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 *H. 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 analyzed 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.
The comparison of the karyotypes of the different
anja
The comparison of the karyotypes of the different
anja
54, 55 and 56) and is living in the area of Ranomafana-Ki-
characterized by a chromosomal polymorphism (2N =

54, 55 and 56) [6], restricted to the bed of the Alaotra lake,
in one metacentric chromosome ;

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) Maroantssetra, e) Alaotra lake, f) Maromiza, g) Tsimbazaza zoo, h) Ranomafana, i) Ambolomavo) Kianjavato, k) Andohahela, l) Mandena.

Abbreviations : HSI = Hapalemur simus, HAU = H. aures, HGM = H. griseus meridionalis, HGssp a = H. griseus ssp a, HGssp b = H. griseus ssp b, HGGO = H. griseus griseus, HGG = H. g. griseus, HGA = H. g. aalatensis, 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 a02 is present in h, i and j; HGssp a07 in h and i; HGssp a03 in h and j; HGssp a07 in h and i; HGssp a10 in h and i.

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 determine 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   | HGssp | 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    |
| HGM   | 0.13002 | 0–0.00563 | 48    | 46    | 34    | 36    | 39     |
| HGssp | 0.11797 | 0.12893 | 0–9   | 0     | 23    | 21    | 21     | 25    |
|     | 0.14524 | 0.15338 | 0–0.02565 | 10    | 28    | 29    | 34     | 32    |
| HGA   | 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    |
| HG    | 0.09098 | 0.09431 | 0.06558 | 0.06558 | 0.07747 | 0–0.00281 | 3    | 9     | 10    |
| HHG   | 0.11075 | 0.10393 | 0.08459 | 0.02640 | 0.00281 | 0–4   | 3      | 5     |
| HGG  | 0.09098 | 0.09098 | 0.06240 | 0.06240 | 0.00281 | 0–0.01133 | 10    | 11    |
| HGSSpa | 0.09098 | 0.09098 | 0.06240 | 0.06240 | 0.00563 | 0.00847 | 0–0.002868 | 13    |
| HGO  | 0.12087 | 0.11049 | 0.10473 | 0.09719 | 0.02580 | 0.02875 | 0–0.01705 |
| 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 | 0–32   |

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 HGSSpa (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 HGSSpb (0–0.02855), (in this subclade, the sequence of HGM 02 and HGSSpb 01 are identical). Larger distances ranging from 0.10473 (HGSSpb/HGM) to 0.15338 (HGM–HGSSpb/HGG–HGSSpa) separate S1 from S2 (Table 1).

In order to confirm the position of *H. griseus* sp 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 (HGSSpa), 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 (HGSSpb), 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, HGSSpa 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].
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 chromosom 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-
ver, the HGsspR haplotypes are mixed with those of HGM. Taking into account these results, HGsspR should be considered as belonging to the group of HGG, HGA and HGO, and HGsspR 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 result 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 mitochondrial DNA from HGM into the HGssp population could have occurred through a matrilinear process resulting in the HGsspR 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
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*

### 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* (HS)/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 aaloitensis* (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                 |
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 individuals 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. alaotrensis captured in Ambato as well as three H. g. alaotrensis which are 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 (~9mm²) 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 then 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 MgCl₂ (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’-CCATCCAACATTCAGCATGTGA-3’) and Pr182 (5’-CCCTCAGAATGATTTGTCCTCA-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 MgCl₂ and the following set of primers: Pr179 (5’-AAACTAGTTAGATACCCCTATT-3’) and Pr180 (5’-AAGACCGCGCCGATGTTG3’). 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 then 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 (HGGs)** | 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) 4.0b4a [19]. Genetic distances (d) measured in neighbor-joining analyses were calculated using the Kimura two parameter method [18] with the following formula: \(d = \frac{1}{2} \ln \left[ \frac{(1-2P)Q}{(1-2Q)} \right] \), with \(P=\text{transitions/positions scored and } Q=\text{transversions/positions scored.} \) 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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