region (Colombian Amazon), 68 samples of *Ateles fusciceps robustus*, several from the Atrato River basin (Chocó Department in the Colombian Pacific coast), and others from Alto Sinú in the Colombian Cordoba Department, 14 samples of *Ateles belzebuth belzebuth* from La Maca-

rena mountains and from several Colombian and Peruvian Amazon areas, 19 samples of *Ateles hybridus* from the middle Magdalena River and the Catatumbo regions in Colombia as well as several samples from the neighbor region of Maracaibo Lake in Venezuela, 30 samples of *Ateles chamek* from diverse regions in Peru (Loreto Department in the Peruvian Amazon on Marañon River) and in Bolivia (Beni Department on Madidi and Beni Rivers), 14 samples of *Ateles geoffroyi vellerosus* from diverse areas of Guatemala, 29 samples of *Alouatta palliata* from diverse areas of Costa Rica and the Choco at the Pacific Colombian coast, and, finally, 9 samples of *Aotus nancymae* coming from the Loreto region (Amazonas), exactly from the Iquitos island (San Pedro) and from the Yanayaky river.
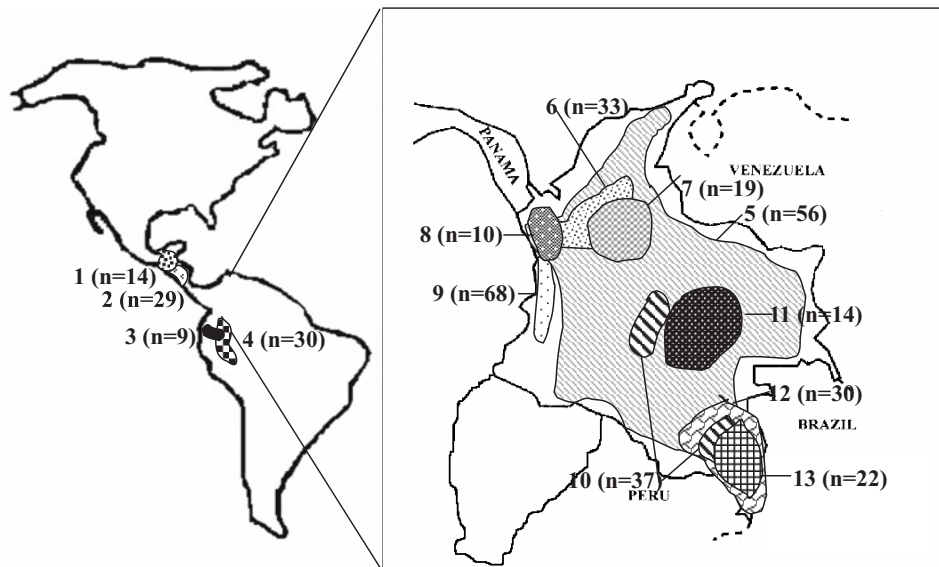
Fig. 1: Geographical distribution of the 13 Neotropical Primate species studied herein. n = sample sizes; 1 = *Ateles geoffroyi vellerosus*; 2 = *Alouatta palliata*; 3 = *Aotus nancymaae*; 4 = *Ateles chamek*; 5 = *Alouatta seniculus seniculus*; 6 = *Cebus albifrons versicolor*; 7 = *Ateles hybridus*; 8 = *Cebus capuccinus*; 9 = *Ateles fusciceps robustus*; 10 = *Saimiri sciureus*; 11 = *Ateles belzebuth belzebuth*; 12 = *Lagothrix lagotricha lagotricha*; 13 = *Cebus apella apella*.

The molecular markers employed were 10 hypervariable microsatellite loci. Four of them, the AP6, AP40, AP68 and AP74 loci, were developed for *Allouata palliata* by ELLSWORTH and HOELZER (1998), and their respective repetition motifs are (TG)11, (TG)4CA(TG)6, (TG)17 and (TG)19, whereas the microsatellite D5S111, D5S117, D6S260, D8S165, D14S51 and D17S804 were developed for humans and previously used in *Saimiri boliviensis* (ROGERS et al., 1995; ZHONG et al., 1995).

The DNA extraction was carried out by using Chelex resine with the procedure of WALSH et al. (1991). The PCR volume reaction was as follows. The final volume re-

action was of 50 μl, with 4 μl of MgCl2 3mM, 5 μl of Buffer 10x, 2 μl of dNTPs 0.04 mM, 8 pmol of each primer, 1 unit of Taq Polymerase, 17 μl of H_2O and 20 μl of DNA. The reaction was carried out in a Perkin Elmer Geneamp PCR System 9600. The temperatures employed were as follows: 95° C for five minutes, 30 cycles of one minute at 95° C, one minute at the optimal annealing temperature (47° C for AP6, 57° C for AP40, 50° C for AP68, and 52° C for AP74, D5S111, D5S117, D6S260, D8S165, D14S51, D17S804), and one minute at 72 °C. Finally, five minutes at 72° C. The amplification productions were stored at 4° C until its use. A 3 % agarose gel, stained with Etidium Bromure, was employed to ratify which samples really amplified. To obtain the size alleles of the microsatellite analyzed, the amplified products were run in a 6 % denaturant polyacrilamyde gel in a Hoefer SQ3 sequencer vertical camera, which let us to discriminate until one unique base-pair. After of a 2-3 hours migration depending of the sizes of the markers analyzed and with 35 W as a constant, the gels were stained with $AgNO_3$. The molecular weight markers used to size the alleles were ϕ X174 cut with Hind III and Hinf I.

Once the genotypes were obtained, the first statistic to be calculated was the unbiased expected heterozygosity (= gene diversity) (Nei, 1978), which expression to be calculated is $H = 2n (1 - \sum_i x_i^2)/(2n - 1)$, where n is the number of individuals analyzed and x_i is the allele frequency of the *ith* allele analyzed. This statistic is especially important due to its independence from evolutionary events, such as selection favoring, or not favoring, homozygous and/or heterozygous, or the reproductive pattern used. Contrarily, the average expected heterozygosity can be influenced by certain stochastic processes as gene drift (NYGREN and RASMUSSON, 1980; RUIZ-GARCIA, 1991).

The second genetic population analysis has been focusing on the detection of recent bottleneck events in the Primate species studied. The term recent means that these species have gone throughout a bottleneck event $2N_e$-$4N_e$ generations ago, being N_e the effective number of these species. To carry out this analysis, the recently derived theory, generated by CORNUET and LUIKART (1996), LUIKART and CORNUET (1998) and LUIKART et al. (1998), was employed. The populations, which have experienced a recent bottleneck, simultaneously lost the allele number and the expected levels of heterozygosity. Nevertheless, the allele number (k_O) is reduced faster than the expected heterozygosity. Therefore, the value of the expected heterozygosity calculated throughout the allele number (H_{eq}) is lower than the obtained expected heterozygosity (H_e). This excess of the expected heterozygosity, regard to that obtained throughout the number of alleles, has been demonstrated under the infinite allele model (KIMURA and CROW, 1964), although it is not so clear under a step-wise mutation model (OHTA and KIMURA, 1973). The microsatellite markers employed herein, although probably nearest to the second mutational model, do not strictly follow and as soon as a marker slightly departs from the step-wise model toward the allele infinite model, the excess of the expected heterozygosity will be fast put forward as a consequence of a bottleneck event. For neutral markers, in a population in mutation-gene drift equilibrium, there is an equal probability that a given locus has a slight excess or deficit of heterozygosity regard to the heterozygosity calculated from the number of alleles. On the contrary, in a bottlenecked population, a big fraction of the loci analyzed will exhibit a significant excess of the expected heterozygosity. To measure this probability, four diverse procedures

were used as follows: a sign test, a standardized difference test, a Wilcoxon´s signed rank test (LUIKART and CORNUET, 1998; LUIKART et al., 1998) and a graphical descriptor of the shape of the allele frequency distribution. A population, which did not suffered a recent bottleneck event, will yield a L-shape distribution, such as expected in a stable population in mutation-gene drift equilibrium, meanwhile a recently bottlenecked population will show a mode-shift distribution. The Wilcoxon´s signed rank test is feasibly the most powerful and well-evidenced test when the number of loci analyzed is low, such as it is in the current case.

Thirdly, the effective numbers of each species were calculated by means of two different mutational models. For the infinite allele model (IAM), the equation used was

$N_e = H/[4\mu(1 - H)]$, where H is the average expected heterozygosity for all the loci employed and μ is the mutation rate per generation. For the step-wise mutation model (SMM), the equation employed was

$Ne = [1 - (1 - H)^2]/[8\mu(1 - H)^2]$. Although there is not any Neotropical primate estimation for mutation microsatellite rate per generation, several estimates obtained for humans and other mammals were used to calculate these effective numbers, which could be noteworthy important to understand the genetic conservation status of each one of the species analyzed. For humans, several estimates for dinucleotide repetitions, such as those used herein, ranged from 5.6×10^{-4} until 10^{-5} (STRAUB, 1993). ELLEGREN (1995) determined for dinucleotide repetitions in pigs a estimation of 7×10^{-5}. SERIKAWA (1992) obtained, for 134 microsatellite loci in rats, a value of 1.5×10^{-4}. Thus, to obtain a wide range of feasible effective numbers in the primates analyzed, the mutation rates employed in this work were among 5.6×10^{-4} to 7×10^{-5}. Recently, RUIZ-GARCIA et al. (2003a) have demonstrated for four *Alouatta* species (*A. palliata*, *A. seniculus*, *A. macconnelli* and *A. caraya*), by means of maximum likelihood simulations of the parameter and comparisons with the divergence times proposed by CORTÉS-ORTIZ et al. (2003) among these *Alouatta* species, that the 7×10^{-5} mutation rates are more probable for the microsatellites used than the other extreme mutation rate. Therefore, the effective numbers obtained with this mutation rate could be more fitted to the reality than the estimates obtained with the 5.6×10^{-4} mutation rate. Recall that the effective numbers calculated in this way represent the average number of reproductive individuals throughout all the history of the species and they are not necessarily the current effective numbers.

Results

In three Tables all the results obtained are presented. Table 1 shows the average gene diversity levels of the 13 Primate species studied by means of 10 DNA microsatellites, including the effective number estimations with different mutation rates per generation and with two extreme opposite mutation models for each one of these species. Table 2 yields the total population sizes of the studied species assuming two different ratios ($N_e/N = 0.15$ and 0.5) and Table 3 presents the results of the possible recent bottlenecks in the 13 Primate species analyzed.

Table 1: Expected heterozygosity (H) and effective numbers by means of two extreme mutation models (IAM = Infinite Allele Model; SMM = StepWise Mutation Model) with two mutation rates per generation feasible in mammals (5.6 x 10^{-4} and 7 x 10^{-5}).

Species	H	Effective numbers			
		5.6x10^{-4}		7x10^{-5}	
		IAM	SMM	IAM	SMM
Cebus albifrons	0.4516	368	519	2941	4152
Cebus apella	0.3774	271	353	2165	2821
Cebus capuccinus	0.2396	141	162	1125	1303
Saimiri sciureus	0.5206	485	748	3878	5984
Aotus nancymae	0.2750	169	201	1355	1612
Alouatta seniculus	0.6380	787	1480	6294	11841
Alouatta palliata	0.4518	368	519	2943	4156
Ateles fusciceps r.	0.6106	700	1249	5600	9991
Ateles belzebuth b.	0.5381	520	823	4161	6584
Ateles hybridus	0.5469	539	864	4311	6912
Ateles chamek	0.5264	496	772	3970	6176
Ateles geoffroyi	0.4384	348	485	2788	3876
Lagothrix lagotricha	0.5380	520	823	4159	6580

Table 2: Global population size numbers estimated by means of two extreme mutation models (IAM = Infinite Allele Model; SMM = StepWise Mutation Model) with two mutation rates per generation typical in mammals (5.6 x 10^{-4} and 7 x 10^{-5}). UE = Upper estimate obtained assuming a N_e/N ratio = 0.15. LE = Lower estimate obtained assuming a N_e/N ratio = 0.50.

Species		Population sizes estimated			
		5.6x10^{-4}		7x10^{-5}	
		IAM	SMM	IAM	SMM
Cebus albifrons	UE	2453	3460	19427	27680
	LE	736	1038	5882	8304
Cebus apella	UE	1807	2353	14433	18807
	LE	542	706	4330	5642
Cebus capuccinus	UE	940	1080	7500	8687
	LE	282	324	2250	2606
Saimiri sciureus	UE	3233	4987	25853	39893
	LE	970	1496	7756	11968
Aotus nancymae	UE	1127	1340	9033	10747
	LE	338	402	2710	3224
Lagothrix lagotricha	UE	3467	5487	27727	43867
	LE	1040	1646	8318	13160
Alouatta seniculus	UE	5247	9867	41960	78940
	LE	1574	2960	12588	23683

Species		Population sizes estimated			
		5.6x10^{-4}		7x10^{-5}	
		IAM	SMM	IAM	SMM
Alouatta palliata	UE	2453	3460	19620	27707
	LE	736	1038	5886	8312
Ateles fusciceps r.	UE	4667	8327	37333	66607
	LE	1400	2498	11200	19982
Ateles belzebuth b.	UE	3467	5487	27740	43893
	LE	1040	1646	8322	13168
Ateles hybridus	UE	3593	5760	28740	96080
	LE	1078	1728	8622	13824
Ateles chamek	UE	3307	5147	26467	41173
	LE	992	1544	7940	12840
Ateles geoffroyi	UE	2320	3233	18587	25840
	LE	696	970	5576	7752

Table 3: Recent bottleneck genetic analyses by means of the CORNUET and LUI-KART (1996) theory. Four tests were developed to analyze posible recent bottlenecks in the 13 Neotropical Primate species studied. These analyses were applied to two extreme mutation models (IAM = Infinite Allele Model; SMM = StepWise Mutation Model). * Significant probabilities which agree quite well with a recent bottleneck. ** Significant probabilities in favor of an evolutionary event contrary to a recent bottleneck (population expansion, population subdivision, hybridization, etc).

Cebus albifrons (no recent bottleneck evidence)			
Sign Test		Standardized Differences Test	
IAM	SMM	T$_2$= 0.182	T$_2$=-2.476
p= 0.6010	p=0.4250	p= 0.4238	p= 0.0066**
Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.5000	one tail for H excess p= 0.5000	Normal L-shaped distribution	
Cebus apella (no recent bottleneck evidence)			
Sign Test		Standardized Differences Test	
IAM	SMM	T$_2$= -0.0262	T$_2$=-0.762
p= 0.5868	p=0.6886	p= 0.3967	p=0.229
Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.8750	one tail for H excess p= 0.8750	Normal L-shaped distribution	
Cebus capuccinus (no recent bottleneck evidence)			
Sign Test		Standardized Differences Test	
IAM	SMM	T$_2$= -0.057	T$_2$=-1.067
p= 0.4290	p=0.3820	p= 0.4772	p=0.1429
Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.5000	one tail for H excess p= 0.5000	Normal L-shaped distribution	

Saimiri sciureus (no recent bottleneck evidence)			
SIGN TEST		**Standardized Differences Test**	
IAM	**SMM**	T_2= 1.041	T_2=0.029
p= 0.6060	p=0.5920	p= 0.1490	p=0.4886
Wilcoxon Test		**Mode-Shift**	
one tail for H excess p= 0.5000	one tail for H excess p= 0.5000	Normal L-shaped distribution	
Aotus nancymaae (some evidence of recent bottleneck, but weak)			
Sign Test		**Standardized Differences Test**	
IAM	**SMM**	T_2= -0.226	T_2=-0.667
p= 0.7165	p=0.6550	p= 0.4105	p=0.2491
Wilcoxon Test		**Mode-Shift**	
one tail for H excess p= 0.5000	one tail for H excess p= 0.5000	Shifted*	
Alouatta seniculus (no recent bottleneck evidence)			
Sign Test		**Standardized Differences Test**	
IAM	**SMM**	T_2= 0.423	T_2=-1.732
p= 0.5676	p=0.1907	p= 0.3360	p=0.0415**
Wilcoxon Test		**Mode-Shift**	
one tail for H excess p= 0.3710	one tail for H excess p= 0.9023	Normal L-shaped distribution	
Alouatta palliata (no recent bottleneck evidence)			
Sign Test		**Standardized Differences Test**	
IAM	**SMM**	T_2= -0.806	T_2=-2.813
p= 0.5679	p=0.2038	p= 0.2100	p=0.0024**
Wilcoxon Test		**Mode-Shift**	
one tail for H excess p= 0.7187	one tail for H excess p= 0.9765	Normal L-shaped distribution	
Lagothrix lagotricha (strong evidence of recent bottleneck)			
Sign Test		**Standardized Differences Test**	
IAM	**SMM**	T_2= 2.126	T_2=1.623
p= 0.0206*	p=0.0415*	p= 0.0167*	p=0.0423*
Wilcoxon Test		**Mode-Shift**	
one tail for H excess p= 0.0156*	one tail for H excess p= 0.1563	Shifted*	
Ateles fusciceps robustus (no recent bottleneck evidence)			
Sign Test		**Standardized Differences Test**	
IAM	**SMM**	T_2= 0.859	T_2=-3.495
p= 0.4851	p=0.0281**	p= 0.1950	p=0.0002**
Wilcoxon Test		**Mode-Shift**	
one tail for H excess p= 0.0625	one tail for H excess p= 1.0000	Normal L-shaped distribution	
Ateles belzebuth belzebuth (no recent bottleneck evidence)			
Sign Test		**Standardized Differences Test**	
IAM	**SMM**	T_2= 0.301	T_2=-0.628
p= 0.5633	p=0.5218	p= 0.3816	p=0.2649

Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.5625	one tail for H excess p= 0.9062	Normal L-shaped distribution	
Ateles hybridus (no recent bottleneck evidence)			
Sign Test		Standardized Differences Test	
IAM	**SMM**	$T_2= 0.726$	$T_2=-0.192$
p= 0.5442	p=0.5680	p= 0.2340	p=0.4237
Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.1562	one tail for H excess p= 0.5625	Normal L-shaped distribution	
Ateles chamek (some evidence of recent bottleneck, but weak)			
Sign Test		Standardized Differences Test	
IAM	**SMM**	$T_2= 1.008$	$T_2=0.291$
p= 0.2955	p=0.3351	p= 0.1567	p=0.2855
Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.3125	one tail for H excess p= 0.3125	Shifted*	
Ateles geoffroyi (some evidence of recent bottleneck)			
Sign Test		Standardized Differences Test	
IAM	**SMM**	$T_2= 1.722$	$T_2=1.120$
p= 0.0916	p=0.1498	p= 0.0425*	p=0.1314
Wilcoxon Test		Mode-Shift	
one tail for H excess p= 0.0312*	one tail for H excess p= 0.0312*	Normal L-shaped distribution	

Cebus albifrons

The mean expected heterozygosity for the microsatellite loci applied to this species was H = 0.4516, which is a medium value for this kind of molecular markers. Recall that only one subspecies of *C. albifrons* out 8-9 that are morphologically defined for Colombia (HERNÁNDEZ-CAMACHO and COOPER, 1976) has been studied in the current work. Probably, if we carry out a global study of all *C. albifrons* taxa presented at Colombia, the gene diversity could be considerably higher. Different basic trends are visible in the results obtained for this species which are extensible for all other species. The effective numbers obtained with the 5.6×10^{-4} mutation rate clearly misrepresent the possible real values for this and all the other species. For instance with the IAM, this value was only 368 meanwhile with SMM the value was also extremely small (519). Although the current census values do not necessarily to fit with the effective number estimates, the values obtained by means of the 7×10^{-5} mutation rate seem to agree better with the expected values according with the current knowledge we have. These values ranged from 2941 to 4152 depending of the mutation models chosen. Taking two feasible extreme ranges of N_e /N for Neotropical Primates (0.15 and 0.5, as I will extensively comment on discussion) and the most probably mutation rate, the average historical total sizes for this species could oscillate from 5882 and 27.680 individuals in northern Colombia. As I will discuss in brief the highest estimate is also the most probable. In Table 3, overall tests to detect

possible recent bottlenecks showed that this species did not reveal any significant trend in favor of this event. For instance, to cite only one example, the expected heterozygosity obtained at AP74 (H_e = 0.919) agreed quite well with that found throughout the allele number observed (k_o = 21) for the allele infinite mutational model (H_{eq} = 0.914), although was slightly lower to that expected under a step-wise mutational model (H_{eq} = 0.942). All tests employed did not reveal any bottleneck trend. The graphic descriptor showed a normal L-shaped distribution, which is compatible with a demographical stable population.

Cebus apella

The average expected heterozygosity for the microsatellite loci applied on this species was H = 0.3774. This value could be considered low for this kind of molecular markers and it is the third lowest gene diversity found for the 13 Primate species reported herein, although this is the Neotropical Primate with the largest distribution in South-America. Additionally, this species in one of the most effective Primate colonizers in the Neotropics (AYRES, 1986), which could be expected that its gene diversity was greater than found. However, as all samples were coming from a determined area of the Colombian Amazon, the gene diversity level determined could represent more a local condition than a generalized value for all the species. The most probable effective numbers ranged from 2165 to 2821 (7×10^{-5} mutation rate), which represents a total population number for this species the Colombian Amazon ranging from 5882 to 27680. The bottleneck analysis showed uncertain results for this sample of *Cebus apella*. For all the markers studied, only the D5S117 locus showed a H_e = 0.545 higher than the Heq for the allele infinite model (H_{eq} = 0.319, p = 0.0530) as well as for the step wise model (H_{eq} = 0.358, p = 0.075), being these probabilities borderline to the statistical signification. Nonetheless, the global probabilities of the sign, standardized differences and the Wilcoxon tests did not detect evidence of recent bottlenecks. Contrarily to this point of view, the graphic descriptor showed a shifted-mode distribution for *C. apella*, which could be an indication of a recent bottleneck. Therefore, no clear evidence is obtained in favor or against of a recent bottleneck on this species at the Colombian Amazon.

Cebus capucinus

The average expected heterozygosity was H = 0.2396, a value extremely low for DNA microsatellites. This value was significantly lower than those found for the other two *Cebus* species studied in Colombia. Indeed, this was the lowest gene diversity value obtained for the 13 species studied. The most feasible effective numbers ranged from 1125 to 1303 individuals, which could represent from 2250 to 8687 individuals as total population number estimates

Although the geographical distribution of *C. capucinus* in Colombia is considerable lower than that determined for the other two *Cebus* species in this country as well as the gene diversity levels and the effective numbers were the lowest found for the 13 species studied herein, the bottleneck analysis did not reveal any recent bottleneck event affecting to this species. No statistic test was significant as well as the graphic descriptor yielded a normal L-shaped distribution, which effectively suggest that the samples studied come from a population in mutation-gene drift equilibrium.

Saimiri sciureus

The average expected heterozygosity was H = 0.5206, which enables to estimate, throughout the commented mutational models, effective numbers ranging from 3878 to 5984 as the most probable values. This represents among 7756 to 39893 as total population sizes for the Colombian territory analyzed. There was not any evidence that the *Saimiri sciureus* sample analyzed proceeded from a bottlenecked population, since neither statistic nor the graphic descriptor (normal L-shaped distribution) provided significant results.

Aotus nancymae

The average expected heterozygosity was only 0.275, the second lowest value found in the present study. The most feasible effective number estimates ranged from 1355 to 1612 individuals, which represents a total population size ranging from 2710 to 10747 individuals if we consider the 7×10^{-5} mutation rate as the most consistent.

The polymorphic markers analyzed for this species did not show any evidence of a provenience from a bottlenecked population. However, the graphic descriptor revealed a shifted mode distribution. At the present, we can not discard the possibility that this shifted mode distribution could be product of the extreme small sample size employed for this species.

Alouatta seniculus

The gene diversity level found for this species sampled throughout all the Colombian territories where this howler monkey is distributed was 0.638, being this value the highest found for the 13 species studied. Translate this value to the most feasible effective numbers, they ranged from 6294 to 11841. The correspondent total population sizes oscillated from 12588 to 78940 individuals. This means that this was the species with the highest historical population size of all the species studied, although the geographical distribution where the samples were obtained was one of the highest analyzed. Although several individual markers showed some significant deviations, being these the cases of D5S111 ($H_e = 0.720$, $H_{eq} = 0.533$, p = 0.0484 for IAM), which is consistent with a recent bottleneck, and AP40 ($H_e = 0.301$, $H_{eq} = 0.585$, p = 0.0332 for SMM) and D14S51 ($H_e = 0.140$, $H_{eq} = 0.481$, p = 0.0316), which showed a significant contrary trend to a recent bottleneck, global test did not indicate that this species in the current Colombian territory have gone throughout a recent bottleneck. The graphic descriptor yielded a normal L-shaped distribution contrary to a recent bottleneck as well.

Alouatta palliata

The gene diversity was 0.4518 for this other howler monkey species, which was lower than the value obtained for the red howler monkey, although some of the DNA microsatellite markers employed were constructed specifically for the Central American species. The most feasible consequent effective numbers ranged from 2943 to 4156 individuals, representing them between 5886 and 27707 exemplars as total population size. Therefore, the *A. palliata* population taken together from Costa Rica and Colombia has been historically among two and threefold times lower than the historical populations of *A. seniculus* in Colombia. Only one individual marker,

D5S111, showed a significant deviation among H_e and H_{eq} for the SMM ($H_e = 0.384$, $H_{eq} = 0.709$, $p = 0.0058$) but showing evidence of a contrary bottleneck event. No global test showed evidence of a recent bottleneck affecting this species, but some of them (standardized differences test for SMM: $T_2 = -2.813$, $p = 0.00245$; Wilcoxon test for SMM: Probability (one tail for H deficiency) = 0.03906) yielded significant evidence in favor of some event which is opposite to a recent bottleneck. The graphic descriptor offered a normal L-shaped distribution.

Ateles fusciceps robustus

The average gene diversity for this species reached a value of 0.6106, the second highest obtained after *Alouatta seniculus* and no significantly different. The historical effective numbers calculated throughout the gene diversity estimate ranged from 5600 to 9991 individuals, which corresponded to values oscillating from 11200 to 66607 animals as total population sizes, despite that this species has a relatively narrow distribution in Colombia. However, the samples obtained were surveyed across all its distribution range of this species in that Neotropical country. In the bottleneck analysis, several markers, such as AP68 and AP74, presented significant values but by contrary reasons to a recent bottleneck, especially for the SMM. No global tests detected any recent bottleneck in this species. Nevertheless, several of these tests were significant by opposite events to a recent bottleneck (Sign test for SMM: $p = 0.02818$; Standardized differences test for SMM: $T_2 = -3.495$, $p = 0.00024$). Furthermore, the graphic descriptor presented a normal L distribution related with a stable population.

Ateles belzebuth belzebuth

The gene diversity level in this species reached an amount of 0.5381, which corresponds to effective numbers oscillating from 4161 to 6584. The total population numbers determined ranged from 8322 to 43893 individuals. These values probably represents the population sizes of this species in the Colombian and Peruvian Amazon and surrounding areas. No evidence of a recent bottleneck was determined for this species. No global tests nor the graphic descriptor revealed any trend in favor of a recent bottleneck.

Ateles hybridus

The expected heterozygosity value obtained was 0.5469, very similar to that determined for *Ateles belzebuth* although its geographical distribution is extremely more reduced. The exemplars surveyed represent all the geographical distribution of this species in Colombia and Venezuela. The effective numbers ranged from 4311 to 6912, which represented a total population size oscillating from 8622 to 46080 individuals for the most probable mutation rate. Such as the previous species no evidence of recent bottlenecks was determined for *A. hybridus*

Ateles chamek

The gene diversity level was 0.5264, which was practically undifferentiated from the values obtained in the other two previous species of *Ateles*, although the samples herein studied were from very diverse areas from Peru, Bolivia and Brazil. The effective numbers ranged from 3970 to 6176, which represent a total population sizes oscillating from 7940 to 41173. All global test did not detect any recent bottleneck.

Nonetheless, the graphic descriptor presented a mode-shift distribution, which could be compatible with a recent bottleneck, although some other events could determined a mode-shift distribution as we will briefly comment in the discussion.

Ateles geoffroyi vellerosus

The gene diversity level of this species was the lowest of all the *Ateles* species herein studied (H = 0.4384). The most feasible effective numbers ranged from 2788 to 3876, which determined total population sizes oscillating from 5576 to 25840. Therefore, the Central American *Ateles* species was that which presented the lowest sizes of all *Ateles* species reported herein. Some bottleneck analyses revealed some positive significant trends in this species. The standardized differences test for IAM was significant (T_2 = 1.722, p = 0.04254) as well as the Wilcoxon test (p = 0.03125). However, the graphic descriptor presented a normal L distribution. Hence, some contradictory results on the importance of bottlenecks affecting to this species was obtained.

Lagothrix lagotricha

The gene diversity level of this species was quite similar to that obtained for the major part of *Ateles* species studied in this work (H = 0.538). The historical effective numbers of the Colombian Amazon *L. lagotricha lagotricha* seem to be enough elevated (4159-6580), which mean a total population size ranging from 8318 to 43867 individuals.

Such as it was shown, none of the species studied clearly showed to go throughout to any recent bottleneck. Perhaps, *Cebus apella*, *Ateles chamek* and especially *Ateles geoffroyi* showed certain evidence in favor of recent bottlenecks. However, contrarily, a noteworthy exception was the case of *Lagothrix lagotricha* in the Colombian Amazon, which showed a very clear case of a recent bottleneck. The major part of the markers studied for this species (AP6, AP74, D5S111, D5S117, D6S260 and D17S804) displayed He values higher than the Heq expected throughout the respective allele numbers observed. The sign test presented significant probabilities with IAM (p = 0.0206), as well as with the SMM (p = 0.0415). Furthermore, the standardized differences test (p = 0.01675) and the Wilcoxon test (p = 0.00781) offered significant probabilities with IAM and borderline significant probabilities in the case of SMM. Adding credence to this, the graphic descriptor clearly revealed a shifted mode distribution. Thereby, the unique species out 13 studied herein, which with any doubt clearly showed to go throughout a significant recent bottleneck was *Lagothrix lagotricha* in the Colombian Amazon. Nevertheless, I want to bring out the fact that this species displayed a relatively high average expected heterozygosity by using 10 DNA microsatellite markers.

Discussion

Heterozygosity levels

The expected heterozygosity levels obtained by means of microsatellite loci are indeed considerable higher than those found by means of other genetic markers, such as isoenzymes. A few examples, including several of the species and genera

studied herein, are enough. Sampaio *et al.*, (1991a) determined a heterozygosity value of 0.015 for 55 individuals of *Cebus apella paraguayanus* at 18 different biochemical loci. This species displayed a low heterozygosity at the CAII locus (H = 0.039), as well (SAMPAIO et al. 1991b). Herein, I reported a heterozygosity level, for *Cebus apella apella*, of 0.377. Although, this is a low gene diversity for markers such as DNA microsatellites, this value is around 10 and 25 higher than the levels obtained with different isoenzymes. SILVA et al. (1993) studied diverse taxa of *Saimiri* and they reported the following heterozygosity levels: 0.027 for *Saimiri sciureus sciureus*, 0.047 for *S. boliviensis*, and 0.013 for *S. ustus*. Similar results were obtained by VANDBERG et al. (1990) with two species of *Saimiri* (0.084 and 0.042, respectively). The levels of heterozygosity reported herein corresponding to 37 specimens belonging to two Colombian subspecies, *S. sciureus macrodon* and *S. sciureus albigena*, showed a value of 0.521, which is among 6 and 40 times higher than the values reported by those authors with isoenzymes. SAMPAIO et al. (1991a) reported, however, heterozygosity levels more similar to those obtained with microsatellite loci, to employ the CAII locus, for *Saimiri boliviensis peruvensis* (H = 0.1513), for one *Saimiri ustus* population (H = 0.1968) and for other *Saimiri ustus* population (H = 0.4227). *Aotus nancymae* was a conspicuous exception. At the biochemical locus, CAII, the expected heterozygosity level was higher (H = 0.4812) than that reported herein by using microsatellite loci (H = 0.275). The microsatellite value obtained for *Alouatta seniculus* (H = 0.638) was significantly higher than all the microsatellite diversity values obtained in the Primates studied herein with exception of those of *Lagothrix lagotricha* and four *Ateles* species (*A. fusciceps, A. belzebuth, A. hybridus* and *A. chamek*). The gene diversity of *A. palliata* (H = 0.452) is significantly lower than the value obtained for *A. seniculus*, which puts forward that the heterozygosity levels herein reported are not the product of differences among heterologous and homologous microsatellite, because the homologous species, in this case *A. palliata*, has lower gene diversity levels than *A. seniculus, Lagothrix lagotricha* and four *Ateles* species. Nonetheless, its gene diversity level was higher than the values obtained for two species of *Cebus* and one species of *Aotus*. Other authors with different genetic markers have also identified high values of heterozygosity in diverse *Alouatta* species. MELO et al. (1992) reported a value of 0.061 to analyze 50 individuals of *Alouatta belzebul belzebul* using 18 isoenzymes and plasmatic proteins, which is a high value for a primate with this type of markers. Adding credence to this, isoenzyme results at the CAII locus showed considerable heterozygosity levels in *A. belzebul* (H = 0.058) and in *A. seniculus* (H = 0.116) (SAMPAIO et al. 1991b). In the outstanding work carried out by POPE (1992), the mean heterozygosity values within *Alouatta seniculus* troops, surveyed in Venezuela, ranged from 0.057 to 0.135, whereas the mean heterozygosity in woodland populations was 0.106 and that in gallery populations reached 0.089 based on 29 biochemical loci. Identically, SAMPAIO et al. (1996) showed that *A. seniculus* exhibits an average heterozygosity of 0.10 by means of protein electrophoresis. These values are among the highest discovered for a primate with this kind of markers. In the same way, the four *Alouatta belzebul* populations studied by SCHNEIDER et al. (1991) displayed levels of heterozygosity ranging from 0.043 to 0.077 by means of 13 biochemical loci. Indeed, CORTÉS-ORTIZ et al. (2003) determined that *A. belzebul* showed the highest levels of mtDNA sequence diversity regard to other *Alouatta*

species, although the divergence among haplotypes was slight. Therefore, the high levels previously found with isoenzyme markers were ratified using DNA microsatellites applied to *Alouatta*. Contrarily, FIGUEIREDO et al. (1998) showed that the mitochondrial COII nucleotide sequences in *A. seniculus* have a very low variation although only two samples were identical. The genetic distances among the haplotypes determined by FIGUEIREDO et al. (1998) in *A. seniculus* such as it was aforementioned was very low (0-1.15 %). Therefore, meanwhile the nuclear diversity seems very high for *Alouatta seniculus* (isoenzymes and microsatellites), the mitochondrial COII gene diversity is more restricted, which could be a proof in favor of some type of constrictive natural selection affecting this mitochondrial gene.

Also it is remarkable the fact that the *Alouatta* species studied with the lowest heterozygosity was *A. palliata* (Central American species) and this was also previously found with isoenzyme markers. MALGREM (1979) and MALGREM and BRUSH (1978) for 170 individuals captured in Costa Rica, by means of 20 biochemical loci, only found a H = 0.01, meanwhile the other Central American *Alouatta* species, *A. pigra*, from Belize also only showed a value of H = 0.021 by using 36 allozyme loci and only 5.6 % of these loci were polymorphic (JAMES et al., 1997). The same authors also revealed very scarce mtDNA gene diversity for this Central American *Alouatta* species. These results are congruent with four historical population crashes in recent times (devastating hurricanes and yellow fever epidemic). Hence, a parallel dynamic in the contrasting heterozygosity levels between the Central and the South American howler monkeys was revealed by microsatellites as it was previously revealed by isoenzymes. Nevertheless, the differences among the microsatellite gene levels were less extreme among the Central American species regard to *Alouatta seniculus* than those estimated with isoenzymes and blood groups in past decades. Furthermore, needless to say that the microsatellite loci were conspicuously more polymorphic than the traditional biochemical loci and they could be therefore the target of population genetic studies on primate species, which could reveal some of the most outstanding evolutionary events modulating the genetic structure of neotropical primate species. For instance, the DNA microsatellite in *A. seniculus* yielded among 4.7 and 11 times more gene diversity than isoenzyme or protein markers.

Effective numbers, population sizes and recent bottleneck.

Practically, no estimates of N_e/N of Neotropical Primates are present in bibliography. The unique estimate found by the author was that for *Leontopithecus rosalia* determined by DIETZ et al. (1999). In that case, $N_e/N = 0.15$. This value was similar to that obtained by FRANKHAM (1995) by means of the average across of 192 published estimates of the N_e/N ratio from 102 species of insects, mollusks, amphibians, reptiles, birds, mammals and plants. The average comprehensive estimates of N_e/N, including the effects of fluctuations in population size, variance in family size and unequal sex-ratio, averaged only 0.10-0.11. However, other N_e/N ratios have been calculated for Cercopithecoidea Primates, being these values as follows: *Cercocebus galeritus* ($N_e/N = 0.19-0.29$), *Macaca fuscata* (0.65) and *Macaca mulatta* (0.45-0.53) (KINNAIRD and O´BRIEN, 1991; NOZAWA, 1972; HARPENDING and COWAN, 1986). Like there is no consensus among these ratio estimates, other theoretical procedures to calculate this ratio were employed. Throughout the effective number esti-

mates, which rely in two different mutational models, I calculated N_e/N ratios by means of theoretical population genetics. The expression, $V(k) = (4N - 2 N_e - 2)/(2N_e)$, where $V(k)$ is the variance of the progenies, is useful to begin. Because this variance in many organisms, included primates, is usually greater than two ($V(k) > 2$), thereby $N_e < N$, being N_e the effective numbers and N the population size. If we consider that the population sizes are constant (of course, it is difficult to say that it will be always true in wild), then the breeding progeny, when reach sexual maturity, had a k = 2 (being k, the mean number of individuals for progeny). If progeny was counted at an earlier stage, k should be greater than 2 and, as $V(k)/k$ is related to k, the obtained $V(k)/k$ must be adjusted to a ratio of k =2 (CRAWFORD, 1984). Additionally, if we consider that the survival of each little monkey is random and independent to that of its siblings, therefore the expression $V(k)/k = 1 + (k/k´)[(V(k´)/k´) - 1]$, where k´ and $V(k´)$ are the mean and the variance of progenies enumerated at an early stage of the life-cycle, is useful to calculate a relation among N_e and N. Conversely, if the progeny average decreased from k´ to k* by the death of entire lineages and subsequently to k by individual death independently of the lineage survival, the equation $V(k)/k = 1 + (k/k*)[(V(k´)/k´) + k´- k* - 1]$ is useful as well to estimate the proportions between N_e and N. Both models are feasible in diverse New World primates, although not identically in function of the species considered, of course. However, regardless, if the data are absolutely true for all the primate species considered in the current work, let me attempt an useful exercise. Imagine for the second model that the individual death is a mix of individual probability and entire lineage survival probability, which is the most secure in the case of Neotropical primates, and k = 2, k´= 1, k* = 2 and $V(k´)$ = 5, for instance, being $V(k)/k$ = 4 and as result, N_e/N = 0.5. Therefore, to obtain the widest spectrum of real total population size estimates, I used a N_e/N ratio ranging from 0.15 to 0.5. In fact, the average value of all primate species studied by FRANKHAM (1995) was 0.422, which is within the interval I employed. Certainly, other organisms have higher ratios, such as *Drosophila* (N_e/N = 0.71-1.0) (CROW and MORTON, 1955), *Homo sapiens* (N_e/N = 0.60-0.95) (CHARLESWORTH, 1980), *Mus musculus* (N_e/N = 0.65) (CHARLESWORTH, 1980), *Sciurus carolinensis* (N_e/N = 0.59) (CHARLESWORTH, 1980) or *Odocoileus virginianus* (N_e/N = 0.4-0.5) (RYMAN et al., 1981), but the employed ratios seem the best for Neotropical Primates. It is noticeable to compare these historical total size estimates of the 13 Neotropical Primates studied herein with the status of these species classified by IUCN and other governmental institutions. Nevertheless, firstly, I want to comment what of these estimates could be nearest to reality. Such as it was commented in Material and Methods, I employed two extreme microsatellite mutation rates, 5.6×10^{-4} (upper value) and 7×10^{-5} (lower value). Our previous molecular analyses studying the evolutionary dynamics of microsatellites in Neotropical Primates have revealed for four *Alouatta* species (RUIZ-GARCÍA et al., 2003a), although for five *Ateles* species (RUIZ-GARCIA et al., 2003b) this affirmation is not so clear, that the average mutation rates of the 10 microsatellite employed is significantly nearer to 7×10^{-5} than to 5.6×10^{-4}. Therefore, the total population estimates are probably more realistic for the first mutation rate than for the second one. Furthermore, I adopted two extreme mutation models (IAM and SMM). In the same two previous cited works with *Alouatta* and *Ateles*, we determined that 91 % of mutations in these 10 microsatellites evolved by SMM, meanwhile only 9 % of mutations

followed a model more related with IAM. Thus, the SMM model seems more probable in the Primates studied than the IAM. Finally, I employed two extreme feasible values of the N_e/N ratio (0.15 and 0.5). Really, the unique ratio directly measured in field for a Neotropical Primate was that cited for *L. rosalia*. Thus, I consider that 0.15 could be more realistic than 0.5. Taken all these considerations together, I believe that the highest total population estimates showed herein are the most nearest to the reality. If some of these estimates are extremely higher than the possible current census values, it could be an indication of population declination in recent times regard to that which was the average total population size throughout the natural history of a given species.

Cebus albifrons

Taken the 7×10^{-5} mutation rate and the SMM as the most appropriate values, the most feasible total population sizes oscillated from 8304 to 27680. Therefore, the upper and more optimistic limit could be around 28.000 individuals at northern Colombia. Recall that the *C. albifrons* subspecies analyzed here (*versicolor*) was classified by UICN (1996) as DD (unknown status), although the Colombian government (CIGC) classified this subspecies as vulnerable (VU). However, this result, jointly with the fact that recent bottleneck was not discovered for this subspecies, let us to think that its situation is not especially dangerous.

Cebus apella

The most feasible total population size for this species in the Colombian Amazon area studied ranged from 5642 to 18807 animals. This turns out to be about an upper limit of 19000 individuals in the current Colombian Amazon. Note that this species was classified as at low risk (LR) by UICN (1996) and by CIGC. Although, a shift-mode distribution was detected for this species, the remainder tests did not detect any evidence of recent bottleneck. Therefore, the no-existence of a well-evidenced recent bottleneck, let us to think that its situation is not dangerous. Clearly, the gene diversity levels of this population are clearly better (although its gene diversity was not very high) than the levels that we have detected in other *Cebus apella* susbspecies, such as the Brazilian *C. apella xanthosternus* and *C. apella robustus* (RUIZ-GARCÍA, unpublished) which indeed are classified by IUCN (1996) in CR (critically endangered) and VU (vulnerable), respectively.

Cebus capucinus

The most feasible total population sizes for this species oscillated from 2606 until 8687 individuals. Therefore, the highest and more probable historical total size for the Colombian Pacific population of this species is around 9000 animals. By examining this value, it is clearly lower than all those estimates for the other two *Cebus* species studied in Colombia and commented above. Note that this *Cebus* species has a more restricted geographic area dispersion in Colombia than the two species cited above. Nevertheless, UICN (1996) and CIGC classified this species as at low risk (LR). No recent bottleneck was detected, therefore, its situation seems not to be dangerous, although the habitat destruction (by its more narrow geographical distribution) could be more dangerous for this species than for the other two *Cebus* species in Colombia. It could be important to determine the gene diversity levels, effective and total numbers as well as the feasible incidence of recent bottlenecks in other subspe-

cies of *C. capucinus*, such as *C. capucinus limitaneus*, *C. capucinus imitator* and especially *C. capucinus curtus* from the Gorgona Island in Colombia.

Saimiri sciureus

The most feasible total population sizes for this species ranged from 11968 to 39893 animals, being the upper limit around 40.000 exemplars. Note, however, the existence within our samples of two forms belonging to two diverse geographical subspecies (*S. sciureus macrodon* and *S. sciureus albigena*), which could inflate the heterozygosity levels. Therefore, the historical total population size of 40.000 individuals could be inflated as well. Nevertheless, the bottleneck analysis did not detect any significant number reduction. Consequently, these results agree quite well with the status offered by UICN (1996) and CIGC, being this status at low risk (LR). This is a very common species, considered as a pet, in a big fraction of Colombia where lives.

Aotus nancymae

The most probable total population sizes for this species ranged from 3224 to 10747 individuals. That is, the upper limit was established around 11000 specimens in the area of the Peruvian Amazon where this species was sampled. This species was classified by UICN (1996), and by CIGC, at low risk (LR). However, we must kept in mind the strong impact that certain persistently hunted Peruvian Amazon areas, where the *A. nancymae* samples were obtained, are gone throughout, especially, around the Amazon region near to Iquitos and the Ucayali River. Note that this Peruvian region is characterized by a noteworthy hunter and deforestation actions. For instance, BODMER (1995) reported that the estimated annual (1990-1991) harvests of Primates, in this area, ranged from 3.252 to 8.536 for small-bodied primates and from 39.026 to 200.821 for large-bodied primates. This means, that each year, hunting accounts for 4-15 % of the populations of small-bodied primates, whereas populations of large-bodied primates are decreasing because hunting accounts for 49-89 % of these primates removed from the Loreto Peruvian region. Another striking data is offered by REDFORD and ROBINSON (1991). AMONG 1962 and 1967, the number of live monkeys exported from Iquitos were 183.664 individuals. In fact, we must recall that a total of 8.173 primates were captured in this Peruvian area during a ten-year period for biomedical research, and *A. nancymae* is one of the most frequent. Certain degree of care is required for this species, especially because its gene diversity level and the total population size was the lowest I found. Furthermore, a shifted-mode distribution was determined which is related with a recent bottleneck, although the other tests did not detect this phenomena. Nonetheless, this was the lowest sample size employed. Therefore, more analyses on this species are required to determine its real gene status.

Alouatta seniculus

The most plausible total population estimates for the red howler monkeys in Colombia ranged from 23682 to 78940. Thereby, the most optimistic upper limit could be around 79000 individuals, being this estimate the highest found for the 13 species studied. Although this species is very difficult to maintain in captivity, its geographical distribution thoroughly Colombia is very wide. UICN (1996) considered this spe-

cies at low risk (LR), although CIGC classified it as LR/vulnerable. The high levels of heterozygosity encountered for this species in this, and in other studies (SCHNEIDER et al., 1991; POPE, 1992), could be related with the fact that they are, jointly with *Ateles* and *Lagothrix*, the body-largest primates in the geographical area analyzed, which could influence their ability to cross ecological barriers as well as to confer the ability to have a lot of diversified food habits, furthermore to be the most fecund of them with females displayed twice as many pregnancies as the other genus considered. Thus, the high levels of heterozygosity in this species may provide the means by which this species has been able to adapt quickly to new environmental habitats and its population sizes are very large. Which is clear, is that *Alouatta seniculus* in Colombia does not go throughout of any population bottlenecks. This result seems very strong for the *A. seniculus seniculus* subspecies studied herein but no necessarily for other subspecies, such as *A. seniculus insulanus*.

Alouatta palliata

The most probable total population size for this species oscillated from 8312 until 27707 individuals, being therefore the most real optimistic value around 28000, which is 3 times lower than the amount determined for the red howler monkey in Colombia. Indeed, the value obtained could be inflated because I have introduced in the analysis individuals from Costa Rica and from the Colombian Pacific area, which are considered two different subspecies (*A. palliata palliata* and *A. palliata aequatorialis*, respectively). However, this fact did not substantially increase the gene diversity levels nor the population sizes regard to that found in *A. seniculus* from Colombia. Otherwise, four out of 10 microsatellites employed were originally developed for *A. palliata*, but this fact did not show that this species had the highest gene diversity levels as has been claimed for homologous microsatellites. In fact, these markers are more diverse in other species (heterologous) than in *A. palliata* (homologous), which shows that the microsatellite employed are very useful for conservation population genetics tasks and that the heterozygosity levels are not biased by the species origin of the microsatellites. Another interesting feature is that some of the bottleneck analyses revealed significant trends in favor of contrary events to a recent bottlenecks. This could mean that a population expansion has characterized to *A. palliata*, which seems no to be the case, or Wahlund effect (= subdivision effect), which is an indirect proof in favor that *A. palliata palliata* and *A. palliata aequatorialis* are really two different gene pools. In general, our bottleneck results agree with the IUCN (1996) classification, being both subspecies determined as in low risk (LR). Nevertheless, other authors have claimed the dangerous situation of certain populations of *A. palliata* and *A. pigra* in Central America. A lot of populations of these species crashed due different yellow fever epidemics. For instance, a crash of the *A. palliata* population in Panamá in 1949 reduced this population until only 239 individuals. Although the population grew to more than 1500 individuals in middle 70s, the loss of isoenzyme genetic variability was a fact. Nevertheless, the hypervariable DNA microsatellites seem not be affected by so radical gene diversity lost as other less variable molecular markers. However, *A. palliata* could be in a recent future a seriously threatened species by forest destruction within their small distributions as well as hurricanes and epizootic epidemics (RYLANDS et al., 1997). Maybe, the slightly lower heterozygosity levels detected for this species regard to *A. seniculus*

could adequately agree with these facts. My own analyses with other populations of Central American *Alouatta* from Panama supposedly belonging to other species, *A. coibensis coibensis* and *A. coibensis trabeata* (FROEHLICH and FROEHLICH, 1986, 1987), reveals extreme low gene diversity levels (RUIZ-GARCIA, unpublished). These gene diversity levels are intensively related with the restricted distribution of the first subspecies to the islands of Coiba (518 km^2) and Jicaron (13 km^2) and the second one to the Azuero peninsula in Panama. Furthermore, in these islands the hunting, road-building for logging and forest destruction threat the survival of these *Alouatta* populations. The same happens for the second subspecies. The National Park of Cerro Hoya (34092 ha) retains the last forest in the cited peninsula with the only population of these howler monkeys, in a region which has been severely degraded due to permanent pasture and excessive hunting.

Ateles fusciceps robustus

The most probable total historical size for this species assuming a 7 x 10^{-5} mutation rate ranged from 19982 to 66607 individuals. Thereby, the most optimistic real upper limit could be around 67.000 individuals. UICN (1996), as well as CIGC, classified this taxa as vulnerable (VU). Indeed, IUCN (1991) classified this group as endangered (EN). However, the population size estimates are the second largest, even similar or larger than the values detected in other primate species at low risk. It is interesting to note that the Conservation Assessment and Management Plan for Primates (1992) reported among 1000 and 3000 exemplars on wild for this *Ateles* taxa. Hence, the great difference among the theoretical calculated total sizes reported herein (20000-67000) and the censuses (3000) could be understood by several explanations as follows. The first is related with possible diversifying natural selection by hitchhiking (linkage with other codifying genes) affecting one or more of the microsatellites studied. This explanation could occur in an identical way in *Alouatta* and *Lagothrix*, being these heterozygosity estimates probably correlated with the ability to conquer new habitats as well as by the fact to be habitat generalists. Thereby if this phenomena is present, this could overestimated the population sizes shown herein. However, a second most plausible explanation is related with the presence of two different gene pools within this subspecies (RUIZ-GARCIA and ALVAREZ, 2003; RUIZ-GARCIA et al., 2003b), one typical of the Pacific area of Colombia and other at the left hand of the Magdalena River and northern Atlantic coast of Colombia. This last gene pool could have hybridized with populations of *Ateles hybridus* from the right hand of the Magdalena River. This surely augments the level of gene diversity in this subspecies, which could inflate the population sizes. Third, that this species had suffered a strong bottleneck, but not recently, and posteriori gene flow from other *Ateles* taxa, which considerably augmented the gene diversity but without parallely increasing the population sizes. Recall that the analysis employed only has enough power to detect recent bottlenecks. On the contrary, if the bottleneck was ancestral the procedure employed has not capacity to detect it. As mentioned above, the recent bottleneck analysis did not detect any evidence of this trend in this species. Contrarily, some significant test evidenced strong events against the detection of recent bottlenecks. This is an additional proof in favor of the existence of two gene pools inside the current geographical distribution of *A. fusciceps robustus*. Therefore, there is not a clear systematic differentiation inside this taxa and more

exhaustive analyses are required to determine the exact status of these *Ateles* populations.

Ateles belzebuth belzebuth

The total population size of this species in the Colombian and Peruvian Amazon oscillated from 13168 to 43893 individuals, being thus the most optimistical real estimate of 44000 exemplars. This is a species classified as vulnerable (VU) by UICN (1991, 1996). Nonetheless, the historical total sizes seem high and no test nor graphic descriptor detected any evidence of recent bottlenecks. If really this theoretical historical population value is higher than the census ones, such as it could be (1000-3000 individuals following the Conservation Assessment and Management Plan of Primates, 1992), the same explanations offered for the previous *Ateles* taxa could be invoked. Additionally, for this species the sample size employed was small and this could affect the power of the bottleneck tests.

Ateles hybridus

The most probable total sizes for this taxa ranged from 13824 until 46080, being the upper estimate around 46000 animals. This value is practically identical to that observed for *A. belzebuth belzebuth*, although its geographic distribution is clearly smaller. Both species practically shared the same alleles for the microsatellites studied with similar frequencies, which argues against the findings made by COLLINS and DUBACH (2000) with mtDNA genes. Data shown elsewhere (RUIZ-GARCIA et al., 2003b), indicated an intense genetic relationships among *A. belzebuth* and *A. hybridus* and the separation time between both taxa is recent and for this reason the major part of the gene variability accumulated in *A. belzebuth belzebuth* has been retained in *A. hybridus*, although the current population size of *A. hybridus* is clearly smaller than that reported herein (theoretical value, 46000 individuals against the value from the Conservation Assessment and Management Plan for Primates, 1992, 100-1000 individuals). In fact this taxa was classified by IUCN (1991, 1996) and CIGC as endangered (EN). All the explanations offered for the previous two *Ateles* taxa could be applied to *A. hybridus* to try to discuss the discrepancies between the theoretical and the census values. Furthermore, an additional explanation could be invoked. The average microsatellite mutation rates per generations in *Ateles* could have changed regard to the other Primates studied, being these mutation rates more related to the 5.6×10^{-4} value than to the 7×10^{-5} value, which is the best for the remainder genera analyzed. If we take this other mutation rate, the historical total population sizes for *A. fusciceps robustus* (IAM: 1400-4667; SMM = 2498-8327), *A. belzebuth belzebuth* (IAM: 1040-3467; SMM: 1646-5487), *A. hybridus* (IAM: 1078-3593; SMM: 1728-5760) agree quite well with the censuses values, 1000-3000, 1000-3000 and 100-1000, respectively. In fact, this could be an excellent explanation to understand why the recent bottleneck analyses did not detect this phenomena in these three *Ateles* taxa. The population sizes in the *Ateles* populations were constantly always small or very small since their oldest origins and no from recent times.

Ateles chamek

The most feasible population size estimates taken the 7×10^{-5} mutation rate oscillated from 12352 to 41173 individuals. Thus the upper limit is around 41000 individ-

uals, which in this case agrees quite well with the censuses for this species (10000-30000 animals following the Conservation Assessment and Management PLAN, 1992). In complete agreement, IUCN (1996) classified this species in low risk (LR). It could mean that the fact, or facts, that cause disagreement among theoretical and censuses values for the other three described *Ateles* species, were not present in *A. chamek*. Recall that several authors (MEDEIROS et al., 1997) recognized to this species as the original *Ateles* form from which derived all the other taxa. Maybe, the mutation rate in this ancestral and large *Ateles* population presents more resemblance to the other Neotropical Primates, meanwhile the Colombian *Ateles* species studied (*A. fusciceps*, *A. belzebuth* and *A. hybridus*) this mutation rate has been accelerated by gene drift during the origins of these species from *A. chamek*. No tests detected any recent bottleneck for this species but the graphic descriptor was shifted-moded, which could denote some trend for a recent bottleneck in this species as well as the possibility of several different gene pools inside the samples analyzed. Recall that animals from the Loreto region in the Peruvian Amazon, from the Beni region in the Bolivian Amazon and some individuals sampled in the Rondonia area from Brazil were included in the present study.

Ateles geoffroyi vellerosus

The plausible theoretical population size estimates for this taxa ranged from 7752 to 25840 (an upper estimation around 26000), which is a significant lower value than those obtained for the other *Ateles* taxa studied considering the 7×10^{-5} mutation rate. This subspecies is considered vulnerable (VU) by IUCN (1991, 1996). If we take the 5.6×10^{-4} mutation rate, these values could oscillate from 970 to 3233. Additionally, some of the recent bottleneck tests detected this phenomena affecting this Central America subspecies. Therefore, attention to the genetic conservation of this subspecies should be a priority.

Lagothrix lagotricha

The most probable theoretical population size for the *L. lagotricha lagotricha* subspecies ranged from 13160 to 43867 animals. Thereby, the most optimistic upper limit could be around 44000 individuals. This is an acceptable average historical population size but apparently contrasts with the fact that this was the unique species, which undoubtedly showed to be gone throughout a recent and striking bottleneck. Effectively, this agrees quite well with its classification by several institutions. IUCN (1991) classified this species as vulnerable (VU), whereas CIGC, and especially INDERENA (other Colombian institution), overall considered the genus *Lagothrix* as endangered throughout all the Colombian territories. Contrarily, IUCN (1996) classified this subspecies as in low risk (LR). This last classification seems unfortunate. This species is the primate more used as meet resource by colonists, but especially by Indians in several countries such as Colombia, Perú, Ecuador and Brazil. For instance, the 230 inhabitants of three Waorani villages in Ecuador killed 562 *Lagothrix lagotricha* in less than a year (REDFORD, 1992). Besides, its beautiful presence converted them as one of the most desired primate pets, therefore the hunters killed the mothers to obtain the sons. If the situation of this subspecies, *L. Lagotricha lagotricha*, has been therefore detected as problematic because it is gone throughout a genetic bottleneck, revealed by this microsatellite study, the situation

of the other Colombian subspecies, not introduced in this work, *L. lagotricha lugens*, which has a very more narrow geographical distribution area as well as the parallel habitat destruction of the Andean piedmont, could be severely critical, in disagreement to that claimed by DEFLER (1996), who affirmed that the situation of this Colombian endemic subspecies should be categorized as vulnerable only. Why, however, did the heterozygosity estimates to be so high in this species and in other genera such as *Alouatta* and *Ateles*? A similar result was found for the brother genus of *Lagothrix*, the endangered *Brachyteles arachnoides* from Brazil (POPE,1996). Only 650-750 individuals are known to remain in wild distributed in 15-18 isolated populations. Virtually all these isolated populations contained less than 25 individuals. In addition, a census conducted in 1970 indicated the existence of at least 3.000 animals but 20 years later this number was around 750 animals. Nevertheless, an allozyme diversity analysis, by means of 32 loci, showed that this genus, together with *Alouatta*, have the highest levels of heterozygosity of whatever primate (H = 0.08-0.12). The explanations for this apparent paradox could be the same for the results obtained for *Lagothrix* in the Colombian Amazon. The individuals could live in relatively large social groups and its reproductive structure has characteristics, which could minimize the N_e/N ratio. Otherwise, as it was claimed for *Alouatta* and *Ateles*, the woolly monkey occurs in a variety of habitats, ranging from high-montane rain forest at 3.000 meters above sea level until the Amazon at the sea level. In addition, as it was shown by DEFLER and DEFLER (1996), although this species is mainly folivorous, a broad fruit and flower assortments are included as food resources. Several authors have claimed that a folivore species must physiologically detoxify a wide amount of secondary compounds, and high levels of heterozygosity in enzymatic route genes could help in this task. By simple linkage these genes could force to microsatellite loci to be highly polymorphic. In any event, *L. lagotricha* can be hidden genetic perils by recent bottlenecks, although its population size estimates were considerable. Therefore, a special attention is demanded by the preservation of this species.

Obviously, in no case with the exception of some *Ateles* form, the population estimation sizes obtained for the 13 species analyzed reached the critical situation shown by other neotropical primate taxa, such as is the case of *Saimiri oerstedi oerstedi* in Costa Rica with 2.500-3.000 animals, *Cebus apella margaritae* in Margarita Island with 250-300 individuals, *Leontopitheus rosalia* with 500 individuals, *Leontopithecus chrysopygus* with 1000 individuals or *Leontopithecus caissara* with 260 individuals and the quoted case of *Brachyteles arachnoides* with 750 animals, all of them in Brazil. This does not mean that the situation of the 13 species analyzed herein could be not taken in care in future conservation plans in the Neotropics.

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RELATIVE HUMIDITY, AMBIENT TEMPERATURE, AND URINE WASHING BEHAVIOR IN BOLIVIAN SQUIRREL MONKEYS, *SAIMIRI BOLIVIENSIS BOLIVIENSIS*.

Ruiz JC

Abstract

In *Saimiri* sp sweating is restricted to the palms and soles, and is not significant to maintain thermal balance. So they have to rely principally on thermoregulatory behaviors. In the present work, it was observed that the frequency of urine-washing behavior (which is defined as urination on the palm of the hand, followed by wiping the hands against the sole of the foot) varied in relation to temperature and relative humidity. Nineteen adult females and four adult males *Saimiri boliviensis boliviensis* were observed for 18 months. There was a significant negative correlation with relative humidity in adult females and males and a significant positive correlation with temperature in adult females and males. It is suggested that one of the functions of urine-washing is as a thermoregulatory behavior which varies in frequency with relative humidity to facilitate optimal evaporative cooling.

Introduction

Since *Saimiri* sp. only have ecrine sweating glands in the palms and in the soles (MACHIDA et al., 1967), the loss of heat through sweating is minimal and makes thermoregulatory behaviors very important in coping with high ambient temperatures (ADAIR, 1985).

Thermoregulatory behaviors include searching for cool and shaded places, adoption of postures that help optimize convection and heat conduction (ADAIR, 1985), sneezing (SCHWARTZ and ROSENBLUM, 1985) and urine-washing (urinating on the palm of the hand and rubbing that hand on the sole of the foot), as is carried out by several species of primates (CLARK, 1978; HARCOURT, 1981; HEYMANN, 1995; MILTON, 1975, 1985; ROBINSON, 1979). Urine-Washing likely functions to promote evaporative cooling (SCHMIDT and SEITZ, 1967) since its frequency is positively correlated with ambient temperature in *Saimiri* sp. (SCHWARTZ and ROSENBLUM, 1985) and in *Cebus apella* (ROEDER and ANDERSON, 1991) and negatively correlated with relative humidity in *Cebus apella* (CAROSI and ROSOFSKY, 1999).

The thermoregulatory effectiveness of this behavior in *Saimiri* sp. would be limited by the relative humidity (which set the limit of evaporation), regardless of the ambient temperature. Aiming to determine the effectiveness of urine-washing as a thermoregulatory behavior, the frequency of this behavior was correlated in this study with the levels of ambient temperature and relative humidity.

Material and Methods

Nineteen adult females and four adult males were observed for one year and a half. The group composition varied over the course of the study since the pregnant females and the dependent infants were separated from the original group. The adult ages varied from 6 to 15 years old; nine females and one male were born in captivity at the Argentinian Primate Center (CAPRIM) and the rest were of Bolivian origin but held captive for 9 years at CAPRIM.

The animals were caged in reproductive groups (1 or 2 males with 4 to 6 females) in outdoor cages of 15 m^3. The cages were furnished with branches and nests. The feeding consisted of commercial food ("Carghill Monos"), supplemented with seasonal fruit and water *ad libitum*. The environmental characteristics of the region where CAPRIM is located (mean ambient temperature= 21.7° C, range –2.8° C -42.4 °C, and 1,200 mm mean annual precipitation without rainy season) permitted the animals to be kept in external cages without heating and subjected them to weather changes.

The animals were observed from 2 to 5 times per week, between 9:00 am and 2:00 pm. The observer was in visual contact with the animals. One hour per group was the sampling period and the "All Occurrence of Some Behaviors" method was used (ALTMANN, 1974). Urine-washing was the behavior selected. Before each period, the ambient temperature and the relative humidity were observed.

The observations totaled 362 hours and the Multiple Linear Regression was used to relate the frequency of urine-washing with the ambient temperature and the relative humidity.

During the observation time, the average ambient temperature was 23.6° C (range 8-36° C) and the mean relative humidity was 67 % (range: 18-100 %).

Results

2,445 cases of "Urine-washing" were observed. The mean frequency of "Urine-Washing" was 2.2667 ± 2.827/female/hour (range 0-14), and 0.3876 ± 0.8367/male/hour (range 0-6).

There were no seasonal variations either in females (Mann Whitney test, U_1=240.5, U_2=155.5, n=40, p=0.2535) or in males (Mann Whitney test, U_1=15, U_2=21, n=12, p=0.6889).

There was a significant negative correlation with relative humidity in adult females and males (rs=-0.3101, p<0.001; rs=-0.1971, p<0.01, respectively) and a significant positive correlation with temperature in adult females and males (rs=0.1712, p<0.001; rs=0.2506, p<0.001, respectively).

Discussion

Different explanations have been suggested to account for the presence of urine-washing behaviors: An aggressive behavior (*Cebus apella*, KLEIN, 1979); as a sign of dominance (male *Saimiri sciureus*, CASTELL and HEINRICH, 1971; TALMAGE-RIGGS and ANSCHEL, 1973); as a part of intragroup communication (*Galago demidovii*, CHARLES-DOMINIQUE, 1974; *Brachyteles arachnoids*, MILTON, 1985;

Alouatta palliata, MILTON, 1975; JONES, 2003; *Cebus apella* UENO, 1991; and *Loris tardigradus*, GOONAN, 1993); as a mean of conveying information about reproductive status (*Saimiri sciureus* females, LATTA et al., 1967; CASTELL and HEINRICH, 1971; HENNESY et al., 1978), and as hygienic (CASTELL and MAURUS, 1967, cited by CARDLAND et al., 1980), marcatory (BALDWIN, 1968, 1969; SEITZ, 1969; CANDLAND et al., 1980) and as "displacement activity" (BALDWIN, 1968, 1969). Urine-washing has been proposed as a type of thermoregulatory behavior in *Cebus nigrivitattus* (ROBINSON, 1979), *Cebus apella* (ROEDER and ANDERSON, 1991; CAROSI and ROSOFSKY, 1999; CAROSI et al., 2001) and *Saimiri* sp. (SCHMIDT and SEITZ, 1967; SCHWARTZ and ROSENBLUM, 1985; RUIZ, 1992).

Although none of the functions attributed to urine-washing have been excluded, this study confirms that adult *S. b. boliviensis* urine-wash more frequently when ambient temperature is high, as has been suggested by SCHMIDT and SEITZ (1967), and experimentally shown by SCHWARTZ and ROSENBLUM (1985).

STITT and HARDY (1971) studied the thermoregulation of *S. boliviensis* with partitional calorimetry and found that this species can dissipate heat through sweating in the palms and the soles, but not enough to maintain the thermic balance to an ambient of 38° C. Because of this, these monkeys primarily make use of thermoregulatory behaviors, which have been defined as "those voluntary actions that control the thermic characteristics of the air-skin interphase and facilitate, for this reason, the body temperature regulation to a stable level" (ADAIR and ADAMS, 1983).

With the exception of CAROSI and ROSOFSKY (1999) in their work in *Cebus apella*, in previous studies the importance of the humidity levels was not considered, since the relative humidity would set a limit to the thermoregulatory effectiveness of this behavior by limiting the levels of evaporation (regardless of the ambient temperature) due to the decrease of the evaporative cooling. The frequency of urine-washing would be expected to decrease when relative humidity was high and so, other heat-eliminating methods should have to increase.

In this study, females urine washed significantly more than the males. So, the males would need to make more efficient use of other behaviors to eliminate heat, such as hanging from branches with the tail suspended, increasing the evaporation surface, searching for shades places, making contact with good thermal conducting surfaces, and sneezing (as suggested by SCHWARTZ and ROSENBLUM, 1985).

In conclusion:

1. Urine-washing in adult male and female *S. b. boliviensis* is a thermoregulatory behavior and its modifications can be explained by the modifications in the ambient temperature, and the relative humidity.
2. In spite of the low Regression Coefficient, the very high significant confidence (more than 0.0002 %) indicates that one important function of "Urine-Washing" is thermoregulation of which the frequency is positive related to ambient temperature and inversely related to relative humidity.
3. There is a sexual dimorphism in the frequency of urine-washing. It is possible that the males make more efficient use of other thermoregulatory behaviors than the males.

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ADENOCARCINOMA OF THE UTERUS IN A COMMON MARMOSET (*CALLITHRIX JACCHUS*).

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Abstract

An uterine adenocarcinioma observed in a 35 months old female marmoset is described. Key features of the tumour were glandular structures lined by a cuboidal to columnar epithelium. They were embedded in solid sheets of epithelial cells with ovoid to pleomorphic nuclei. In the walls of the glandular structures abundant vacuoles with eosinophilic content (PAS negative) could be observed. Due to focal invasion of the myometrium the neoplasm was classified as adenocarcinom.

Introduction

The common marmoset (*Callithrix jacchus*) is now being increasingly used in many fields of biomedical research, including that of toxicology. Especially if only small amounts of a drug are available for toxicological testing, common marmosets are the species of choice. However, there exists only little information on non-neoplastic background pathology of this species (OKAZAKI et al., 1996, TUCKER, 1984) and also little is known about incidences and types of spontaneous neoplasms. Tumours observed up to now in common marmosets included undifferentiated carcinoma of the nasal cavity (BASKERVILLE et al., 1984), squamous cell and undifferentiated carcinoma of the nasopharyngeal region (BETTON, 1983; MCINTOSH et al., 1985), adrenal and hepatic myelolipomas (KAKINUMA et al., 1994), adenocarcinoma of the small intestine (CHALIFOUX, 1990; BRACK, 1998), bronchial adenoma (BRACK, 1996), ovarian teratoma (HAWORTH et al., 2003) and paratrichial sweat gland adenocarcinoma (KHAN et al., 1999). In the present paper to our knowledge we report for the first time on an uterine adenocarcinoma in a common marmoset.

Case report

The tumour was observed in a 35 months old female common marmoset (weight 361g) that was part of a toxicity study (low-dose group). The animal was humanely euthanized at terminal kill and a complete necropsy was performed. No macroscopic lesions of the uterus were observed at necropsy. Samples of all organs were fixed in 10 % neutral buffered formalin, embedded in paraffin wax and sectioned at a nominal thickness of 5 μ. Slides were stained with H.E., PAS reaction and Goldner stain. Microscopically, the neoplasm obliterated nearly the complete lumen of the uterus with only a small fluid filled central lumen remaining (Fig. 1). One major constituent

of the neoplasm were glandular structures lined by a cuboidal to columnar (partly multilayered) epithelium with a vesicular, round to ovoid nucleus (Fig. 2). Another key feature of the epithelium was the abundant presence of variably sized round vacuoles with a strongly eosinophilic content that partly masked cytoplasm and nuclei of these cells (Fig. 3). Vesicle content stained negative in PAS reaction. Multifocaly the epithelium of the glands was missing and eosinophilic round vacuoles had accumulated in their lumen. Glandular structures were separated by solid sheets of epithelial cells that had ovoid or pleomorphic nuclei. Slight infiltration of these areas by polymorphonuclear granulocytes was found scattered all over the tumour. One to two mitotic figures were seen in a high-power field. In a central area of the tumour a small minor portion with numerous small densely packed blood-filled vascular channels revealing thickened basement membranes and hypertrophic endothelial cells (bulging into the vascular lumen) occurred. At one site the neoplasm invaded the myometrium indicating its malignancy.
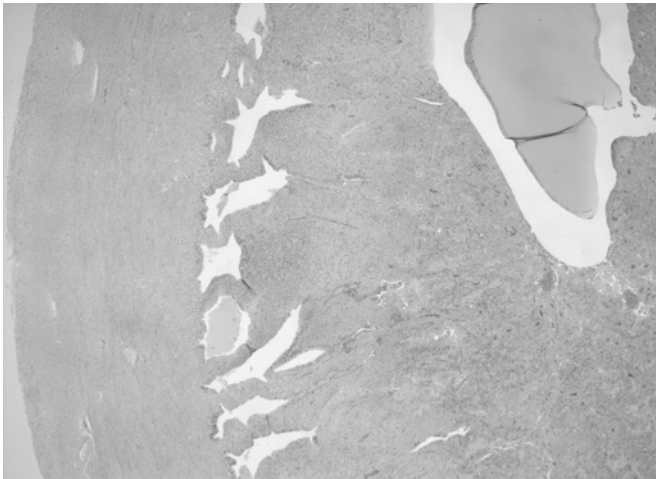

Fig. 1: Uterine adenocarcinoma obliterating the uterine lumen. H.E. x 30
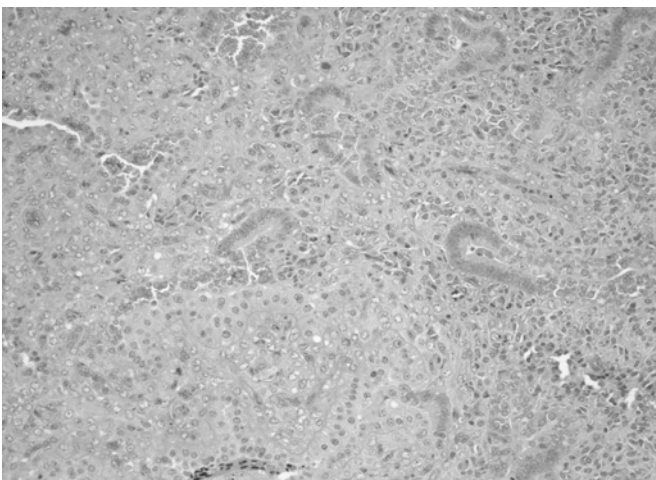

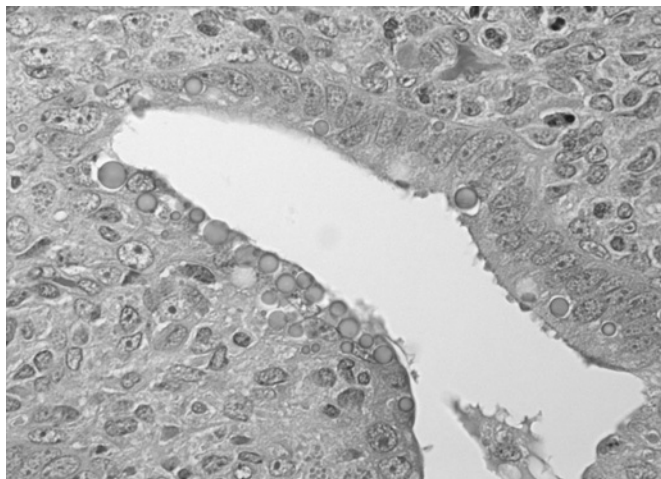Fig. 2: Glandular structures lined by a cuboidal to columnar epithelium. H.E. x 300

Fig. 3: Glandular epithelium filled with strongly eosinphilic vacuoles. H.E. x 600

Discussion

Few reports on uterine tumours do exist in primates. Most neoplasms were observed in rhesus monkeys reflecting the former widespread use of this species in biomedical research. Tumour types seen in rhesus monkey include a choriocarcinoma (LINDSAY et al., 1969), a sarcoma (PLESKER et al., 2002), an adenocarcinoma (STROZIER et al., 1972), a hemangioma (DiGIACOMO and McGANN, 1970), leiomyomas (DiGIACOMO and McGANN, 1970; TAKAYAMA et al., 2000) and a leiomyosarcoma (COOK et al., 2004). Sporadic cases observed in other primate species were an epitheloid leiomyosarcoma in a pig-tailed macaque (BIRKEBAK et al., 1996), an endometrial carcinoma in a squirrel monkey (CHALIFOUX, 1993) and leiomyomas in a spider monkey (BINHAZIM et al., 1989). In our own colony of cynomolgus monkeys an adenoma, a leiomyoma and an uterine polyp were observed (unpublished data). The age of the animals in the cited reports was between 12 and 32 years confirming the well-known experience that tumour incidence dramatically increases with age. In contrast the uterine tumour reported here was rather unusual because it was found in a young adult female (based on a marmoset life expectancy of 8-12 years).

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