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Research article

Phylogenetic relationships between *Hapalemur* species and subspecies based on mitochondrial DNA sequences

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Abstract

Background: Phylogenetic relationships of the genus *Hapalemur* remains controversial, particularly within the *Hapalemur griseus* species group. In order to obtain more information on the taxonomic status within this genus, and particularly in the cytogenetic distinct subspecies group *of Hapalemur griseus*, 357 bp sequence of cytochrome b and 438 bp of 12S mitochondrial DNAs were analyzed on a sample of animals captured in areas extending from the north to the south-east of Madagascar. This sample covers all cytogenetically defined types recognized of the genus *Hapalemur*.

Results: Phylogenetic trees and distances analyses demonstrate a first emergence of *Hapalemur simus* followed by *H. aureus* which is the sister clade of the *H. griseus* subspecies. *Hapalemur griseus* is composed of 4 subspecies separated into two clades. The first contains *H. g. griseus, H. g. alaotrensis* and *H. g. occidentalis*. The second consists of *H. g. meridionalis*. A new chromosomal polymorphic variant from the region of Ranomafana, *H. griseus ssp*, has been analysed and was found in both clades.

Conclusions: Our results support the raising *of H. g. meridionalis* to the specific rank *H. meridionalis*, while neither cytogenetic nor molecular evidences support the raising *of H. g. alaotrensis* to a species rank despite its morphological characteristics. The new cytotype *H. g. ssp* which has been previously characterized by cytogenetic studies contains animals clustering either with the group *of Hapalemur griseus griseus* or with that of *Hapalemur meridionalis*. This suggests the existence of an ancestral polymorphism or an introgression of mitochondrial DNA between subspecies.

Background

The genus *Hapalemur* of the Malagasy Prosimian family Lemuridae comprises three clearly different species: *Hapalemur griseus*[1], *H. simus*[2] and *H. aureus*[3]. Classi-

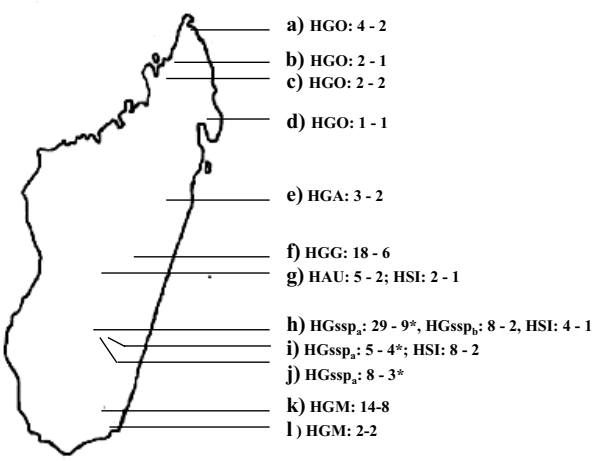


Figure 1
Map of Madagascar showing the capture locations and/or the origin of the different *Hapalemur* species and subspecies, a) Analamera, b) Ambato, c) Ambakoany, d) Maroantsetra, e) Alaotra lake, f) Maromiza, g) Tsimbazaza zoo, h) Ranomafana, i) AmbolomavoJ) Kianjavato, k) Andohahela, l) Mandena. Abbreviations : HSI = *Hapalemur simus*, HAU = *H. aureus*, HGM = *H. griseus meridionalis*, HGssp$_b$= *H. griseus ssp$_b$*, HGssp$_a$= *H. griseus ssp$_a$*, HGG = *H. g. griseus*, HGA = *H. g. alaotrensis*, HGO = *H. g. occidentalis*. The first number behind each taxon represents the number of animals captured and the second, the number of haplotype found in this area. *The haplotype HGssp$_a$02 is present in h, i and j; Hgssp$_a$03 in h and j; Hgssp$_a$07 in h and i; HGssp$_a$10 in h and i.

fication of different types within the *H. griseus* group has been controversial, particularly in the light of the variations existing in coat colour [4]. Systematic cytogenetic studies led to the description of four subspecies of *H. griseus* characterised by different karyotypes: *H. g. griseus* (HGG) (2N = 54) [5,6], initially considered as the most widespread and supposed to live in the Eastern forest of Madagascar from the north to the south [7]; *H. g. occidentalis* (HGO) (2N = 58) [6] restricted in the north-west and the eastern part of Madagascar as east as Maroantsetra [4,8]; *H. g. meridionalis* (HGM) (2N = 54) [9] found in the south, near Fort Dauphin, and differing from *H. g. griseus* in one metacentric chromosome ; *H. g. alaotrensis* (HGA) (2N = 54) [6], restricted to the bed of the Alaotra lake, with a karyotype similar to HGG containing more juxtacentromeric heterochromatin, and characterized by a larger body size [4]. In addition, a new variant of HG, *H. griseus ssp* (HGssp), has recently been described. This type is characterized by a chromosomal polymorphism (2N = 54, 55 and 56) and is living in the area of Ranomafana-Kianjavato [8].

The comparison of the karyotypes of the different *Hapalemur* led to a phylogenetic tree characterised by an earlier emergence *of H. simus* (HSI), followed by *H. aureus* (HAU) which represents the sister clade of the different groups of the *H. griseus* subspecies [8,10]. As molecular studies were fruitfully used for taxonomic investigations of several taxa, including lemurs [11–15], and as no large morphological differences could be found between the cytogenetically close subspecies of *H. griseus*, mitochondrial cytochrome b and 12S DNA analyses were performed in order to determinate more accurately their species and/or subspecies status.

Results

A total of 115 animals covering all the genus *Hapalemur* were captured in bamboo forests extending from the north to the south of Madagascar. The different capture areas of each species and subspecies as the number of animals are indicated in Fig. 1.

The PCR amplification of the cytochrome b resulted in a 357 bp fragment. Alignments using blast search demonstrated that this fragment corresponds to the beginning of the mitochondrial cytochrome b gene. All the sequences were aligned and the substitutions were scored. Animals sharing the same sequences were regrouped into a same haplotype. These different haplotypes are summarised in Fig. 1 and table 2. Consensus sequences constructed with the most common sequence in each species and subspecies (not shown) demonstrated that transition substitutions occur more frequently than transversions (an average of eight transitions for 1 transversion) and gaps are absent. As expected [16], most of the substitutions occur on the third position of the codon (54 out of 64 substitutions), while the first position changed nine times and the second once. The portion amplified comprises the four invariant codons considered necessary for cytochrome b function [17]. Changes were sometimes noticed in the third base of the codon in these positions, but the amino acid remains the same so that the functionality of the cytochrome b is not affected.

Kimura two parameter distances [18] and absolute distances calculated with the different haplotypes (Table 1) as phylogenetic trees constructed with the computer program Pylogenetic Analysis Using Parsimony (PAUP) 4.0b.4a [19] (Figs 2, 3, 4, 5) were used to compare the different *Hapalemur* species and subspecies. Neighbor-joining analyses of cytochrome b haplotypes demonstrated that *H. simus* emerged first followed by *H. aureus* which is the sister clade of the *H. griseus* subspecies. Inside *H. griseus* subspecies, two subclades are present and the *H. griseus ssp* (HGssp) are distributed in both the subclades (Figs 2, 3). For this reason, the *H. griseus ssp* were separated in HGssp$_a$ and Hgssp$_b$. The first subclade (S1) contains HGA, HGG, HGssp$_a$ and HGO. The second subclade (S2) is composed of HGM and HGssp$_b$. Maximum parsimony

Table 1: Lower and higher values of Kimura two parameters distances (under the diagonal) and absolute distances (above the diagonal) between haplotypes of *Hapalemur* species and subspecies. For abbreviations see Figure 1.

	HSI	HAU	HGM	HGsspb	HGA	HGG	HGsspa	HGO
HSI	**0–7**	36	38	38	30	30	30	31
	0–0.01991	42	46	42	35	36	39	40
HAU	0.11024	**0–2**	41	42	31	30	30	34
	0.13002	**0–0.00563**	48	46	34	36	36	39
HGM	0.11797	0.12893	**0–9**	0	23	21	21	25
	0.14524	0.15338	**0–0.02565**	10	28	29	34	32
HGsspb	0.11797	0.13290	0.00000	**0–5**	22	21	21	25
	0.13085	0.14524	0.02855	**0–0.01414**	26	27	32	30
HGA	0.09098	0.09431	0.06558	0.06558	**0–1**	1	2	4
	0.11075	0.10393	0.08459	0.07747	**0–0.00281**	3	9	10
HGG	0.09098	0.09098	0.06240	0.06240	0.00281	**0–4**	3	5
	0.11075	0.11075	0.08789	0.08092	0.00847	**0–0.01133**	10	11
HGsspa	0.09098	0.09098	0.06240	0.06240	0.00563	0.00847	**0–10**	5
	0.12087	0.11049	0.10473	0.09719	0.02580	0.02875	**0–0.02868**	13
HGO	0.09431	0.10445	0.07522	0.07522	0.01133	0.01421	0.01421	**0–6**
	0.12469	0.12117	0.09767	0.09055	0.02883	0.03180	0.03781	**0–0.01705**

analyses (data not shown) demonstrated the same topology, unless the bootstrap support for S1 dropped from 94 to 66.

The use of the Kimura two parameter method (Table 1) shows inside S1 a very close distance between HGA and HGG (ranging from 0.00281 to 0.00847), while a larger distance occurs between HGG and $HGssp_a$ (0.00847 to 0.02875) as well as between HGG and HGO (0.01412 to 0.03180). In S2 (Table 1), short distances are observed between HGM and $HGssp_b$ (0–0.02855), (in this subclade, the sequence of HGM 02 and HGssp 01 are identical). Larger distances ranging from 0.10473 ($HGssp_a$/HGM) to 0.06240 (HGM-$HGssp_b$/HGG-$HGssp_a$) separates S1 from S2 (Table 1).

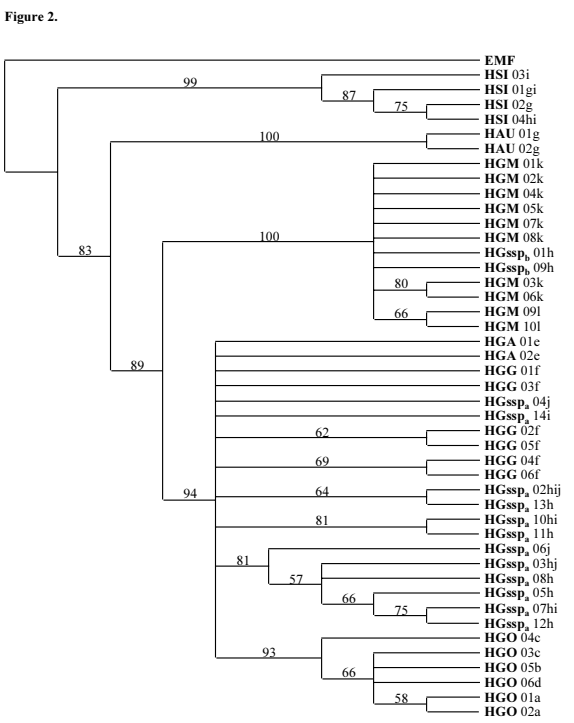
In order to confirm the position of *H. griseus ssp* inside the *H. griseus* subclades, a portion of 12S mitochondrial DNA was amplified and sequenced. The resulted 438 bp fragments were aligned, and the sequences were analyzed with the program PAUP. The trees obtained with HGO, HGG, HGssp and HGM 12S sequences (Figs 4, 5) also demonstrated a separation of HGssp into two subclades. The first ($HGssp_a$), which is composed of animals clustering with HGO and HGG when cytochrome b sequences are analyzed (Figs 2, 3), also clusters with HGO and HGG when 12S sequences are employed (Figs 4, 5). The second ($HGssp_b$), constructed with animals clustering with HGM when cytochrome b sequences are analyzed (Figs 2, 3), also clusters with HGM when 12S sequences are employed (Figs 4, 5).

Discussion

During the last decade, comparisons of mitochondrial DNA sequences have been very useful for the analyses of the phylogenetic species relationships including lemurs [11–15,20]. In our study, cytochrome b and 12S mtDNA sequences were used, in order to clarify the position of each species and subspecies in the genus *Hapalemur*. Despite the fact that we analysed only short sequences, the genetic distances are considered to be relevant because of the large number of animals involved, since large sample size reduces errors in the estimation of evolutionary relatedness [11,21].

Comparisons of cytochrome b sequences demonstrated that pairwise genetic distances between *H. simus* and *H. aureus* and the other *Hapalemur* (distances ranging from 0.09098 to 0.15338) are the highest. The corresponding phylogram also shows that these taxa are well separated. Our molecular data thus strongly support the species status for *H. simus*, *H. aureus* and *H. griseus*.

Among the *H. griseus*, the genetic divergence observed between *H. g. meridionalis* and the HGG, HGA, $HGssp_a$ and HGO (ranging from 0.06240 to 0.10473) is markedly higher than all other values of the same level of intra *H. griseus* comparison (ranging from 0.00281 to 0.03781). The phylogram also clearly separates the *H. g. meridionalis* from other *H. griseus*. This high genetic divergence in the ranging of those observed between species of lemurs, would support the classification *of H. g. meridionalis* in a separate species "*H. meridionalis*" despite the relatively small chromosomal differences observed [9].



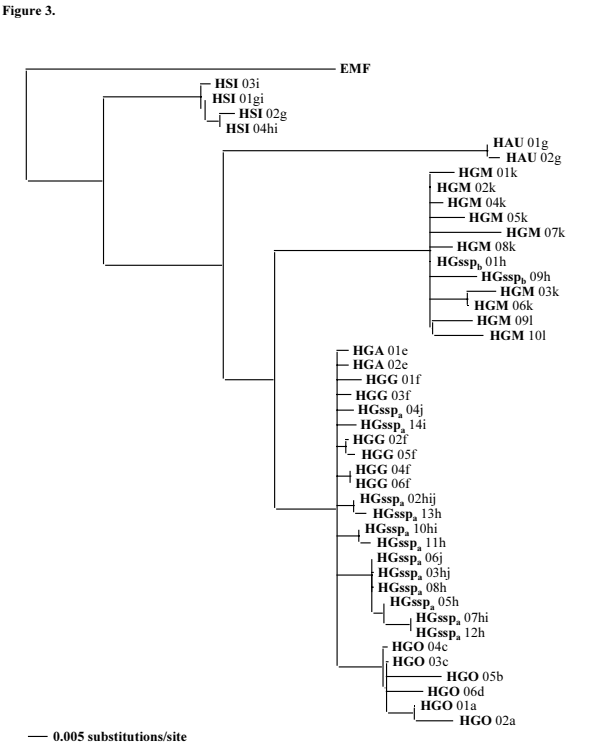


Figure 2
Phylogenetic tree based on 357 bp sequences of the cytochrome b gene. Bootstrap method with neighbor joining search and Kimura two parameter distance correction obtained with 10000 replications are used. Only nodes with a bootstrap value greater than 50% are indicated. The tree is rooted using an *Eulemur macaco flavifrons* (EMF) sequence and the comparisons are made between the haplotypes. The letter behind each taxon indicates the capture locality. For abbreviations see Figure 1.

Figure 3
Phylogram based on 357 bp sequences of the cytochrome b gene. Bootstrap method with neighbor joining search and Kimura two parameter distance correction obtained with 10000 replications are used. The tree is rooted using an *Eulemur macaco flavifrons* (EMF) sequence and the comparisons are made between the haplotypes. The letter behind each taxon indicates the capture locality. For abbreviations see Figure 1.

Lower distances (ranging from 0.01133 to 0.03781) are observed between *H. griseus* and *H. g. occidentalis.* Moreover, these two forms cluster in the same clade. So, despite the cytogenetic differences existing between these two forms, which differ by two chromosomal rearrangements [10], we propose the maintenance of the subspecies status for *H. g. occidentalis.*

On the basis of our analyses, *H. g. alaotrensis* is indistinguishable from *H. g. griseus.* The very short genetic distances found between *H. g. griseus* and *H. g. alaotrensis* (0.00281 to 0.00847) suggests a combination of these two groups into a single subspecies. However, the differences of the body sizes [4] and the differences in the content of heterochromatin found in both karyotypes [22] support the separation *of H. g. griseus* and *H. g. alaotrensis* in separate subspecies [23], but in no case in species apart as it has been previously proposed [24].

The systematic position of HGssp is more difficult to establish when cytogenetic and molecular data are compared. Cytogenetic data demonstrated the existence of an unique chromosomal polymorphic subspecies characterized by two karyotypes, 2N = 56 and 2N = 54, and their hybrids 2N = 55 [8]. In the view of molecular data, HGssp contains two groups, $HGssp_a$ and $HGssp_b$, separated by an important genetic distance (0.06240–0.09719) in the range of those observed between species. Each of these two groups contains both karyotypes, 2N = 56 and 2N = 54, as well as their hybrids. The $HGssp_a$ are separated from HGG, HGA and HGO by a genetic distance in the range of those observed between subspecies (0.00563–0.03781), while $HGssp_b$ appears similar to HGM. Moreo-

Figure 4.

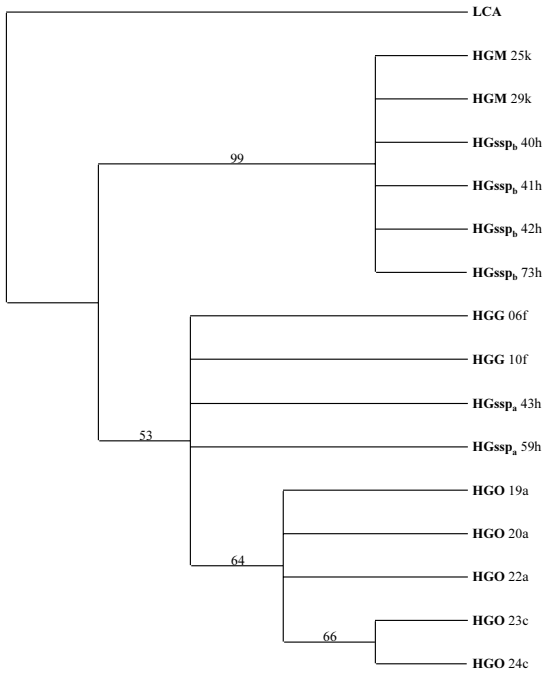


Figure 5.

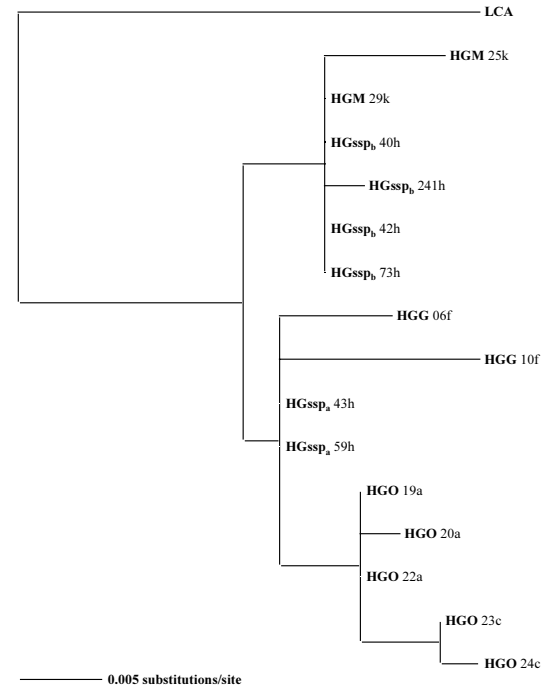


Figure 4
Phylogenetic tree based on a 438 bp 12S mitochondrial DNA sequences. Bootstrap method with neighbor joining search and Kimura two parameter distance correction obtained with 10000 replications are used. Only nodes with a bootstrap value greater than 50% are indicated. The tree is rooted using a *Lemur catta* (LCA) sequence (EMBL, accession n°A Y012130) The letter behind each taxon indicates the capture locality. For abbreviations see Figure 1.

Figure 5
Phylogram based on a 438 bp 12S mitochondrial DNA sequences. Bootstrap method with neighbor joining search and Kimura two parameter distance correction obtained with 10000 replications are used. The tree is rooted using a *Lemur catta* (LCA) sequence (EMBL, accession n°A Y012130) The letter behind each taxon indicates the capture locality. For abbreviations see Figure 1.

ver, the HGssp$_b$ haplotypes are mixed with those of HGM. Taking into account these results, HGssp$_a$ should be considered as belonging to the group of HGG, HGA and HGO, and HGssp$_b$ as belonging to the group of HGM.

The existence of two well-separated clades among the *Hapalemur griseus ssp* originating from the Ranomafana region could possibly resulted from either recent mitochondrial DNA introgression or ancestral polymorphism. As the boundaries between HGM and HGssp are still unknown, we could hypothesize that the limits could be close to Ranomafana, allowing hybridization between these two forms. A transfer of mitochrondrial DNA from HGM into the HGssp population could have occurred through a matrilinear process resulting in the HGssp$_b$ haplotype. New investigations in areas located in the south of Ranomafana should thus allow the finding of HGM populations containing introgressed HGssp mitochondrial DNAs, unless this transfer occurs only from HGM to HGssp. A second hypothesis is that an ancestral polymorphic population containing both HGM and HGssp haplotypes and a chromosomal polymorphism have diverged in two separated populations. In the population of Ranomafana, the HGM and HGssp haplotypes as well as the chromosomal polymorphism were maintained. In the second population, only the HGM haplotype remained present, and a gain of a large block of heterochromatin gave rise to the karyotype characteristic of the HGM [9].

The comparisons of the phylogenetic trees based on mitochondrial DNA sequences with those previously obtained from cytogenetic data are only partially concordant. In both trees, *H. simus* emerges first, followed by *H. aureus* and then by the different *H. griseus* forms. The cytogenetic

Table 2: Haplotypes and GenBank accession numbers of Hapalemur cytochrome b sequences.

Species and subspecies/number of individuals	Haplotype/number of individuals	GenBank accession numbers
Hapalemur simus (HSI)/14	01/3	AJ428977
	02/1	AJ428978
	03/4	AJ428979
	04/6	AJ428980
Hapalemur aureus (HAU) / 5	01/4	AJ428957
	02/1	AJ428958
Hapalemur griseus meridionalis (HGM)/16	01/1	AJ428959
	02/1	AJ428960
	03/1	AJ428961
	04/1	AJ428962
	05/1	AJ428963
	06/1	AJ428964
	07/1	AJ428965
	08/7	AJ428966
	09/1	AJ428967
	10/1	AJ428968
Hapalemur griseus ssp (HGssp) / 50	01/7	AJ429054
	02/12	AJ429055
	03/3	AJ429056
	04/2	AJ429057
	05/6	AJ429058
	06/1	AJ429059
	07/6	AJ429060
	08/5	AJ429061
	09/1	AJ429062
	10/2	AJ429063
	11/2	AJ429064
	12/1	AJ429065
	13/1	AJ429066
	14/1	AJ429067
Hapalemur griseus alaotrensis (HGA) / 3	01/1	AJ428969
	02/2	AJ428970
Hapalemur griseus griseus HGG)/18	01/1	AJ428971
	02/12	AJ428972
	03/1	AJ428973
	04/1	AJ428974
	05/1	AJ428975
	06/2	AJ428976
Hapalemur griseus occidentalis (HGO) / 9	01/2	AJ428982
	02/2	AJ428983
	03/1	AJ428984
	04/1	AJ428985
	05/2	AJ428986
	06/1	AJ428987
Eulemur macaco flavifrons (EMF)/11	01/11	AJ428981

data allow to propose an evolutionary tree in which *H. g. occidentalis* emerges first, followed by *H. g. griseus*, and *H. g. meridionalis*[10], whereas on the tree based on cytochrome b and 12S sequences, *H. g. meridionalis* appears as a sister clade of the other *H. griseus*. This difference may be related to the short distances observed inside each clade which allowed no branching order.

Conclusions

Our molecular studies of the *Hapalemur* genus raise the question of the classification of *H. g. meridionalis* in the species status *H. meridionalis*. They also confirm the subspecies status *of H. g. occidentalis* and the absence of arguments in favour of the classification of *H. g. alaotrensis* as a separate species. The sequencing of the *Hapalemur griseus*

ssp originating from Ranomafana reveals animals clustering either with the *H. meridionalis* or with the group of *Hapalemur griseus griseus*. No monophyletic clade could be determined in this new cytotype, so that the taxonomic status of the *Hapalemur griseus ssp* remains undefined. As our molecular data did not match the branching sequence within the *H. griseus* group based on cytogenetic data, further investigations including nuclear DNA will be necessary in order to resolve this issue.

Materials and methods

Animal studied

Six survey were organised from 1997 to 2001 allowing the capture of 115 animals in bamboo forests extending from the north to the south of Madagascar. Animals were captured using blowpipe projections and then sexed, weighted and measured. Skin samples were cut off under general anaesthesia with a 2 mg/kg injection of ketamine solution (Ketalar® Parke-Davis) and conserved deep frozen in liquid nitrogen. The different capture areas and the number of animals are indicated in Figure 1. As the *H. griseus* taxonomy is essentially based on cytogenetic criteria, karyotypes were made in order to confirm the species and subspecies rank. From the north to the south, the following subspecies were caught: a total of nine *H. g. occidentalis* (four at Analamera (a), two at Ambato (b), two at Ambakoany (c), and one at Maroantsetra (d)). Three *H. g. alaotrensis* at the Alaotra lake (e); 18 *H. g. griseus* at Maromiza (f); 50 *H. griseus ssp.* (37 at Ranomafana (h), five at Ambolomavo (i), eight at Kianjavato (j)); 16 *H. g. meridionalis* (14 in Andohahela (k) and two in Mandena (l)). In addition, 12 *H. simus* (four from Ranomafana (h), and eight from Ambolomavo (i)), as well as two *H. simus* and five *H. aureus* from the Zoological Park of Tsimbazaza (g) were studied. Animals were released at their capture location, immediately after recovery from anaesthesia, except two *H. g. occidentalis* captured in Ambato as well as three *H. g. alaotrensis* which are kept in the Zoological Park of Mulhouse and one *H. g. occidentalis* from Maroantsetra which is kept in the private Zoological Park of Mandraka. As outgroup, we used a sample *of Eulemur macaco flavifrons*, from one of the individuals captured in the Sahamalaza forest for an earlier study [25].

DNA extraction

DNA samples were extracted from the skin biopsies using the standard proteinase K digestion followed by a phenol chloroform extraction as described by Sambrook *et al.*[26] with minor modifications. Small pieces of skin (~9mm^2) were suspended in 200µl of extraction buffer (Tris 0.2M pH 8.4; KCl 0.5M; proteinase K: 5 mg/ml), and incubated at 37°C overnight. The samples were than heated at 95°C and mixed with an equal volume (200µl) of phenol/chloroform (1/1). After centrifugation (6 mn at 8500 g) the aqueous part was precipitated with 2 volumes of absolute ethanol at -20°C in presence of 1/10 (V/V) of ammonium acetate 5M. After centrifugation (5 mn at 8500 g), pellets were rinsed with 200µl of 70% ethanol and dried at 37°C. Pellets were resuspended in sterile double-distilled water and the concentration of the DNAs were measured by absorption at 260 nm. DNA samples were then stored at -30°C.

Amplification conditions

Cytochrome b

The polymerase chain reaction (PCR) was employed to generate a double-stranded fragment of 357 bp corresponding to a part of the mitochondrial cytochrome b gene. Each amplification was performed in the presence of 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5mM $MgCl_2$ (Gibco BRL), 200 µg BSA, 0.5 µM of each primer, 5U of Taq DNA polymerase (Perkin Elmer Cetus), 200 µM of each dNTP (Boehringer Mannheim) and 360ng of template DNA in a volume of 200 µl. The following primers derived from those described by Kocher *et al.*[27] were employed: Pr181 (5'-CCATCCAACATGTCAGCATGATGAAA-3') and Pr182 (5'-CCCTCAGAATGATATTTGTCCTCA-3'). Reactions were done in a Perkin Elmer Cetus DNA thermocycler 480 as follows: predenaturation (7 mn at 93 °C) and 35 cycles of denaturation (30 s at 94°C), annealing (45 s at 42°C), extension (1 mn 30 s at 72°C), followed by a final extension step (10 mn at 72°C).

12S mitochondrial DNA

The same PCR conditions than those described for the cytochrome b amplifications were applied, with a concentration of 2 mM $MgCl_2$ and the following set of primers: Pr179 (5'-AAACTAGGATTAGATACCCTATTAT-3') and Pr180 (5'-AAGAGCGACGGGCGATGTGT-3'). Amplifications were performed in a Perkin Elmer Cetus DNA thermocycler 480 as follows: predenaturation (10 mn at 94°C) and 40 cycles of denaturation (30 s at 94°C), annealing (45 s at 45°C), extension (1 mn 30 s at 72°C), followed by a final extension step (10 mn at 72°C).

Sequences

Amplification products were electrophoresed on 1.3% agarose gels in TBE buffer (Tris base 87 mM, boric acid 89 mM, EDTA 2 mM, pH 8.0) in the presence of 0.5 µg/ml of ethidium bromide. Electrophoresis were performed at 200 mA for 2 h in TBE. Gels were than examined and photographed under UV light with a Polaroid system. The major bands on the gels were cut and centrifuged (8500 g 10 mn) in order to recover the DNA fragments [28]. The fragments were then precipitated with 0.1 volumes of ammonium acetate 5 M and 2 volumes of absolute ethanol at -20°C. After centrifugation (8500 g 10-mn), the pellets were air dried, resuspended in 50 µl of sterile bidistilled water and sequenced on an automatic ABI PRISM sequencer with the Taq dye deoxy terminator cycle sequenc-

ing kit. Each sample was sequenced from 5' to 3' and 3' to 5'.

Cytochrome b sequences were aligned and for each taxa, similar sequences were grouped under one haplotype. These haplotypes and the EMBL GenBank accession numbers are listed in table 2. EMBL GenBank accession numbers for the 12S sequences are listed in table 3.

Table 3: GenBank accession numbers of Hapalemur 12S sequences.

Species/subspecies	N°	GenBank accession numbers
Hapalemur griseus meridionalis (HGM)	25	AJ429205
	29*	AJ429206
Hapalemur griseus ssp (HGssp)	41	AJ429207
	43	AJ429208
	59	AJ429209
Hapalemur griseus griseus (HGG)	06	AJ429210
	10	AJ429211
Hapalemur griseus occidentalis (HGO)	19 **	AJ429212
	20	AJ429213
	23	AJ429214
	24	AJ429215

* 12S sequence of HGM29 is similar to HGssp40, HGssp42 and HGssp73. ** 12S sequence of HGO19 is similar to HGO22.

The aligned sequences were analysed using neighbor-joining and maximum parsimony methods with the computer program Pylogenetic Analysis Using Parsimony (PAUP) 4.0b.4a [19]. Genetic distances (d) measured in neighbor-joining analyses were calculated using the Kimura two parameter method [18] with the following formula: d=-1/21n [(1-2P-Q)Xsqr(1-2Q)], with P=transitions/positions scored and Q=transversions/positions scored. For neighbor-joining and maximum parsimony analyses, bootstraps of 10000 replicates were performed to examine the relative support of each relationship in the resultant topologies. Maximum parsimony trees were calculated via fast stepwise addition with random addition sequence.

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