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GENETIC ANALYSIS OF *LEPILEMUR MUSTELINUS RUFICAUDATUS* POPULATIONS USING RAPD MARKERS

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ABSTRACT.- The genetic heterogeneity within and among natural populations of the brown weasel lemur, *Lepilemur mustelinus ruficaudatus,* was studied by random amplification of polymorphic DNA (RAPD). The populations are located in western Madagascar and have been subject to different degrees of fragmentation. By using six random primers in arbitrarily primed (AP-) PCR a total of 153 RAPD products were compared in a sample of 48 individuals. In the data at hand, there was no effect of population size on genetic variability. However, on the basis of nucleotide diversity the degree of molecular variability reflects a trend towards non-random mating behavior.

KEY-WORDS.- *Lepilemur mustelinus ruficaudatus,* Prosimiae, RAPD, Nucleotide Diversity, Population Genetic Structure, Habitat Fragmentation

RESUME.- L'hetérogénéité génétique à l'intérieur et entre des populations naturelles du Lémurien *Lepilemur mustelinus ruficaudatus* a été étudiée par l'amplification au hasard de ADN polymorphique (RAPD). Les populations sont localisées dans la région occidentale de Madagascar et ont subi différents niveaux de fragmentation. Par l'utilisation de six marqueurs arbitrairement marqué (AP-) PCR un total de 153 produits RAPD ont été comparés dans un échantillon de 48 individus. Dans les données disponibles aucun effet de taille de la population sur la variabilité génétique n'a été observé. Cependant, basé sur la diversité des nucléotides, le degré de variabilité moléculaire observé reflet des tendances vers un comportement d'accouplement non hasardeux.

MOTS-CLES.- Lepilemur mustelinus ruficaudatus, Prosimiae, RAPD, Nucléotide, Diversité, Structure génétique de la population, Fragmentation de l'habitat

INTRODUCTION

Over the last few centuries the forests of Madagascar became increasingly fragmented (BURNEY & MACPHEE, 1988; NELSON & HORNING, 1993a,b). However, there are not only man-made fragments, but the range of many species is subdivided by natural barriers such as the central high plateau or rivers. These barriers probably had important consequences for the evolution of the present biogeographic pattern, but the underlying mechanisms are poorly understood (BATTISTINI & RICHARD-VINDARD, 1972;

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TATTERSALL, 1982; RICHARD & DEWAR, 1991; MARTIN, 1995; GANZHORN, submitted). In order to understand effects of habitat fragmentation on the differentiation of lemur species, we studied effects of natural and man-made fragmentation on genetic characteristics of *Lepilemur mustelinus ruficaudatus*.

MATERIAL AND METHODS

ANIMALS, MATERIAL SAMPLING AND DNA ISOLATION

Lepilemur mustelinus ruficaudatus of four different populations were captured from their day shelters in 1993 and 1994 near Morondava in western Madagascar. The locations of the sites are shown in figure 1. « Kirindy » represents a large forest bloc with a substantial population of *Lepilemur*. The two fragments « Manamby North/North » and « Manamby North/South » have been cut off from the northern forest bloc some 30-40 years ago. The resulting fragment was then subdivided by a new road around 1987. It is unclear to what extent this road represents an effective barrier for dispersal. « Manamby South » is south of the Morondava River. At present, this looks like a clear barrier for *Lepilemur*, at least in the small area where there is still forest left on either side of the river. More population statistics are given in Table I.

Table I. Geographical coordinates, size and population statistics for subpopulations of *Lepilemur mustelinus ruficaudatus*;P = mean and standard error of nucleotide diversity within populations calculated according to CLARK and LANIGAN (1993)

	Kirindy	Manamby North/North	Manamby North/South	Manamby South	Manamby combined
Abbreviation	Kirindy	MNN	MNS	MS	Manamby
Lat./Long.	20°04'/44°40'	20°24'/44°50'	20°26'/44°50'	20°28'/44°49'	
Size [km^2]	300	6-10	6	50	
Population size	60.000	100-200	60-110	3.000	
P	0.011±0.0027	0.012±0.0028	0.014±0.0030	0.007±0.0023	0.017±0.0038
Sample size	20	6	16	6	28

Skin samples were obtained by ear clipping from 48 individuals of *Lepilemur*. In some cases blood samples were added to the corresponding skin biopsy. The biopsies were used for DNA isolation after having been kept at ambient temperatures for up to 10 months in Queen's buffer (SEUTIN *et al.*,1991). DNA was isolated by standard

procedures (SAMBROOK *et al.*, 1989). DNA recovery was 5.0 to 30µg depending on biopsy size and blood admixture. The DNA obtained appeared high-molecular weight (>50kbp) and undegraded independent of the storage time in Queen's buffer as judged by electrophoresis in 0.5% agarose gels.

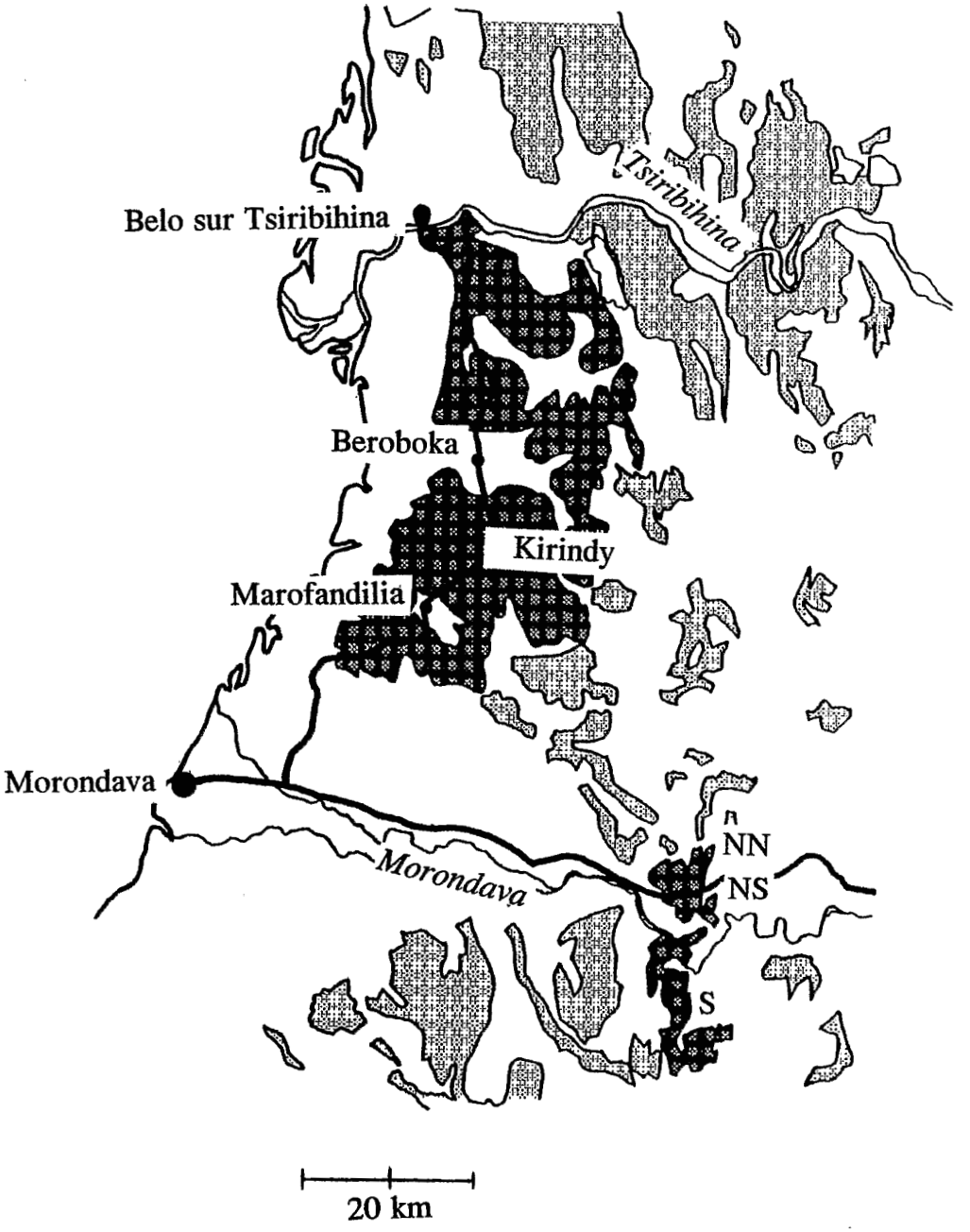

Fig. 1. Location of study sites in western Madagascar. The highlighted areas represent primary forest. Study sites are shaded darker than other forest. NN, NS, and S mark the forests called Manamby North/North, Manamby North/South, and Manamby South, respectively. Names in italics mark rivers.

RAPD - FINGERPRINTING

A total of 40 random 10-mer primers (Operon Technologies, Alameda,CA, USA, set A and H) were initially tested to find out the most informative ones. As the majority of primers produced clearly distinguishable patterns of amplification products on DNA of *Lepilemur,* we used the primers A01, A02, A04, A18, H14 and H17 for further analysis (Table II). Each amplified 20 to 31 bands with molecular weights from 0.3 kb to 3.0 kb. To ensure comparability and reproducibility of polymorphisms, at least two samples from previous amplifications were repeated in subsequent PCRs. Negative controls, containing no target DNA, were included in each PCR. Only bands were scored that appeared distinct, and reproducible. The size and distribution of RAPD bands was estimated using pBR 328/Bgl II and Hinf I digested DNA (Boehringer, Mannheim, Germany) as a molecular weight size marker, which was included twice in each electrophoretic run. The size of a particular RAPD product was calculated individually for each DNA sample. Details of RAPD-conditions are described by LEIPOLDT *et al.* (submitted,a).

Table II. Nucleotide sequence of random primers used for RAPD on genomic *Lepilemur* DNA

Primer designation	Nucleotide sequence
OPA - 01	5'- CAGGCCCTTC - 3'
OPA - 02	5' - TGCCGAGCTG - 3'
OPA - 04	5' - AATCGGGCTG - 3'
OPA - 18	5' - AGGTGACCGT - 3'
OPH - 14	5' - ACCAGGTTGG - 3'
OPH - 17	5' - CACTCTCCTC - 3'

STATISTICAL ANALYSIS

RAPD bands at each position on the gel were scored as either present or absent. The banding patterns were converted into a presence/absence matrix for each DNA sample and each primer separately. « 0 » means absence and « 1 » indicates presence of a particular band. Using the six random primers A01, A02 , A04, A18, H14 and H17 a total of 153 bands were obtained. The data matrix for all six primers was used for the

statistical analysis. On the basis of this matrix the nucleotide diversity (P) within populations and the nucleotide divergence (d) between populations were calculated using the RAPDDIP program written by CLARK and LANIGAN (1993). Nucleotide diversity was calculated on the basis of the cumulated data for all six primers.

RESULTS

RAPD profiles were produced on 48 samples of total DNA using six different single primers (Table II). Initially, we screened 40 single decamer primers with one DNA sample: four of them repeatedly failed to amplify any product, 26 reproducably allowed the amplification of multi - band RAPD patterns. For the analysis of the whole sample we chose arbitrarily two primers (OPA-04, OPH-14) which yielded medium numbers of banding profiles (20 - 23), and four primers (OPA-01, OPA-02, OPA-18, OPH-17) which produced a high number of bands (26 - 31). In total, 153 RAPD bands were observed: 129 and 137 in the samples from Kirindy and Manamby, respectively. Seventeen bands proved to be specific for Kirindy, 25 bands were Manamby-specific, *i.e.* were uniquely observed in only one of the two populations (Table III).

Table III. Total number, number of common and population-specific RAPD bands obtained with different primers on *Lepilemur* DNA

	Number of bands scored in			Population specific	
	Total sample	Kirindy	Manamby	Kirindy	Manamby
OPA - 01	31	24	28	3	7
OPA - 02	26	20	24	3	7
OPA - 04	23	21	21	2	2
OPA - 18	26	25	23	3	1
OPH - 14	20	18	17	3	2
OPH - 17	27	21	24	3	6
Total	153	129	137	17	25

The nucleotide diversity within the population is higher in the pooled sample of Manamby (1.7%) than in Kirindy (1.1%). The nucleotide divergence between these two populations of *Lepilemur* is 1.7% (Table IV). If the Manamby data are split into subgroups corresponding to the forest fragments, the mean nucleotide diversity varies between 0.7% and 1.4% in the samples from Manamby South (MS) and Manamby North/South (MNS), respectively. The population from Manamby North/North (MNN) shows an intermediate value of 1.2%. Comparison of the Manamby subgroups shows only slight differences (1.1 - 1.4%) of nucleotide divergence (d). The values of

divergence between the Manamby populations are almost identical to the d - values obtained by comparing the complete Kirindy data to the data of each of the Manamby subgroups (1.2 - 1.4%). Thus, except for the lower nucleotide diversity in the *Lepilemur* - population of Manamby South, genetic heterogeneity on the nucleotide level does not differ considerably within and between the populations.

Table IV. Nucleotide divergence (mean and standard error) between populations of *Lepilemur*, calculated according to CLARK and LANIGAN(1993); abbreviations as listed in Table I.

	MNS	MS	Kirindy
MNN	0.014±0.003	0.011±0.003	0.014±0.003
MNS		0.013±0.004	0.014±0.002
MS			0.012±0.002
Manamby			0.017±0.003

DISCUSSION

Since its first description as a method to detect genetic polymorphism that can be used as genetic markers in gene mapping and cloning of specific chromosomal regions (WILLIAMS *et al.*, 1990; MICHELMORE *et al.*, 1991; TELENIUS *et al.*, 1992), RAPD has been widely used for genetic analyses to characterize genomes from various sources (WELSH & MCCLELLAND, 1993; WILLIAMS *et al.*, 1993). The advantages of RAPD in the study of genetic variation over methods like RFLP -, VNTR - analysis, DNA - fingerprinting and DNA - sequencing are that (1) the number of loci that can be investigated is almost unlimited due to the large number of different random primers, (2) the genomes are analyzed randomly, *i.e.* representatively without discrimination between coding and non-coding sequences, and (3) analysis can be done without prior knowledge of DNA sequences of a particular organism. Practical disadvantages have been reported, though, such as nongenetic artifacts in RAPD patterns (RIEDY *et al.*, 1992; ELLSWORTH *et al.*, 1993) that could make interpretation difficult especially in pedigree analysis and thus demand methodological precautions to guarantee reproducibility and exclusion of artifactual bands (WILLIAMS *et al.*, 1993; BIELAWSKI *et al.*, 1995).

The present study applied RAPD to relate genetic diversity in free ranging populations of *Lepilemur mustelinus ruficaudatus* (*L.m.r.*) to differing degrees of habitat fragmentation. Six random primers produced 153 bands, some of which were specific for subpopulations. The nucleotide diversity within the total group of all 48 individuals is 0.014. If the sample is analyzed separately depending on the origin of the animals, similar P - values were obtained in the population of Kirindy (0.011) and the pooled samples from Manamby (0.017). The three subpopulations from Manamby had values between 0.007 and 0.014. Based on these values, fragmentation of the habitat of *L.m.r.* does not seem to have induced markable changes in the genetic structure of the populations analyzed.

The genetic variability of *L.m.r.* populations is similar to nucleotide diversities of 0.011 and 0.009 found in natural populations of *Microcebus murinus* and *M. rufus*, respectively (LEIPOLDT *et al.*, submitted,a). Comparable data for other free-ranging mammals are lacking, but compared to nucleotide diversity values of 0.001 to 0.003 in North American snake populations (GIBBS *et al.*, 1994), genetic diversity appears high in *L.m.r.* populations. This could be a general feature of *Lepilemur* spp. and possibly also for other lemur species which show substantial variability even in their karyotypes (RUMPLER & ALBIGNAC, 1978; ISHAK *et al.*, 1992). Genetic studies on *Lepilemur* populations of the same study area performed on 22 enzyme loci by protein electrophoresis can help to interpret the RAPD data (LEIPOLDT *et al.*, submitted,b). It suggests that the high degree of genetic diversity as identified by RAPD, can be the result of a process of genetic differentiation not visible morphologically.

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