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RAPD (Random Amplified Polymorphic DNA) profiles of ten macaque species

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Abstract — Here, we report on the RAPD profiles of 61 individuals belonging to 10 macaque species. We used 23 different PCR primers on each sample and found an average of 17 bands per primer. The RAPD profiles appear to be highly reproducible because we found no differences in the amplification patterns produced by the DNA extracted from hair or blood. Strikingly, each species had a unique RAPD pattern homogeneously shared by all individuals. Comparisons between taxa showed that variability in the RAPD pattern was low, and the Sm index was below 0.601. Cluster analysis led to a division of the macaques into two main clusters: One with M. *sylvanus* and M. *silenus* and the other with M. *arctoides, mulatta, fascicularis, nemestrina, tonkeana* and *fuscata. Macaca nigra* and M. *radiata* were positioned outside of these clusters. Gene flow may explain the zoogeographic pattern present in the RAPD profiles. The lack of within-species variability suggests the operation of founder effects and strong genetic drift, which may have been particularly strong in the case of peripherally placed species such as M. *radiata, fuscata* and *nigra.* The position of M. *tonkeana* is divergent from all the commonly accepted taxonomic and phylogenetic schemes. This result suggests that the RAPD technique is not always able to reveal the "true" phylogenetic relationships within the genus *Macaca.* The nature of genetic variation uncovered by the RAPD method is still unclear, and prudence should guide inferences about nucleotide divergence, population structure and phylogeny based solely on RAPD markers.

Key words: *Macaca*, molecular taxonomy, RAPD.

INTRODUCTION

The taxonomy and phylogeny of the genus *Macaca* continue to provoke discussion even if the classification proposed by FOODEN (1980) is commonly used. There is not even agreement on the exact number of species. Although the morphological, ecological and ethological variability within the genus is striking, there is apparently no barrier to hybridization between taxa in captivity. Interspecific gene flow has also been frequently reported in nature (FOODEN 1964; GROVES 1980; MELNICK and KIDD 1985; CAMPERIO *et al.* 1989; FOODEN and LANYON 1989). Apparently, it is difficult to propose definitive phylogenetic and taxonomic conclusions.

A number of bio-molecular studies have focused on the genus *Macaca*. Recently, Melnick and co-workers reported on nuclear loci (MELNICK and KIDD 1985; MELNICK 1988) and restriction enzymes analysis of the mitochondrial genome, mtDNA (MELNICK and HOELZER 1992; HOELZER and MELNICK 1992; MELNICK *et al.* 1993; HOELZER *et al.* 1994; ROSENBLUM *et al.* 1997; MORALES and MELNICK 1998). ZHANG and SHI (1993a,b) also used restriction enzymes to analyze mtDNA, while HAYASAKA *et al*. (1996) sequenced a portion of the mitochondrial genome.

In this work, we have analyzed nuclear DNA using the RAPD (Random Amplified Polymorphic DNA) technique (WELSH and MCCLELLAND 1990; WILLIAMS *et al.* 1990).

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This PCR-based technique relies on single short, (5-15 base) random oligonucleotides primers. The amplified DNA fragments are then visualized on agarose or acrylamide gels, and the bands are scored as present or absent. A RAPD-pattern is therefore the product of all the DNA fragments from a particular individual amplified using a single primer of random sequence.

These fragments have a dominant Mendelian inheritance. The RAPD technique does not require previous information regarding the sequence, employs a very small quantity of DNA and is economical in terms of both cost and labor. This method may be useful to analyze species in which information on the genome is scarce, as is the case for many primate species.

The level of variability detected by this technique changes depending on the species analyzed. However, it has been stated that the differences between species are high compared to those between individuals of the same species (PEREZ *et al.* 1998). We have used the RAPD technique to analyze specimens from ten *Macaca* species, representing, according to Fooden, the four main subgroups *(silenus-sylvanus, fascicularis, sinica* and *arctoides).* Our purpose has been to gather RAPD data on macaques, to test their usefulness for an assessment of genetic variability in macaque species and to exploit the results so as to interpret macaque phylogeny, taxonomy and evolution.

MATERIALS AND METHODS

We collected hair and blood samples from various European zoological gardens and the Institute of Anthropology, University of Zurich, Switzerland (see Table 1). The Zurich samples were from wild animals of known geographic origin. According to information provided by keepers, the zoo specimens were sampled to avoid animals known to be related.

The DNA of each individual was extracted from three hairs, incubating the roots (cut with sterile scalpels) for 1-2 h at 55 °C in 400 1 of a buffer containing Tris-HCl 10 mM pH 8, KC1 50 mM, Nonidet-P 40 0.45% (v/v), Tween 20® 0.45% (v/v), and 0.024 mg proteinase K (Boehringer Mannheim).

For some samples, DNA was extracted from EDTA-treated whole blood, using the following procedure: 500 l of whole blood were added to an equal volume of sucrose 0.32 M, Tris-HCl 10 mM (pH 7.5), Triton X 100 1% (v/v). After centrifugation at 12,000 rpm for 10 min., the pellet was washed several times with 0.5 ml of a solution containing KCl 50 mM, Tris-HCl 10 mM (pH 8.3), Nonidet P40 0.45% (v/v) and Tween 20® 0.45% (v/v). The pellet was then incubated at 55 °C for 75 min. with 0.03 mg of proteinase K. Each sample was analyzed using 25 different primers, 20 derived from Kit L of 10-mer primers from Operon Technologies (Alameda, California) and 5 ranging in length from 20 to 25 nucleotides; each primer was used singly, as is usual in the RAPD technique, which differs from the classical PCR reaction precisely because of the use of a single primer of random sequence (see Table 2 for the complete list and sequence of primers employed).

TABLE 1 — Complete list of the species, source and number of individuals employed in this study.

Species	no.	Source
Macaca arctoides	3	Wuppertal (Germany) zoo
	3	Budapest (Hungary) zoo
	2	Zurich
M. fascicularis	2	Budapest zoo
	3	S. Raffaele (Milano, Italy) hospital
M. fuscata	4	Stuttgart (Germany) zoo
	5	Zurich
M. mulatta	3	Budapest zoo
	6	Zurich
M. nemestrina	5	S. Raffaele hospital
M. nigra	2	Olomouc (Czech Republic) zoo
	3	Boras (Sweden) zoo
M. radiata	3	Usti N. Labem (Czech Republic)
M. silenus	4	Duisburg zoo (Germany)
M. sylvanus	4	Stuggart zoo
	6	Zurich
M. tonkeana	3	Zurich
Papio hamadryas	2	Bratislava zoo (Slovak Republic)

Amplification reactions were performed in a 40 l volume containing Tris-HCl 67 mM pH 8.8, $(NH_4)_2SO_4$ 16 mM, Tween 20 0.01% (v/v), $MgCl_2$ 3mM, 200 M of each dNTP, primer 0.2 M, template DNA 30 l (corresponding to about 100 ng), and 0.725 U of Taq DNA polymerase. After incubation at 90 °C for 60 s and at 95 °C for 90 s, the reaction mixtures were cycled 45 times through the following temperature profiles: 95 °C for 30 s, 36 °C for 60 s and 75 °C for 120 s. The samples were then incubated at 75 °C for 10 min., at 60 °C for 10 min. and held at 4 °C. An MJ (PTC-150) thermocycler and the fastest transitions available between each temperature were used.

Ten microliters of each amplification product were loaded on a 2 % (w/v) agarose gel (Boehringer, Mannheim), with a TAE buffer containing 0.5 mg/ml

TABLE 2 — List and sequence of primers employed in the present study. Those primers which gave rise to a electrophoretic profile unresovable (due to the presence of too many bands very close one to each other) are marked with *.

Name	5' → 3' sequence
p1	GGCATGACCT
p2	TGGGCGTCAA
p3	CCAGCAGCTT
p4	GACTGCACAC
p5*	ACGCAGGCAC
p6	GAGGGAAGAG
p7	AGGCGGGAAC
p8	AGCAGGTGGA
p9	TGCGAGAGTC
p10	TGGGAGATGG
p11	ACGATGAGCC
p12	GGGCGGTACT
p13	ACCGCCTGCT
p14	GTGACAGGCT
p15	AAGAGAGGGG
p16	AGGTTGCAGG
p17	AGCCTGAGCC
p18	ACCACCCACC
p19	GAGTGGTGAC
p20	TGGTGGACCA
BU3	CAACTTCATCCACGTTCACC
BU6	GAAGAGCCAAGGACAGGTAC
APO-B1	ATGGAAACGGAGAAATTATG
APO-B2	CCTTCTCACTTGGCAAATAC
CD4-1*	TTGGAGTCGCAAGCTGAACTAGC

ethidium bromide and electrophoresed at 100 V for 2 h; gels were photographed under a UV transilluminator with a Polaroid camera.

Amplification fragment analysis and band identification were undertaken through a computer-aided visual check by means of Gelreader (NcsA 1991). Bands with the same mobility were considered homologous, regardless of their intensity. For the electrophoresis, a 2% agarose gel was used that can resolve fragments differing by about 50 bp; due to this resolution power, fragments differing by less than 50 bp were considered the same. Bands were scored according to the presence/absence (1/0) characters to obtain similarity matrices based on the simple matching index (Sm), the ratio between the number of common bands and the total number of bands (SNEATH and SOKAL 1973). A pairwise similarity matrix was derived by the simple matching index method. The matrix was then exploited to construct the dendrogram with UPGMA. Raw presence/absence data were also used to derive the Euclidean squared distances employed in the cluster analysis with Neighbor-Joining and Fitch-Margoliash algorithms. The presence/absence data were applied in the Wagner parsimony analysis. Cluster analysis was carried out with the NTSYS version 1.80 and PHYLIP version 3.57c (FELSENSTEIN 1995) packages.

RESULTS

We analyzed 61 samples from 10 macaque species, plus 2 samples from the outgroup *Papio hamadryas* with 23 of the 25 primers initially selected. We scored only sharply defined bands ranging from 0.15 to 1.5 kb, for a total of 400 fragments. Each single primer used in the RAPD assays therefore produced an electrophoretic pattern with an average of about 17 bands. Strikingly, each species had a unique RAPD pattern homogeneously shared by all individuals. No differences were noted between the amplification patterns produced by the DNA extracted from hair and those extracted from blood (Fig. 1).

Comparisons between taxa

Comparisons between taxa showed that variability in the RAPD pattern was low and that the Sm index was below 0.601 (see Table 3). However, the data analysis revealed some clear-cut results. All the dendrograms (Figures 2,3) showed *Macaca nigra* and *M. radiata* in peripheral positions, while the other species were divided into two main clusters. One cluster was

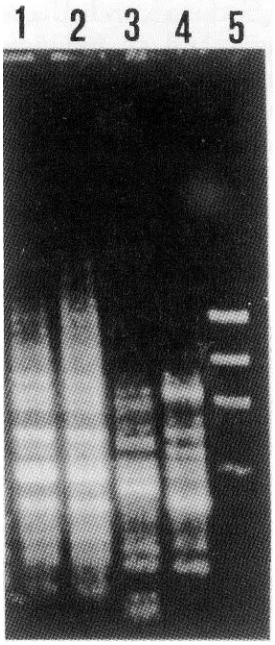

Fig. 1 — Agarose gel picture showing the identity of amplification pattern obtained with DNA hair extracted, lane 1, and Blood extracted (lane 2: both these lanes refer to the same M. *sylvanus* specimen). Lanes 3, 4 and 5 refer respectively to M. *nemestrina*, *M. nigra* and molecular weight marker.

TABLE 3 — Similiraty matrix based on the Sm index. MRA, *Macaca radiata,* MAR, *M. arctoides;* MMU, *M. mulatto.;* MTO, M. *tonkeana;* MNI, *M. nigra;* MFA, *M. fascicularis;* MNE, M. *nemestrina;* MSY, M. *sylvanus;* MSL, M. *silenus;* MFU, M. *fuscata.*

MRA	1.000									
MAR	0.610	1.000								
MMU	0.630	0.779	1.000							
MTO	0.652	0.805	0.761	1.000						
MNI	0.664	0.664	0.677	0.703	1.000					
MFA	0.613	0.735	0.742	0.747	0.633	1.000				
MNE	0.601	0.730	0.744	0.728	0.659	0.764	1.000			
MSY	0.616	0.606	0.613	0.618	0.643	0.620	0.638	1.000		
MSL	0.628	0.666	0.679	0.657	0.693	0.669	0.705	0.686	1.000	
MFU	0.640	0.671	0.711	0.676	0.684	0.688	0.676	0.735	0.696	1.000

formed by M. *sylvanus* and M. *silenus* and the other cluster included all other species (M. *arctoides, mulatto,, fascicularis, nemestrina, tonkeana and fuscata).* It may be noted that M. *fascicularis* is joined to *nemestrina* and *arctoides* is joined to *tonkeana.*

DISCUSSION

LAN *et al.* (1995) showed that RAPD could be applied for a single-species study. Here, we have used RAPD for the first time in a survey comprising several species belonging to the same genus. The most striking result of our work is that geographic differentiation seems to be present not at the population level, but at the species level. Each species had a unique amplification pattern, and all individuals within a species had a homogeneous pattern.

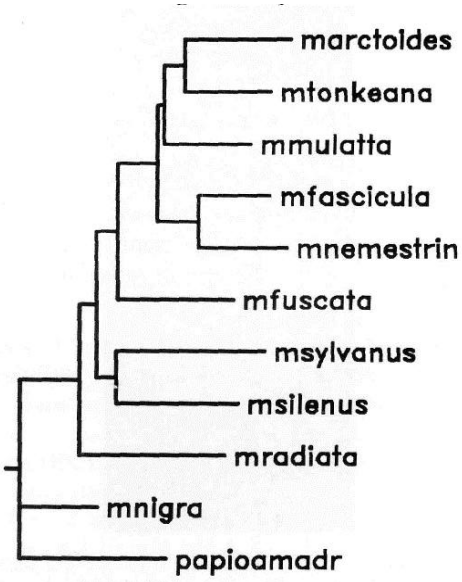

Fig. 2 — Unrooted Neighbor-Joining dendrogram (obtained from the matrix with Euclidean squared distance) with *Papio hamadryas* as outgroup.

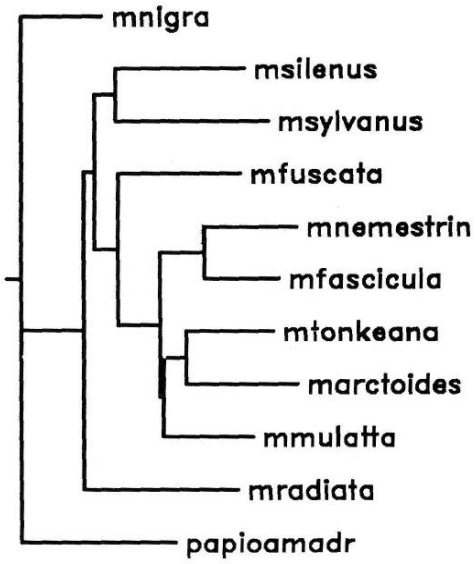

Fig. 3 — Phenogram of Fitch-Margoliash analysis (based on Euclidean squared distance) with *Papio hamadryas* as outgroup.

Although we analyzed only 61 individuals, this species-specificity and homogeneity could very well reflect a peculiar characteristic of the macaque RAPD pattern. We took care to analyze samples from unrelated animals, often (as in the case of the Zurich specimens) coming from different geographic populations of the same species. Furthermore, our sample sizes are greater than those in other recent reports (HAYASAKA *et al.* 1996; MORALES and MELNICK 1998).

In prokaryotes, it is even possible, using the RAPD, to discriminate among different strains of the same species (FEKETE *et al.* 1992; FANI *et al.* 1993); however, in eukaryotes, discrimination seems reliable only at the population level (MASSETI *et al.* 1997). Some researches have in fact demonstrated that discrimination among

individuals is often hampered by technical problems such as band reproducibility (RIEDY *et al.* 1992; AYLIFFE *et al* 1994). The present results suggest that, in higher eukaryotes, the RAPDs may be homogeneous within species and may therefore be helpful in making inferences about between-taxa relationships. However, more data on a number of primate taxa are needed to confirm this hypothesis, since other researches have, on the other hand, demonstrated that discrimination between individuals is possible even in the case of species of mammals (CUSHWA *et al.* 1996).

Up to now, RAPD data have not been used to measure genetic distance. RAPD bands are normally considered as simple Mendelian loci with dominant inheritance (LYNCH and MILLI-GAN 1994), but RAPD bands can also be considered as phenetic markers at the molecular level (STEWART and EXCOFFIER 1996). The phenetic relationships among the RAPD profiles can then be exploited to make inferences about phylogenetic relationships between taxa. We used a cluster analysis (i.e. Neighbor-Joining, Fitch-Margoliash) which does not assume a molecular evolutionary clock, because the sequence and inheritance of amplification fragments are not completely known. Our lack of knowledge about the real significance and genetic basis of the RAPD fragments has permitted the development, at the present time, of only a number of working hypotheses to explain the pattern we have observed within and among macaque species.

One hypothesis is that the pattern of species clustering that we found suggests a zoogeographic differentiation of the RAPD profiles in the genus *Macaca.* Other researchers have concluded that the RAPD profiles appear to divide the samples in relationship to their geographic position (HUFF *et al.* 1993; HAIG *et al.* 1996).

The dendrograms obtained with the RAPDs clearly show the presence of two main groups and two peripheral, isolated species, *M. radiata* and M. *nigra*. The first cluster could be called "continental", as the species included (with the exception of the insular *M. tonkeana* and *M. fuscata)* live in the same continental area ranging from eastern India-Bhutan to the Yunnan and Kwantung provinces of southern China. The second cluster is represented by *M. silenus* and *M. sylvanus.* Our dendrograms differ significantly from the classification of FOODEN (1980), which subdivided the genus into four groups: silenus-sylvanus (M. *silenus, sylvanus, nemestrina* and Sulawesi species), fascicularis (M. *fascicularis, mulatto,, cyclopis, fuscata),* sinica (M. *sinica, assamensis, radiata, thibetana)* and arctoides (M. *arctoides).* Although this classification has been based principally on morphological and ecological criteria, it has also been supported by some genetic studies (MELNICK and KIDD 1985; FOODEN and LANYON 1989; ZHANG and SHI 1993; MORALES and MELNICK 1998). Several hypotheses may help explain these differences. In previous studies, nuclear loci were not directly analyzed (MELNICK and KIDD 1985; MELNICK 1988; FOODEN and LAN-YON 1989). Furthermore, there is often a clear discrepancy between the results inferred from nuclear loci and those from mtDNA (cf. MELNICK and HOELZER 1992; MELNICK *et al.,* 1993; MELNICK and HOELZER 1993). This discrepancy may be due to the fact that the social organization of many *Macaca* species, with a high degree of male dispersal (DITTUS 1975; MELNICK *et al.* 1984; PUSEY and PACKER 1987), causes striking differences in the transmission patterns of mitochondrial and nuclear genes. The result is that mtDNA data overestimate the variability at the inter-population level and underestimate intra-population variability. According to MELNICK and HOELZER (1993, p. 3) "in many instances phylogenetic trees based on nuclear data more accurately reflect species relationships. Trees based on mtDNA, on the other hand, are more likely to reflect ancient biogeographic events, such as parapatry, than to reflect contemporary phylogenetic relationships."

The analysis may provide clues to evolutionary processes that are at work in the genus *Macaca.* In the case of the "continental" group, it could be hypothesized that gene flow may be a mechanism to explain the zoogeographic pattern present in the RAPD profiles. The possibility of interspecific transmission of genetic material has been frequently proposed (FOODEN 1964; CAMPERIO *et al.* 1989; FA 1989) and seems to be supported by the high degree of genetic homogeneity present (i.e. between *M. fascicularis, mulatta* and *nemestrina).*

On the other hand, the unique RAPD signature of each species and the lack of within-species variability suggest the operation of founder effects and strong genetic drift, especially in in-

sular species such as *M. radiata, fuscata* and *nigra*. These evolutionary forces permit clear genetic differentiation even in recently diverged species (FOODEN and LANYON 1989). Another hypothesis is that the peripheral position of these species could reflect extreme peripatric origins (MORALES and MELNICK 1998). This may be the case of *fuscata,* which in all dendrograms is placed just beside the continental cluster containing the other fascicularis group species.

Our work is among the very few efforts (HAYASAKA *et al.* 1996; MORALES and MELNICK 1998) to compare *M. silenus* and *M. sylvanus*. The RAPD technique places these species very close. Based on morphological considerations, an ancient relationship between these two species has been suggested by FOODEN (1980). The RAPD-profile similarity is difficult to explain. It is clearly not due to geographic distribution, and it is surprising because both species are isolated remnant populations in which genetic drift is expected to be an important evolutionary force. It could reflect the fact that both species are considered to have conserved traits close to the ancestral population that gave rise to all other macaque species. However, we are unable to determine which hypothesis is correct: both species may have conserved an ancient RAPD profile. On the other hand, the similarity may indicate a recent common ancestor or merely be the result of convergence. Further comparisons between these species with a wide range of biomolecular methods might help answer these questions.

Both the Neighbor-Joining (NJ) algorithm (SAITOU and NEI 1987) and the Fitch and Margoliash (1967) criterion gave dendrograms with almost the same clustering pattern, including those with *Papio hamadryas* as an outgroup. This result was also obtained with the Wagner parsimony analysis (FARRIS 1970) based on the presence/absence (1/0) character state of RAPD fragments (data not shown).

The marginal position *of Macaca radiata,* the only species in our sample belonging to the sinica group, appears to support the peripheral position of this group (HOELZER *et al.* 1992) and may reflect the effects of genetic drift due to recent, strong genetic isolation. It is reported that *M. radiata* is severely restricted to the mountainous region of southwest India (FA 1989). The inclusion of *M. arctoides* in the continental group agrees with other studies (FOODEN and LANYON 1989; MORALES and MELNICK 1998) and supports the hypothesis of a recent common ancestor or interspecific gene flow in intergradation zones (FOODEN 1964).

The position of *M. tonkeana* inside the continental cluster is highly anomalous and divergent from all the commonly accepted taxonomic and phylogenetic schemes. *M. tonkeana* is always placed nearby *M. arctoides.* However, it is very unlikely that gene flow could explain the RAPD data. On the other hand, we can not rule out that convergence and homoplasy may be present in the RAPD pattern, and thus we urge caution in applying RAPD data to draw hard phylogenetic conclusions. As always, any genetic data must be considered in the whole context of what is known about the phylogeny and evolution of the taxa in question. There may be some unidentified evolutionary forces at work which would help explain the position of *M. tonkeana.* However, it can be noted that the other Sulawesi macaque in our sample, *M. nigra,* as expected, has a peripheral position in the dendrograms.

Gene flow may well explain the RAPD position of *M. mulatta, fascicularis* and *nemestrina,* but a recent common ancestor could also be responsible for the closeness of these species. The position of *Macaca fuscata* compared to the other species of the fascicularis group is highly illustrative. Despite the likely origin of *M. fuscata* from an ancestral population of *M. mulatta,* the distance between these species in the dendrograms suggests that founder effects and genetic drift are the evolutionary forces most likely explaining the substantial genetic differences that have apparently developed in a relatively short time (AQUADRO and KILPATRICK 1981). A quite similar process could be responsible for the isolated position of *M. nigra.*

Finally, the clear contrast between the phylogeny inferred from RAPD data and that derived from nuclear and mitochondrial DNA markers makes it necessary to consider the possibility that the RAPD technique is unable to reveal the "true" phylogenetic relationships within the genus *Macaca.* Some authors have raised questions about the reproducibility of RAPD-PCR reactions (OLIVER *et al.* 1999). However, this does not seem to be a problem here, since we obtained identical profiles for individuals from different biological material

(hair and blood). A more serious issue is the fact that the nature of genetic variation uncovered by the RAPD method is still unclear (PEREZ *et. al.* 1998) The existence of some common selection factors involving this type of marker in some species can not be ruled out (GLAZKO *et al.* 1997). Prudence should guide inferences about nucleotide divergence, population structure and phylogeny based on RAPD markers (PEREZ *et al.* 1998).

CONCLUSIONS

1. Each macaque species had a unique RAPD pattern that was identical in all individuals of that species. This result confirms that genomic variation within and between macaque species is low.

2. The RAPD analysis mainly separated species of the genus *Macaca* on the basis of a zoogeographic differentiation into two main clusters. The first cluster was represented by four species living in a wide continental area with evident zones of intergradation (i.e. *M. mulatta, nemestrina, fascicularis* and *arctoides)* and by the insular species *fuscata* and *tonkeana.* A second cluster linked M. *silenus* and *sylvanus.* The remaining species, M. *radiata* and *nigra,* occupy peripheral, isolated positions in the dendograms.

3. The contrast between unorthodox RAPD clusters and traditional phylogenetic and taxonomic schemes of macaque evolution may be explained by a combination of gene flow, founder effect and genetic drift. Gene flow could be responsible for the closeness of the continental cluster species, while genetic drift and extreme peripatric origins could explain the position of species such as *radiata, nigra* and *fuscata.* Finally the closeness of *silenus* and *sylvanus* could be due to the conservation of traits from the common ancestor that gave rise to the entire genus.

4. There is a clear discrepancy between the phylogeny suggested by RAPD analysis and that inferred from classical molecular markers. The genetic nature and mechanisms underlying RAPD variability remain unclear. Therefore, strict conclusions about the evolutionary history and taxonomy of macaques species solely on the basis of RAPD profiles are not possible at this time. However, RAPD-profile analysis is useful for generating hypotheses concerning phylogeny and the processes and mechanisms that have operated to forge macaque evolution.

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