Genetic Comparison of Wild Populations of *Lepilemur septentrionalis* and *Lepilemur dorsalis* Using RAPD Markers

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ABSTRACT. The genetic polymorphism of Malagasy prosimian populations is studied by using RAPD markers. The analysis includes two *Lepilemur septentrionalis* populations of the area of Analamera separated by a deforested hill crest and one population of *L. dorsalis* from the island of Nosy-Be. The genetic diversity is higher in the two populations of Analamera than in that of Nosy-Be and the level of genetic differentiation is higher between the population of *L. dorsalis* and the two populations of *L. septentrionalis* than between the two populations of *L. septentrionalis* themselves. Despite the hill crest separating the two populations of *L. septentrionalis*, our results demonstrate that they belong to one population. The respective roles of the geographical barriers and the reproductive barrier between the two species, are discussed.

Key Words: Lemurs; *Lepilemur*; RAPD; Genetics; Speciation.

INTRODUCTION

Genetic variability of natural populations is of much concern because it is often related to the ability of a population to respond to environmental changes. Measures of genetic diversity in a natural population can also provide information on the genetic structure of each population and on their evolution.

Different studies comparing the genetic variability of mainland and insular populations have shown that the mainland populations are more polymorphic than the insular ones (ISHIMOTO, 1973; DARGA et al., 1975; RABARIVOLA et al., 1996).

Since the RAPD (Random Amplified Polymorphic DNA) was developed by WILLIAMS et al. (1990), it has been widely used for screening genotypes of different species of plants and animals. This is due to fact that the RAPD assay is rapid (GIESE et al., 1994) and easy to perform on large populations (HARADA et al., 1993), requires a small amount of genomic DNA and generates a large number of markers. Initial cloning and sequencing steps are not necessary since a set of primers is already available. RAPD markers can even be used (MICHELI et al., 1994) for detecting genetic structure at the population level in vertebrates (GIBBS et al., 1994). In addition, in recent studies on primates RAPD markers were used successfully to estimate the effect of the fragmentation of habitat on genetic characteristics (LEIPOLDT et al., 1996).

In this study we compare two nocturnal and endemic species of Madagascar by using RAPD analysis: an insular population of *L. dorsalis* and two mainland populations of *L. septentrionalis*. *L. septentrionalis* individuals were captured in the deciduous forest of Analamera while the *L. dorsalis* were obtained from a humid forest of the Lokobe reserve (Nosy-Be island). We do

know that *L. septentrionalis* generally sleep in tree holes while *L. dorsalis* of Nosy-Be prefer bundles of foliage as daytime resting places. So far little is known of the ecology and social organization of these lemur species.

Our main objective was to detect specific markers for each species, to evaluate the genetic variability within and between the two species and to examine the effects of isolation and habitat fragmentation on their genetic structure.

MATERIAL AND METHODS

ANIMALS

We sampled 35 individuals of *L. septentrionalis* (LSE) and 35 individuals of *L. dorsalis* (LDO) from the mainland forest of Analamera and the island forest of Lokobe (Nosy-Be) respectively (Fig. 1). Individuals of *L. septentrionalis* are divided into two subpopulations according to their capture sites. LSE1 includes 18 individuals captured on the east side of a chain of hills and LSE2 comprises 17 animals from the west side of the hills (Fig. 1A). The 35 individuals of *L. dorsalis* (LDO) were captured on the eastern border of the reserve of Lokobe (Fig. 1B)

Animals were caught in traps constructed with wire-netting and a small ear biopsy was performed on each animal anaesthetized by injection of 0.2 ml of ketamine solution (50 mg/ml; Ketalar® Parke-Davis). These methods are safe and reduce the stress caused by the tissue collection. Samples were collected in small sterilized tubes and immediately stored in liquid nitrogen. After recovery from anesthesia, the animals were released at the capture location.

DNA EXTRACTION AND AMPLIFICATION CONDITIONS

DNA was isolated from the "ear samples" using standard SDS — proteinase K digestion and phenol — chloroform extraction as described by SAMBROOK et al. (1990). Amplifications were performed according to the slightly modified methods of WILLIAMS et al. (1990) by using a Perkin-Elmer 480 Thermal Cycler programmed in the following way: 6 min at 94°C followed by 45 cycles of 30 sec at 93°C, 45 sec at 37°C, 2 min at 72°C, and a final extension step of 10 min at 72°C. From 52 primers (Operon Technologies Inc.) initially screened, the following were potentially informative: OPA1: 5'-CAGGCCCTTC-3'; OPA9: 5'-GGGTAACGCC-3'; OPA10: 5'-GTGATCGCAG-3'; OPA18: 5'-AGGTGACCGT-3'; OPH4: 5'-GGAAGTCGCC-3'; OPH13: 5'-GACGCCACAC-3'; OPH14: 5'-ACCAGGTTGG-3'.

Amplification products were separated according to their size on 1.2% agarose gels using 1x TBE as running buffer. Gels were stained with ethidium bromide and were photographed under UV light using a Polaroid film.

The presence/absence of each fragment was recorded in a binary data matrix (a band was scored as 1 if it was present and as 0 if it was absent).

DATA ANALYSIS

Numerical data corresponding to the RAPD series of each primer were used to evaluate the level of homozygosities in the different populations as well as the nucleotide diversity (π) within each population and also the nucleotide divergence (D) between the different populations.

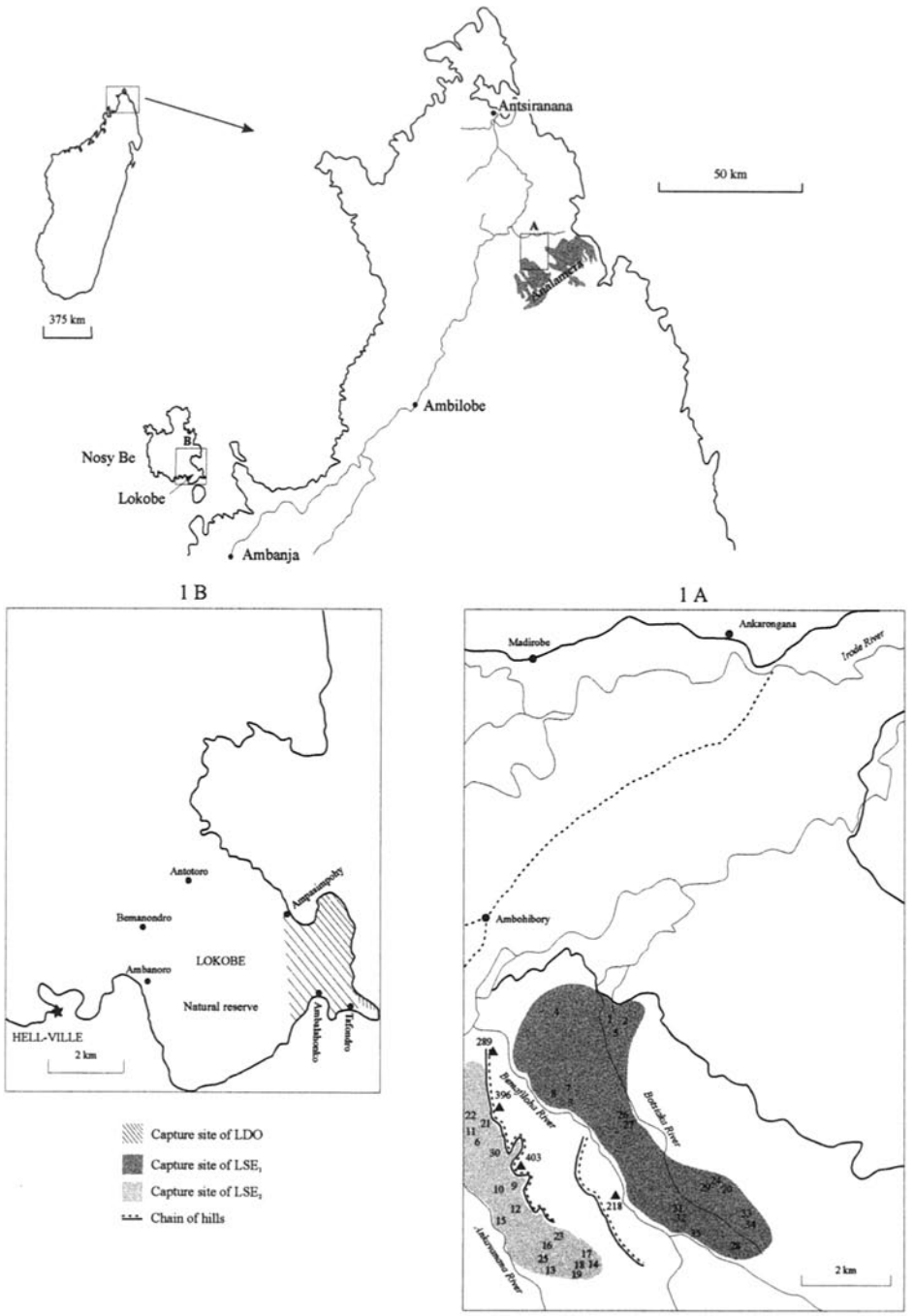


Fig. 1. Map of the North of Madagascar showing the forest of Analamera and the Lokobe reserve of Nosy-Be. 1A and 1B indicate the details of the capture site of *Lepilemur septentrionalis* in the Analamera region and the capture site of *L. dorsalis* on the island of Nosy-Be respectively. In 1A, each number represents a location of capture. The two small rivers, Bemafiloha and Ankavanana, do not represent impermeable frontiers between the animals as they dry out during the dry season.

Homozygosities for each primer were calculated using the following formula:

$$H_i = N_{aa} + (1 - \sqrt{N_{AA}})^2$$

$$H_m = \frac{1}{T}\sum_{i=1}^{T} H_i$$

with N_{aa} = frequency of absent bands at i-th site (frequency of homozygote recessives $=q^2$)
N_{AA} = frequency of present bands at i-th site (frequency of homozygote dominant $= p^2$)
H_i = frequency of homozygote bands at i-th site $= p^2 + q^2$
or $p + q = 1$, thus, $H_i = q^2 + (1-q)^2$ leading to the equation cited above
H_m = mean homozygosity
T = total number of bands.

The D and π values were determined from the matrix data by using the random amplified polymorphic DNA patterns in diploids (RAPDDIP) programme (CLARK & LANIGAN, 1993), which can count the bands shared by two individuals and can estimate the expected proportion of fragments that remain unchanged since the two sequences diverged from a common ancestor. The sequential equations for such estimations are described in CLARK and LANIGAN (1993). The different values were statistically compared by using the mean difference comparison test.

CLUSTER ANALYSIS

Cluster analysis was performed using the STATISTICA® programme (StatSoft, Inc.). In this cluster, each of the possible RAPD band positions was considered to be either identical or non-identical. Thus the proportion of shared band positions between two individuals was used to estimate their RAPD profile similarities. Based on these profiles, individuals were grouped by the UPGMA procedure.

The hierarchic classification is based on unweighted pair-groups among Euclidean distances between two individuals x and y by using the following formula:

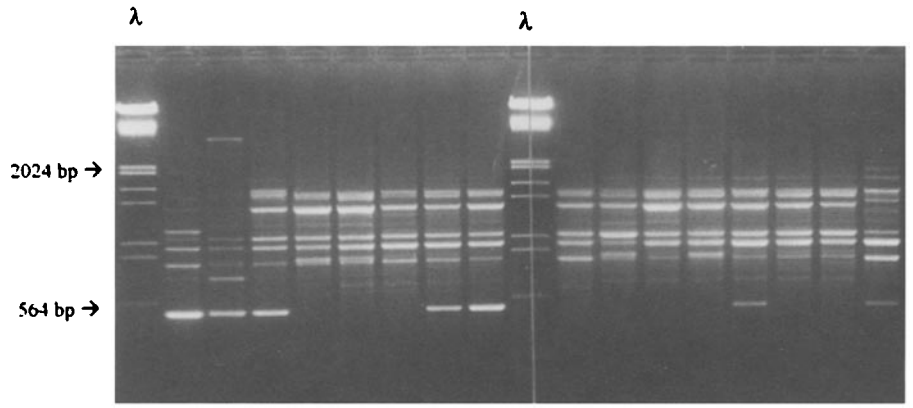
$$d(x, y) = \sqrt{\sum_i (x_i - y_i)^2}$$

with x_i = 1 or 0 for the individual x at site i
y_i = 1 or 0 for the individual y at site i
d = genetic distance.

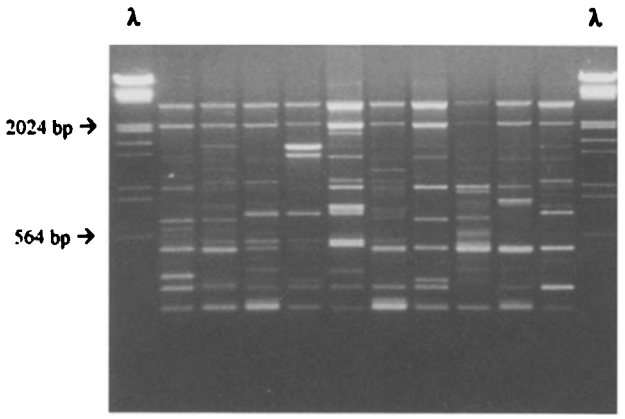
RESULTS

RAPD PROFILES

Examples of RAPD profiles obtained from two different primers are shown in Figure 2. The seven primers used in this study generated a total of 166 bands. In all cases the analysis was restricted to fragment lengths between 300 and 2000 bp, defined by the λ marker present on each gel. Table 1 displays the number of detected bands per primer and monomorphic bands in each population. If bands were present in 100% of individuals in a population, they were called monomorphic dominant. Bands present in all individuals of the three populations were called "common monomorphic dominant" while those found only in a specific population were called



OPH 13



OPA10

Fig. 2. RAPD patterns from two primers (OPA10 and OPH13). λ = Lambda DNA/*Eco*RI + *Hind*III markers.

"monomorphic dominant specific." According to this definition, a "monomorphic dominant specific" fragment for one population has to be considered "monomorphic recessive" for another population, if it is absent in that population. It is worth mentioning that very few RAPD bands of different staining intensity were encountered in this study, but the discrimination of these bands as homozygote (the bold bands) and heterozygote (faint bands) is impossible (LYNCH & MILLIGAN, 1994).

HOMOZYGOSITY AND VARIABLE BANDS

All primers produced different values of H_i within the different populations, varying from one band to another. The average homozygosity (H_m) in each population was calculated (Table 2). The highest value of homozygosity was obtained for LDO, the lowest for LSE1. A band is considered polymorphic, if it is absent at least in one individual surveyed in a single population, or according to HARTL (1988), a band is polymorphic, if H_i is <0.9. Otherwise, it is considered a monomorphic band. Table 2 summarizes these results.

Table 1. Distribution of bands present at 100% (monomorphic dominant band) or absent (monomorphic recessive band) in at least one population.

Primers	Total number of bands	Running number of monomorphic band	Frequency of monomorphic dominant/ recessive bands in the three populations (%)		
			LSE1	LSE2	LDO
OPA 1	25	5	5.6	23.6	0
		6	72.2	64.8	100
		7	0	0	82.9
		11	100	100	100
		12	100	100	100
OPA9	22	2	0	0	45.8
		3	38.9	17.7	100
		6	66.7	29.5	0
		8	0	0	100
		13	83.4	23.6	0
		14	50	58.9	0
		21	61.1	41.2	100
OPA10	26	2	5.6	0	5.8
		8	5.6	5.9	0
		11	50	35.3	100
		15	100	100	0
		20	22.3	17.7	0
		23	38.9	58.9	0
OPA18	22	2	0	0	42.9
		5	0	0	8.6
		7	38.9	41.2	0
		11	0	0	68.6
		12	16.7	0	100
		16	100	100	100
		18	100	100	100
OPH4	25	2	33.4	17.7	0
		3	44.5	17.7	0
		10	0	0	34.3
		13	5.6	29.5	0
		14	83.4	58.9	100
		15	22.3	23.6	0
		16	77.8	82.4	100
		17	100	100	100
		20	16.7	17.7	0
OPH13	24	5	38.9	0	0
		9	16.7	11.8	0
		10	61.2	53	0
		15	55.6	58.9	0
		16	61.2	88.3	100
		17	55.6	11.8	100
		18	100	100	100
		23	50	70.6	0
OPH14	22	11	55.6	23.6	100
		12	38.9	53	100
		15	100	100	100
		18	100	100	100

If a band is present or obsent in only one or two populations, the frequency of its occurrence in the remaining population is also given. LSE1 and LSE2 represent the two populations of *Lepilemur septentrionalis* with 18 and 17 individuals respectively. LDO: *L. dorsalis* population with 35 individuals.

Table 2. Homozygosity values and percentages of monomorphic and variable bands of each population.

	Populations		
	LSE1	LSE2	LDO
H_m (average for 7 primers)	0.65	0.67	0.72
% of monomorphic bands	10.24	10.84	23.49
	16.41*	15.85*	26*
% of variable bands	89.76	89.16	76.51
	83.59*	84.15*	74*

LSE1, LSE2, and LDO: Same populations as in Table 1; H_m: mean homozygosity obtained from seven primers;* values obtained using Hartl formula.

Nucleotide Divergence

With the (RAPDDIP) programme a complete numerical data set of each population was used to evaluate the nucleotide diversity (π) within each population and the nucleotide divergence (D) between the populations of LSE1, LSE2, and LDO. Supposing that a meaningful comparison of the two different species is dependent on considering all individuals of LSE as one population, we recalculated these indices after pooling LSE1 and LSE2 into one whole population of LSE and comparing it with the unique population of LDO (results are given in Table 3). The π values of LES1 and LES2 were not significantly different but the π value of LDO was significantly lower than the π values of LSE (with a 5% probability of type I error). Also the genetic distances D between the two LSE species and LDO were significantly higher than the one found between the two subpopulations of LSE.

Cluster Analysis

The cluster analysis shows that all individuals of the LDO species but one (LDO8) clearly group together, whereas some individuals of the LSE form one basal group and some other form a sister group with LDO (Fig. 3). However, animals caught on both sides of the deforested top of the hills appear to be related in the cluster.

DISCUSSION

Despite the numerous advantages of the RAPD technique (Maki & Horie, 1999), some problems regarding its reliability have been reported (Park & Kohel, 1994). According to our experiments the reaction needs to be optimised and adapted to each species as well as to the quality of the DNA.

Table 3. Averages of genetic variations for the populations.

	π		D
LSE1	$\pi 1 = 0.029 \pm 0.014$	LSE1/LDO	$D1 = 0.035 \pm 0.019$
LSE2	$\pi 2 = 0.028 \pm 0.015$	LSE2/LDO	$D2 = 0.034 \pm 0.017$
LSE	$\pi = 0.030 \pm 0.019$	LSE1/LSE2	$D3 = 0.026 \pm 0.020$
LDO	$\pi 3 = 0.016 \pm 0.001$	LSE/LDO	$D = 0.036 \pm 0.018$

LSE1, LSE2, and LDO are the same populations as in Table 1. LSE = LSE1 + LSE2. π: Intrapopulational diversity; D: interpopulational distances.

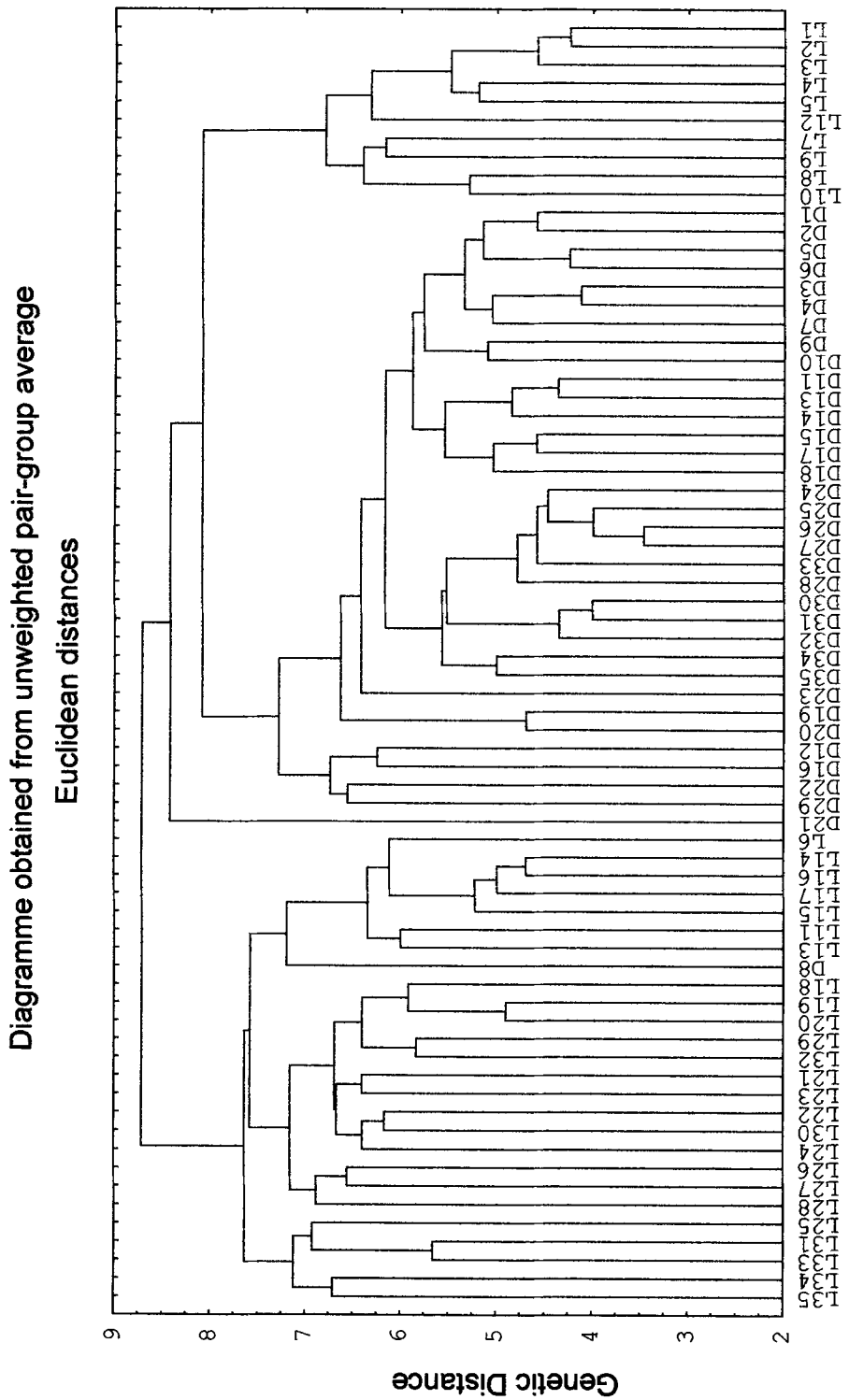


Fig. 3. Cluster analysis of the individuals of the *Lepilemur septentrionalis* (LSE) and the *L. dorsalis* population of Nosy-Be (LDO). The diagram represents the unweighted pair-group average Euclidean distances. The number attributed to LSE and LDO are the same as those indicated on the map (Fig. 1A) displaying the capture site for each animal.

RIESEBERG (1996) found that similarity of fragment size is a good predictor of homology, at least among closely related populations or species. Indeed, monomorphic bands specific either to LSE or to LDO may be regarded as species-specific markers according to the results of BARDAKCI and SKIBINSKI (1994) on tilapia.

GENETIC DIVERSITY

The percentages of variable and monomorphic bands reflect the genetic structure of each population. Thus the LDO population shows a lower diversity (23.49% monomorphic bands) than LSE1 and LSE2 (10.24% and 10.84% monomorphic bands respectively). This low genetic diversity (π) within LDO was expected considering the small capture area in the Lokobe forest and the quasi-sedentary behavior of the lepilemur. On the other hand, LDO is isolated and separated from the mainland population by the sea, which would make them vulnerable to population bottleneck, a process commonly associated with reduced genetic diversity. Small population size and/or small founder may likewise account for low genetic variation. Despite the lack of population size estimations, Analamera ($\sim$34700 ha) seemed to harbor more animals than the Lokobe forest ($\sim$740 ha).

The fact that higher π values within LSE1 and LSE2 populations (0.029 and 0.028 respectively) than within *L. ruficaudatus* (0.007 – 0.018) populations (LEIPOLDT et al., 1996) is surprising since *L. ruficaudatus* is a 'continental' species and the samples were obtained from animals of a larger capture area than our capture areas for LSE. Indeed, neither insularity nor the *a priori* sampling effect explain the small *L. ruficaudatus* π value. The modalities of animal capture could have been debatable: we know that the capture of a larger number of animals can give a reduced value of genetic diversity if the sample contains many related individuals. In addition, different experimental conditions in the two studies, as well as the primer used, may contribute to explain the differences. Thus, to be able to compare different results, some agreements on standardized conditions would be required and the differences related above have to be taken into account with precaution. Nevertheless, additional information, such as environmental heterogeneity for example, could be of help for further discussion on this matter.

GENETIC DIVERGENCE

The relatively high genetic distance between the *L. dorsalis* and the *L. septentrionalis* populations suggests a reduced gene flow between them, either due to the geographical and/or the chromosomal barrier. In one hand the sea separating Nosy-Be from mainland acts as a major barrier to dispersal between LDO and LSE. Thus, a current gene flow across this gap is highly unlikely for this type of animal. Moreover the geographical distance separating them is about 400 km. In this context it is of interest to mention the absence of differentiation between *Microcebus murinus* populations of Morondava and Ampijoroa (about 600 km distant) (NEVEU et al., 1998).

On the other hand, the large number of chromosomal rearrangements occurred during the evolution of these two groups suggests a reproductive barrier between them (ISHAK et al., 1992; RUMPLER, 2000). Indeed, the meiotic studies performed on different inter- and intra-specific lemur hybrids confirm the major role played by chromosomal rearrangements in the establishment of reproductive barriers, especially in the males (RATOMPONIRINA et al., 1988; RUMPLER & DUTRILLAUX, 1990).

The reduced genetic distance between LSE1 and LSE2 is not surprising, because the deforested crest of hills is not a real effective barrier.

In conclusion, our RAPD data show that the genetic distance between the LDO and LSE populations is larger than between the two different populations of the LSE. Different factors such as geographical distance, a geographical barrier (sea) isolating Nosy-Be from mainland at least 10,000 years (BATTISTINI, 1960), or an interspecific barrier due to numerous chromosomal rearrangements may simultaneously or separately play a role. On the other hand, the deforested hill does not represent an effective geographical barrier for the LSE.

The insular and founder effects, the small population size and the bottleneck events were considered separately or simultaneously to reduce the genetic diversity within the LDO population.

Finally, the bands regularly present in all animals of each species could be identified as specific markers. Nevertheless, homology studies would be desirable, at least in the investigation of different species (POWELL et al., 1996).

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