Comparative Medicine
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Vol 54, No 2
April 2004
Pages 193-201

Effects of Geographic Origin on Captive *Macaca mulatta* Mitochondrial DNA Variation

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Partial sequences from mitochondrial (mt) 12S and 16S rRNA genes were analyzed to characterize diversity among captive rhesus macaques (*Macaca mulatta*) originating from various geographic regions. Several nested clades, defined by closely related haplotypes, were identified, suggesting considerable genetic subdivision, probably relics from heterogeneous origins, founder effects, and genetic drift, followed by breeding isolation. The rhesus matrilineages from India differed discretely and markedly from Chinese matrilineages; approximately 90% of the genetic heterogeneity among the combined samples of Indian and Chinese rhesus macaques studied here was due to country of origin. In addition, mtDNA sequences from macaques of China were more diverse than those from rhesus macaques of India, an outcome consistent with China's greater subspecies diversity and with nuclear genotype distributions. Otherwise, the distribution of mtDNA variation within rhesus macaques of China, and especially within those of India, exhibited far less structure and did not conform to a simple isolation-by-distance model.

As the demand for genetically heterogeneous and well-characterized rhesus macaques for biomedical-based research increases, mtDNA haplotypes can be useful for genetically defining, preserving maximal levels of genetic diversity within, and confirming the geographic origin of captive breeding groups of rhesus macaques.

The rhesus macaque (*Macaca mulatta*) is the most frequently used non-human primate model in biomedical research and constitutes the largest domestic population of research primates. Because of the rising demand from the biomedical research community for a steady supply of genetically well-characterized rhesus macaques, research-oriented genetic management is now an integral part of the overall operation of most domestic rhesus breeding facilities (3, 8-10, 21, 22, 44, 55). Strategies for genetic management of domestic rhesus colonies, populations of which are small but genetically heterogeneous, are tailored to monitor distribution of reproductive success, maintain genetic variability, and minimize inbreeding and genetic subdivision. Even under genetic management, despite maintaining high and stable levels of gene diversity, colonies are prone to genetic subdivision due to colony founder effects, genetic isolation, and genetic drift (21). This is exacerbated by the diversity of regions of origin of rhesus macaques used as founders of various captive colonies; the geographic range of *M. mulatta* exceeds that of any primate other than *Homo sapiens* (55), and rhesus from various regions of origin may manifest long-established genetic differences simply due to isolation by distance. Thus, the founding populations of breeding centers that acquired founder stock from foreign exporters in different geographic regions are likely to differ genetically. For example, large differences in frequencies of alleles at autosomal loci, such as allozymes, simple tandem repeats (STRs), or microsatellites, and the *DQA1* and *DQB1* genes among colonies of domestic rhesus originating in India and China, the two principal historic suppliers of rhesus macaques to the United States, have been documented (21, 22, 39, 40, 44, 52). Moreover, rhesus macaques from India and China exhibit autosomal gene pools, each of which reflects appreciable genetic substructuring (27), probably associated with their separate phylogenetic and demographic histories (21, 30, 44). Although assignment programs have been developed (34) that we have adapted to successfully identify the country of origin of rhesus macaques with > 90% accuracy, many nuclear loci are required for this purpose.

Whether variations in allelic representation are mostly attributable to ancestral genetic differentiation among allopatric regional populations, demographic events unique to parapatric subpopulations, or stochastic changes associated with practices of captive breeding, differences in allele frequency distributions might also occur at quantitative trait loci (QTL) that are objects of biomedical research. For example, rhesus macaques originating from China are more resistant to experimentally induced infection with simian immunodeficiency virus (SIV) than are those originating from India (25). Thus, the relative proportion of Indian vs. Chinese rhesus subjects in that group influences the average response to infection of animals in a control or experimental group under study. Although high levels of genetic variability probably ensure long-term viability of captive colonies (36, 37), the contribution of inter-animal additive genetic variances to responsiveness to experimental procedures can obscure correlates under study (11, 16). For this reason, knowledge of the location of origin of rhesus macaques can inform researchers about their suitability as subjects in any given biomedical study.

In the wild, male dispersal and female philopatry ensures close correspondence between maternal kinship and spatial proximity, causing some matrilines to be endemic in some geographic re-

Received: 8/11/03. Revision requested: 10/10/03. Accepted: 12/08/03.
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gions while being less common or completely absent in others (26, 32). Mammalian mitochondrial DNA (mtDNA) is transmitted matrilineally, without recombination, and experiences a rate of mutation up to an order of magnitude higher than that of non-repetitive nuclear loci (2, 20). Accordingly, sequence variation in mtDNA is a potent tool for inferring matrilineal relationships and genetic diversity among rhesus macaques of non-admixed ancestry (30, 47, 51, 53). Melnick and co-workers (28) estimated that > 90% of mtDNA diversity in free-ranging *M. mulatta* results from interpopulational differences. This is in stark contrast to nuclear genetic markers, for which approximately 90% of the diversity occurs within local populations. As animals from various regions within the rhesus macaque's geographic range (e.g. India, Burma, and China) have been used to found various domestic captive colonies as a biomedical research resource in the United States, these colonies might also reflect substantial mtDNA substructure. Although in some instances, the Chinese and Indian rhesus macaques have been indiscriminately cross-bred at some breeding centers, in other instances, even the country of origin of many captive-bred animals is not known. Under such circumstances, mtDNA haplotypes should provide more-sensitive and cost efficient indicators of the geographic origin of rhesus macaques of non-admixed genealogy than do nuclear (e.g. STR) polymorphisms. Moreover, even though captive rhesus breeding groups are typically founded by a group of unrelated adult females, animals not known to be related but with identical mtDNA haplotypes, probably descend from the same ancestral female, notwithstanding the greater contribution of numerous nuclear DNA lineages. One goal of genetic management should be to maintain the representation of all founding matrilines and patrilines in approximately equal frequencies and maximize biogeographic representativeness of these lineages in domestic captive rhesus colonies. Therefore, monitoring the distribution of mtDNA haplotypes in captive rhesus colonies should be an integral part of genetic management of the colonies. As part of a larger effort to increase the mtDNA diversity database for rhesus macaques, the objective of the study reported here was to use partial sequences of the mitochondrial 12S and 16S rRNA genes to investigate the influence from geographic origins on the extent of genetic diversity among captive rhesus macaques.

Materials and Methods

Animals and blood samples. Blood and DNA samples were collected from 28 unrelated rhesus macaques at: the California National Primate Research Center (CNPRC), University of California, Davis; COVANCE Research Products (formerly known as Texas Primate Center, TPC) in Alice, Tex.; the Laboratory Animal Breeders Services (LABS) of Virginia, in Yemessee, S.C.; the Department of Veterinary Resources, University of Miami (UM), Fla.; the Cayo Santiago facility of the Caribbean Primate Research Center, San Juan, Puerto Rico; the Michigan Department of Public Health (MDPH) in East Lansing Mich.; the Deutsches Primatenzentrum, Göttingen, Germany; and the Biomedical Primate Research Centre (BPRC) in Rijswijk, The Netherlands. These colonies are managed in compliance with Institutional Animal Care and Use Committee (IACUC) regulations (or in accordance with the National Institutes of Health guidelines or the US Department of Agriculture regulations [1, 41]) prescribing the humane care and use of laboratory animals. These samples from seven captive colonies purportedly represent three regional populations (i.e., northern India, Southeast Asia, and China [Table 1]). Table 1 indicates that geographic origins of each of these animals differ markedly. Animals from COVANCE are founders that derive from two separate shipments from heterogeneous locales in the Sichuan Province in western China. The CNPRC sample 31437 was acquired from a breeding center in Kunming (southwestern China) whereas CNPRC samples 33607, 33608, and 33617 were purchased from Souchou breeding center outside Shanghai (northeastern China). The founding rhesus from the LABS descend solely from animals trapped at a half dozen sites widely distributed along approximately 500 km of the Himalayan

Table 1. Regional subpopulations/regions/captive colonies which the rhesus 12S rRNA and 16S rRNA genes represent

Indian subcontinent	
Subpopulation northwestern India	
⁻L547—12S rRNA, 16S rRNA	Kashmir, NW India/UM, US (NWI)
84320—16S rRNA only	Kashmir, NW India /UM, US (NWI)
⁻H517—12S rRNA, 16S rRNA	Kashmir, NW India /UM, US (NWI)
⁻I741—12S rRNA, 16S rRNA	Kashmir, NW India /UM, US (NWI)
Subpopulation north central India	
32018—12S rRNA, 16S rRNA	Eastern Uttar Pradesh, N. India/Cayo Santiago, US (NI)
⁻*32038*—12S rRNA, 16S rRNA	Eastern Uttar Pradesh, N. India/Cayo Santiago, US (NI)
⁻AC2E—12S rRNA, 16S rRNA	Western Uttar Pradesh, N. India/LABS, US (NI)
⁻AC85—12S rRNA, 16S rRNA	Western Uttar Pradesh, N. India/LABS, US (NI)
⁻5S7—12S rRNA, 16S rRNA	Western Uttar Pradesh, N. India/LABS, US (NI)
⁻A13N—12S rRNA, 16S rRNA	Western Uttar Pradesh, N. India/LABS, US (NI)
Animals purportedly from India	
*25311—12S rRNA, 16S rRNA	India/CNPRC, US (I) - ?
23007—12S rRNA, 16S rRNA	India/CNPRC, US (I) - ?
51—12S rRNA, 16S rRNA	India/Deutsches Primatenzentrum, Germany (I) - ?
61—12S rRNA, 16S rRNA	India/Deutsches Primatenzentrum, Germany (I) - ?
Southeast Asia	
Subpopulation Burma	
PR523—12S rRNA, 16S rRNA	Burma/BPRC, The Netherlands (B) - ?
PR539—16S rRNA only	Burma/BPRC, The Netherlands (B) - ?
23274—12S rRNA, 16S rRNA	Burma/CNPRC, US (B) - ?
23276—12S rRNA, 16S rRNA	Burma/CNPRC, US (B) - ?
Subpopulation Thailand	
*M9—12S rRNA, 16S rRNA	Thailand/MDPH, US (T) - ?
Subpopulation Vietnam	
24450—12S rRNA, 16S rRNA	Vietnam/CNPRC, US (V)⁻ ?
*24453—12S rRNA, 16S rRNA	Vietnam/CNPRC, US (V)⁻ ?
China	
Subpopulation southwestern China	
RQ579—12S rRNA only	Sichuan, SW China/COVANCE, US (SWC)
RQ554—12S rRNA, 16S rRNA	Sichuan, SW China/COVANCE, US (SWC)
31437—12S rRNA, 16S rRNA	Kunming, SW China/CNPRC, US (SWC)
24670—12S rRNA, 16S rRNA	Kunming (via Russia), SW China/ CNPRC, US (SWC)⁻ ?
Subpopulation northeastern China	
33607—12S rRNA, 16S rRNA	Shanghai, NE China/CNPRC, US (NEC)
⁻⁻*33608*—12S rRNA, 16S rRNA	Shanghai, NE China/CNPRC, US (NEC)
⁻⁻33617—12S rRNA, 16S rRNA	Shanghai, NE China/CNPRC, US (NEC)

? denotes animals, the true location of origin (region, country and locale) of which is uncertain. ⁻Identifies haplotypes 32038 in India: ⁻⁻for 33608 in China and * for 32038 haplotypes derived from animals, the geographic origins of which are not certain. Italics denote regional haplotypes.

foothills of western Uttar Pradesh, whereas those from Cayo Santiago descend solely from animals trapped near Lucknow in central Uttar Pradesh; those from the UM descend solely from animals trapped within a restricted area about 100 km northwest of Jammu, Kashmir, NW India.

Because this study was designed to include a heterogeneous representation of captive rhesus macaques, the within-country proveniences for the others studied are unknown or uncertain, but are assumed to add geographic heterogeneity to the sample of known trapping locations within their country of origin. The alleged country of origin of animals, the exact geographic origins (i.e., locale and region) of which are unknown or uncertain, is assumed to be correct, and these animals are indicated with the symbol '?'. For this study, animals purchased from breeding colonies in Kunming and Souchou are hypothetically considered to have originated in southwest and eastern China, respectively, though not necessarily solely from Yunnan and Jiangsu/Zhejiang provinces. The oft-cited claim that founders of rhesus macaques bred at each of the approximately one dozen rhesus-breeding facilities in China represent a mixture of regional origins (19, 57) also was tested in this study.

The dams of animals 23007 and 25311 were imported to the United States from India (38), but information on the exact location where the animals were caught is not available. According to colony records at the CNPRC, animal 24670 is of Chinese origin, but was imported via the former Sukumi Primate Center in Russia. Since the exact genetic history in Russia of this animal is undeterminable, we accord less confidence to its alleged origins in China. The geographic origins of animals 51 and 61 are known with even less confidence. Their founders are reported to have been exported from India to China for captive breeding, then subsequently purchased from China by the Deutsches Primatenzentrum. However, these animals exhibit class I- and class-II major histocompatibility (MHC) genotypes uncharacteristic of rhesus macaques originating in India, but typical of those from China (41). Therefore, these animals might actually be either of Chinese origin or hybrid descendants of Chinese and Indian progenitors.

The M9 animal also is of unknown origin that descends from a stock representing the last remnants of a large number of rhesus macaques at the MDPH originally imported for use in experimental polio vaccine trials. Because many of the rhesus macaques imported for that purpose originated in Thailand, it is conceivable that this animal is also of Thai origin. Samples PR523, PR539, 23274 and 23276 are from animals originating in Burma; the first two and the last two samples were acquired by the CNPRC and the BPRC, respectively. Samples 24453 and 24450 were drawn from rhesus macaques acquired by the CNPRC from the former Sukumi Primate Center in Russia and are alleged to have originated from Vietnam.

Samples of DNA. The DNA samples from the BPRC and the Deutsches Primatenzentrum were shipped to the Veterinary Genetics Laboratory (VGL) and the Molecular Anthropology Laboratory (MAL), University of California, Davis on dry ice whereas blood samples from all other animals were drawn into anticoagulant-treated vacutainer tubes and (except for samples from CNPRC) shipped on blue ice to the MAL by overnight mail. Using conventional methods, total DNA was extracted from the blood samples. The 12S rRNA partial homologues were then amplified, using degenerate oligonucleotides that were redesigned from the mammalian universal 12S rRNA primers (24): AAACTSGGATTAGATACCCYAYTA and AAGSGCGACGGGCGSTGTGT. The 16S rRNA partial sequences were amplified, using Kocher and co-workers' (24) mammalian universal primers CGCCTGTTTATCAAAAACAT and CCGGTCTGAACTCAGATCACGT. Separate portions of these samples were amplified at the MAL and at the VGL. At the MAL, 1 µl of template DNA from each extraction was added to 2.5 µl of 10X polymerase chain reaction (PCR) buffer, 0.2 m*M* each dNTP, 0.6 m*M* each primer, 0.5 µg of bovine serum albumin (BSA)/µl, 1.5 m*M* $MgCl_2$, and 0.5 U of *Taq* polymerase (Applied Biosystems, Foster City, Calif.) in a final volume of 25 µl. The PCR amplifications were performed under the following conditions: initial denaturation at 95°C for four minutes, then 40 cycles of denaturation at 95°C for 30 sec, annealing at 52°C and 55°C (for the 12S rRNA and 16S rRNA genes, respectively) for 30 sec, and extension at 72°C for 30 sec, followed by a final extension step at 72°C for four minutes.

At the VGL, 25-µl PCR reactions were performed, using 1 µl of template DNA, 2.5 µl of 10X PCR buffer (Applied Biosystems), 0.2 m*M* each DNTP, 1.5 m*M* $MgCl_2$, 0.5 U of AmpliTaq (Applied Biosystems) and 0.6 µ*M* and 0.4 µ*M* each primer, respectively, for 12S rRNA and 16S rRNA amplifications. The PCR cycling parameters at the VGL were: five cycles of initial denaturation at 96°C for two minutes each, annealing at 55 and 58°C (for the 12S rRNA and 16S rRNA genes, respectively) for 50 sec, and extension at 72°C for 45 sec, then 25 cycles of denaturation at 96°C for 30 sec, annealing at 55 and 58°C (for the 12S rRNA and 16S rRNA genes, respectively) for 45 sec, and extension at 72°C for 45 sec, followed by a final extension step at 72°C for 15 min.

Samples with successful amplification were filtered, using a Microcon 100 filter unit (Millipore Corp., Bedford, Mass.). Amplified fragments were cloned, using the TOPO TA kit (Invitrogen, San Diego, Calif.) and following the manufacturer's instructions, because preliminary studies indicated that cloned sequences were cleaner than those obtained from direct PCR analysis. Forward and reverse sequences were generated by use of the ABI 377 (Applied Biosystems) and the Basestation (MJ Research, Woburn, Mass.) DNA sequencers with M13 primers (Invitrogen) and the Big Dye Terminator Cycle Sequencing version 3.0 kit (Applied Biosystems) and by following the manufacturer's recommendations. Electrophenograms were analyzed, using the SeqMan II Module of DNASTAR Inc. (Madison, Wis.). Sequences were aligned using CLUSTAL W (46), and alignments were further improved manually. Hickson and co-workers (18) observed that CLUSTAL W tended to produce accurate alignments even when gap weights were changed in relation to rRNA secondary structures (23).

For each animal, up to three clones were forward and reverse sequenced. Sequencing and PCR errors were eliminated by comparing aligned clonal sequences from the same animals as well as among different animals. Consensus sequences were obtained to further minimize effects from sequencing discrepancies. Twenty-eight 410-bp 12S rRNA consensus sequences and twenty-nine 551-bp 16S rRNA consensus sequences were obtained. The 12S and 16S rRNA partial sequences generated in this study are available from GenBank under accession numbers: AY360266 to AY360318. Only twenty-seven 12S and 16S rRNA sequences were combined to form the 961 base concatenations because not all animals rendered sequences for both genes.

The phylogenetic analyses include 12S and 16S rRNA

Table 2a. Observed number of pairwise substitutions along 961-bp concatenations

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
23274/B?																									
23276/B?	9																								
23007/I?	15	8																							
24670/SWC?	12	9	17																						
31437/SWC	16	15	19	17																					
32018/NI	14	10	6	19	16																				
*32038/NI	16	12	6	21	18	2																			
*AC2E/NI	16	12	6	21	18	2	0																		
*AC85/NI	16	12	6	21	18	2	0	0																	
*25311/I?	16	12	6	21	18	2	0	0	0																
*H517/NWI	16	12	6	21	18	2	0	0	0	0															
*I741/NWI	16	12	6	21	18	2	0	0	0	0	0														
*M9/T?	16	12	6	21	18	2	0	0	0	0	0	0													
24450/V?	14	10	6	19	16	2	2	2	2	2	2	2	2												
*24453/V?	16	12	6	21	18	2	0	0	0	0	0	0	0	2											
33607/NEC	15	14	20	16	5	17	19	19	19	19	19	19	19	17	19										
**33608/NEC	16	15	21	15	6	18	20	20	20	20	20	20	20	18	20	1									
**33617/NEC	16	15	21	15	6	18	20	20	20	20	20	20	20	18	20	1	0								
*L547/NWI	16	12	6	21	18	2	0	0	0	0	0	0	0	2	0	19	20	20							
*5S7/NI	16	12	6	21	18	2	0	0	0	0	0	0	0	2	0	19	20	20	0						
*A13N/NI	16	12	6	21	18	2	0	0	0	0	0	0	0	2	0	19	20	20	0	0					
51/I?	22	19	25	25	12	22	24	24	24	24	24	24	24	22	24	13	14	14	24	24	24				
61/I?	17	12	18	7	18	22	24	24	24	24	24	24	24	22	24	19	20	20	24	24	24	28			
PR523/B?	11	14	18	18	11	17	17	17	17	17	17	17	17	15	17	8	9	9	17	17	17	17	21		
RQ554/SWC	20	19	25	19	10	22	24	24	24	24	24	24	24	22	24	5	4	4	24	24	24	16	24	13	
Sylvanus	55	48	52	51	58	56	58	58	58	58	58	58	58	56	58	59	60	60	58	58	58	56	50	59	62

? denotes uncertain animal origin; asterisks indicate haplotypes

orthologues derived from the published complete mtDNA sequence of the Barbary ape (*Macaca sylvanus* [GenBank accession number AJ309865]) as the outgroup. This sequence was used to root the cladograms because it has been argued that *M. sylvanus* belongs to the *M. silenus-sylvanus* subgroup (13) which is phenetically more distant from the *M. fascicularis* subgroup, to which the rhesus macaque belongs, than are the remaining two subgroups (*M. sinica and actoides*; [6]). More recent data on *Macaca* Y chromosome (47), mtDNA (17, 29), and the nuclear locus *NRAMP1* (7) also suggest that *M. sylvanus* is a sister clade to all other macaque species. The mean number of pairwise differences between concatenated rhesus haplotypes was calculated, using the ARLEQUIN package (version 2.0, [42]). Genetic variation was expressed as the average number of these pairwise differences, or nucleotide diversity (π), which is analogous to gene diversity of nuclear loci (31, 45). The ARLEQUIN package was also used to estimate pairwise F_{st} (43) for regional subpopulations comprising animals from India and China, the exact originating proveniences for which are known (i.e., northwestern India, north central India, southwestern China, and northeastern China) to ascertain the effects of geographic distance and isolation on regional genetic differentiation. The analysis of molecular variance (AMOVA) program from ARLEQUIN was used to compute hierarchal F-statistics (54) to determine the level of genetic differentiation among matrilines within each subpopulation (F_{st}), between each of the two regional subpopulations (F_{sc}) in each country (India and China), and between these two countries (F_{ct}). Maximum likelihood (MLK) with, and without the assumption of molecular clock, and neighbor joining analyses were used to measure genetic distances among 12S and 16S rRNA concatenations, using PHYLIP (version 3.572c, [12]). Molecular clock constraints assume constant rates of nucleotide substitution in each rRNA gene for all taxa (16).

Results

Table 2b. Inter-regional haplotypic distances

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
23274/B?														
23276/B?	9													
23007/I?	15	8												
24670/SWC?	12	9	17											
31437/SWC	16	15	19	17										
32018/NI	14	10	6	19	16									
[11]32038/NI	16	12	6	21	18	2								
24450/V?	14	10	6	19	16	2	2							
33607/NEC	15	14	20	16	5	17	19	17						
[2]33608/NEC	16	15	21	15	6	18	20	18	1					
51/I?	22	19	25	25	12	22	24	22	13	14				
61/I?	17	12	18	7	18	22	24	22	19	20	28			
13. PR523/B?	11	14	18	18	11	17	17	15	8	9	17	21		
RQ554/SWC	20	19	25	19	10	22	24	22	5	4	16	24	13	
Sylvanus	55	48	52	51	58	56	58	56	59	60	56	50	59	62

Superscripts denote number of member sequences represented.

From the 28 rhesus macaques that were sampled, twenty-seven 12S and 16S rRNA sequences were obtained representing thirteen 12S rRNA and ten 16S rRNA haplotypes. The average pairwise differences for each gene were 7.5 and 5.4, and their (nucleotide diversity) values were 0.018 and 0.0098, respectively. The number of pairwise nucleotide differences along the 12S-16S rRNA concatenations is shown in Table 2a, and Table 2b presents the differences among the 26 sequences and 15 unique haplotypes, respectively. A total of 14 unique rhesus 12S-16S haplotypes were observed among all breeding colonies. The *M. sylvanus* sequence exhibited an average difference of 57 from the rhesus sequences, ranging from 48 (Burma: 23276) to 62 (southwestern China: RQ554) bases. The range of nucleotide differences between all pairs of rhesus macaques varied from 0 to 28 nucleotides (i.e., up to 3%), with mean pairwise nucleotide difference of 12.9 (π = 1.3%). The average pairwise difference among only the 14 unique sequences observed, (Table 2b) was 15.4 (π = 1.6%) and might represent an estimate of diversity less influenced by sampling effects than by the former estimate.

Table 3. Subpopulation pairwise levels of genetic differentiation among matrilines within each subpopulation (F_{st}) and nucleotide diversity (π) estimates (based on a Mantel test of 10,000 reiterations)

	1	2	3	π
Northwestern India				0.0000
North central India	-0.154			0.0007
Southwestern China	0.885	0.837		0.0104
Northeastern China	0.966	0.983	0.157	0.0007

Table 4. The AMOVA design and results among regional aggregates

Source of variation	*df*	% Variation	Averaged for significance tests for random distribution of animals (10,100 permutations)
Among regions (India & China) (Fct = 0.903)	1	90.29	*P* = 0.3349
Among subpopulations/ within regions (Fsc = 0.227)	2	2.20	*P* = 0.1998
Among matrilines/ within subpopulations (Fst = 0.925)	10	7.51	*P* = 0.0001
Total	13		

F_{ct} = genetic differentiation between countries; F_{sc} = genetic differentiation between regional populations in each country.
See Table 3 for key.

Among the five animals from China and nine from India, the exact geographic origins of which are known, only six regional haplotypes were observed. Of these, four were unique to China; haplotypes 33607 and 33608 (the latter comprising sequences 33608 and 33617) were found solely in northeastern China, and haplotypes RQ554 and 31437 were present only in southwestern China. Although more Indian than Chinese rhesus macaques were sampled, only two haplotypes were present among them: haplotype 32018 was unique to north central India, and those belonging to haplogroup 32038 (i.e., 32038, AC2E, AC85, 5S7, A13N, H517, I741 and L547) were observed in the northwestern and north central Indian subpopulations. Sample M9, hypothesized to derive from Thailand, also was a member of this 32038 Indian lineage. One additional haplotype from each of the two countries was identified in rhesus macaques, the exact origin of which is unknown.

The range of pairwise differences between Chinese and Indian haplotypes was 16 to 25 bases within a mean of 21.36 and a π value of 2.2%. The average pairwise mtDNA diversity observed among all Chinese rhesus macaques sampled (8.27, π = 0.86%) was higher than that among the Indian rhesus (1.30, π = 0.14%). When only the unique rhesus sequences in Table 2b are used for these estimates to preclude some of the potential influences of sampling, the average pairwise differences among Indian and among Chinese, and between Indian and Chinese rhesus macaques were 4.67 (π = 0.49%), 9.80 (π = 1.02%), and 19.73 (π = 2.05%), respectively.

The Mantel test (Table 3) indicated that genetic structure among the regional subpopulations is congruent with a discrete rather than a clinal distribution between rhesus macaques in India and China, one that did not conform strictly to a simple isolation-by-distance model. Reflecting this discrete variation, mtDNA differentiation between regional subpopulation pairs ranged from inconsequential between regional population in the same country (< 0.16) to very high for populations in different countries (> 0.83). The northeastern Chinese macaque population appeared most genetically disparate from the rest (its pairwise F_{st} values ranged from 0.157 [southwestern China] to 0.983 [north central India]), although rhesus macaques from southwestern China exhibited far greater diversity than those in the other three regions. The AMOVA tests (Table 4) also indicated significant geographic subdivision (90.3% of the total) between countries (India and China) and far less subdivision (amounted to only 2.2% of the total) among the various regional subpopulations within India and China.

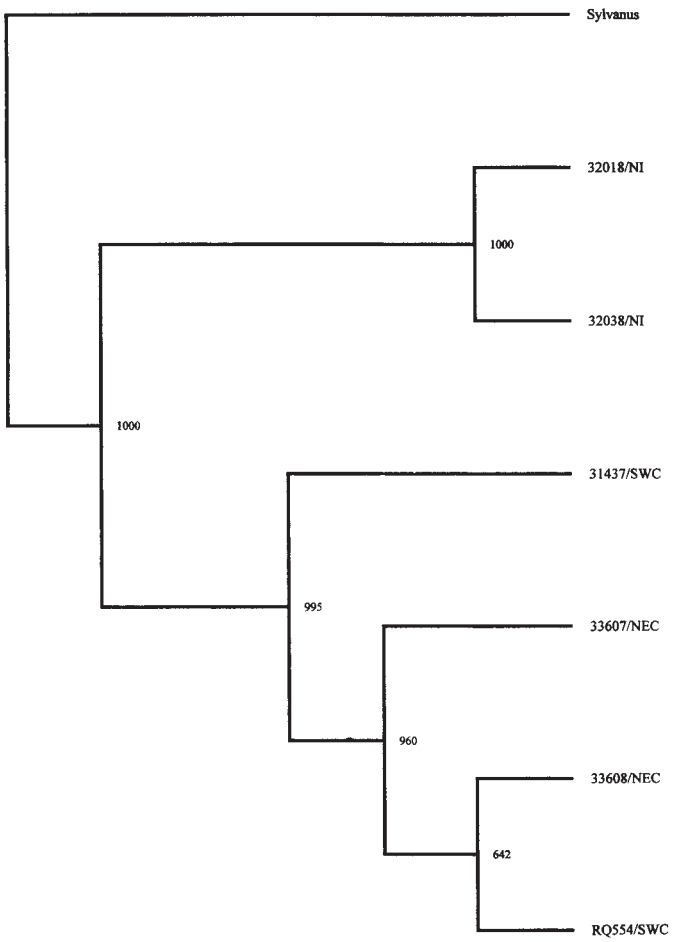

Figure 1. Maximum likelihood (MLK) tree of regional haplotypes. The MLK percentages of the 1,000 bootstrap replicates supporting a particular clade within the MLK tree are indicated at each branch.

Maximum likelihood, with and without the assumption of molecular clock, and neighbor-joining gave identical topologies and similar bootstrap values for the concatenated rhesus haplotypes. Figure 1 shows that the MLK species tree based on the concatenations confirmed the differentiation of Indian and Chinese animals. Bootstrap sampling of these subpopulations, while supporting some local substructuring, strongly supports the sharing of some haplotypes among subpopulations within the same country, especially in India. Despite the weaker support for some external nodes within the Chinese clade, the Indian and Chinese rhesus macaques are separated from each other with strong support based on 100%-majority bootstrap consensus. This indicates that the southwestern and northeastern Chinese macaques are reciprocally monophyletic

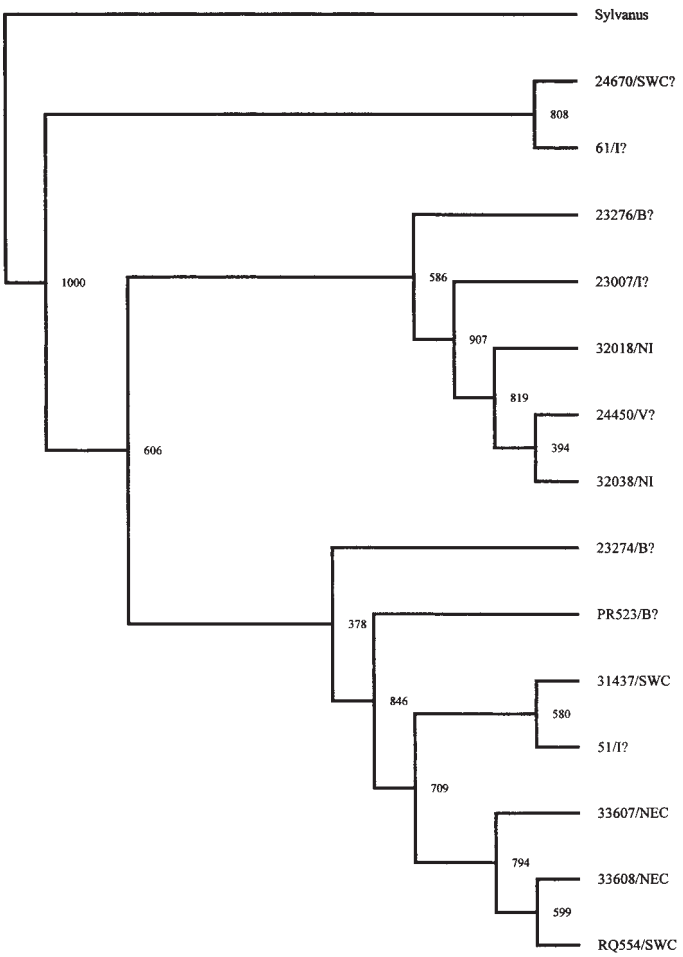

Figure 2. Maximum likelihood tree of captive rhesus haplotypes.

with the north central and northwestern Indian animals. Although all of the northeastern Chinese macaque samples in Fig. 2 (MLK tree involving all rhesus haplotypes) clustered with most southwestern Chinese and some of the Burmese samples, the Indian samples from Kashmir and Uttar Pradesh were thoroughly intermingled with each other, along with the remaining Southeast Asian animals. Haplotype 51, purportedly from India, most resembled that of the Chinese rhesus macaques. Haplotypes 23274 and PR523, allegedly from Burmese rhesus macaques, clustered with most Chinese haplotypes, and one Vietnamese haplotype (24450) clustered with the remaining Indian haplotypes.

Collura and Stewart (5) and van der Kuyl and co-workers (49) reported nuclear integrations of the mtDNA cytochrome b and 12S rRNA genes. Although there are no reports of nuclear 16S rRNA inserts in *Macaca* spp., their existence cannot be discounted (33). There is, however, some evidence that the variation depicted in this study originated from genuine 12S and 16S rRNA genes. On the basis of sequence identity and ML tree constructs (not shown), our 12S rRNA consensus sequences corresponded with rhesus mtDNA haplotypes that were reported by van der Kuyl and co-workers ([48] GenBank accession number: L35203) and Tosi and co-workers ([47] GenBank accession numbers: AF424949 to AF424953) and are orthologous to the ones used by van der Kuyl and co-workers (50) for genetic management of captive-bred primates. Also, our analysis based on 12 and 16S rRNA concatenations confirmed an intraspecific divergence (1.3%) that was comparable to that of 2.5% reported by Melnick and co-workers (28) despite the substantial differences between the two studies in sampling across the rhesus macaque geographic range. Any inclusion of the more-slowly evolving pseudogenes (56) in our analysis would have resulted in a lower estimate of divergence within the rhesus populations.

Discussion

On the basis of morphologic evidence, taxonomists have described several subspecies of *M. mulatta*. Fooden (14) separated the taxon into 13 subspecies; two of these are found in India, six in China, and the remaining in the intermediate regions between these two countries. Groves (15) identified only six *M. mulatta* subspecies, two from Indian and four from China. However, both recognized the separation between those from the Kashmir/Punjab region (i.e., *M. m. villosus* or *M. m. erythrea*) and the other regions of India (*M. m. mulatta*), marked differences between rhesus macaques in western (*M. m. vestita*) and eastern (*M. m. sanctijohannis, M. m. littoralis and/or M. m. tcheliensis*) China, and a greater level of subspeciation among Chinese than among Indian rhesus macaques.

Sequence comparison of the rhesus 12S and 16S rRNA genes reveals significant, and approximately equal degree of intraspecific diversity in both genes. Consistent with the aforementioned morphologic classification, our results indicated marked divergence between Indian and Chinese rhesus macaques and greater divergence of rhesus macaques in China than in India. Each of the haplotypes from Kashmir, alleged to represent *M. m. villosus* (15), was interspersed with *M. m. mulatta* haplotypes from other regions in India. In contrast, most haplotypes of rhesus macaques acquired from breeding centers in northeastern and southwestern China where indigenous rhesus macaques are assigned to the subspecies *M. m. vestita* and *M. m. sanctijohannis*, respectively, fell in separate groups within the Chinese clade. In this study, most variation (> 90%) was distributed between countries (F_{ct} = 0.903), with little genetic substructure occurring within India or China.

The observation of high levels of inter-regional variation confirms an earlier report by Melnick and co-workers (27) that attributed 91% of the total mtDNA diversity to differences among different regional populations of rhesus macaques, with < 10% attributed to differences among subpopulations within regions (i.e., countries). The results of our study and that of Melnick and co-workers (28) agree: that differences at the level of the individual within local subpopulations are low.

Estimates of nucleotide divergence among the Indian and among the Chinese rhesus macaques studied by Melnick and co-workers (28) were 0.4 and 0.7%, respectively, whereas our own corresponding estimates were about 0.14 and 0.86%, respectively. However, when only the unique sequences in these two studies are considered, nucleotidic divergence in Indian and Chinese rhesus macaques of the study reported here (0.49 and 1.02%, respectively) were more similar to those reported by Melnick and co-workers (28): 0.53 and 0.72%, respectively. The difference in estimates of within-region diversity in their and our studies, especially that for Chinese rhesus macaques, the geographic range of which is greater and more complex than that of Indian rhesus

macaques, is probably due, in part, to the greater geographic diversity and size, hence lower sampling error, of our regional samples. The lower Indian values, compared with those of Chinese rhesus macaques in their and our studies, might reflect the effects of haplotype sharing stemming from greater geographic homogeneity of the rhesus macaque home range in India than in China. Although even our own sampling size was not large and our results should be confirmed by study of a larger sample, the higher Chinese values are consistent with the higher level of genetic diversity reported for the Chinese than the Indian rhesus macaques that was based on protein coding (44), STR (21), the MHC loci (39, 40, 52), and morphology/taxonomy, and are consistent with the greater level of subspeciation in that country (14, 15).

The ML tree in Fig. 1 corroborates the reciprocal monophyly between Chinese and Indian rhesus macaques, as indicated by results of the Mantel test; however, the two regional variants of Chinese rhesus fail to form completely separate clades. Together with the weaker bootstrap values, this suggests lack of regional differentiation within China. This Chinese genetic substructure might also reflect effects of alleged admixing of founder stock from different subpopulations in breeding centers in these two regions (19, 57). If so, the degree of mixing of rhesus breeding stock originating from different geographic regions has been exaggerated because Chinese, unlike Indian rhesus macaques, still exhibit moderate levels of genetic subdivision and isolation by distance.

Founder effects and intergenerational genetic drift have undoubtedly occurred in the captive colonies from where the animals included in this study derived, and might contribute to the aforementioned discrepancies. The domestic gene pool that was founded by Indian macaques has been documented to exhibit marked genetic subdivision on the basis of analyses of several protein coding and non-coding autosomal loci (30, 44). The most recent estimates of nuclear genetic subdivision (measured as F_{st}) within the parental generation and among the first-generation offspring cohorts at several domestically bred specific pathogen-free (SPF) Indian rhesus colonies was approximately 0.099, indicating a high level of genetic substructure (21) of captive Indian rhesus breeding colonies in the United States. However, the close similarity of mtDNA haplotypes between Indian rhesus from Kashmir and Uttar Pradesh in the study reported here suggests that genetic subdivisions among the more-derived Indian SPF animals results from stochastic processes associated with captive breeding rather than biogeographic diversity among free-ranging rhesus macaques in India.

The clearest outcome of our study is the stark genetic difference between rhesus macaques of Indian and Chinese origin and their corresponding levels of diversity. On the basis of an assignment index (34), calculations of relative probabilities of STR genotypic associations, using allelic frequencies from Chinese and Indian populations, accurately assigned approximately 90% of Chinese and Indian rhesus macaques to their correct country of origin (data not presented). Our study suggests that the use of mtDNA should increase the success of assignment of non-admixed rhesus to their country of origin to 100% (a specialized panel of STR loci with allele frequencies that differ maximally between Indian and Chinese rhesus macaques has proved effective for identifying rhesus macaques of mixed Chinese/Indian ancestry [21]). This result is consistent with reports of other phenotypic differences between the two regional populations of rhesus macaques. Peng and co-workers (35) reported that Indian rhesus macaques have a narrower postorbital constriction and biorbital width than do Chinese rhesus macaques. This craniometric disparity is concordant with the marked clinical differences between Chinese and Indian rhesus macaques when animals from the respective populations are fitted with cranial implants for biomedical trials. Morphologic differences between individuals in different regional populations also include body weight, size, and shape. Chinese rhesus males tend to be heavier, longer, and taller than Indian rhesus males. As juveniles, Chinese rhesus females are taller and heavier, but as mature adults, Indian rhesus females are longer and heavier, thus causing higher levels of sexual dimorphism among Chinese rhesus adults (4).

Rolfs and co-workers (39, 40) and Viray and co-workers (52) reported major differences in allele frequencies at the MHC class-II *DQA1* and *DQBI* loci between rhesus macaques from India and China, and to a lesser degree between those from either India or China and Burma. These regionally specific traits strengthen the rationale for genetically characterizing exhaustively animal models established for biomedical research.

In some instances, the country of origin of a rhesus may be unknown or mistakenly classified. Animals 51 and 61, for example, were purchased with the belief they were of Indian origin, but unpublished work in our laboratory and at the Deutsches Primatenzentrum (41) indicated that these animals exhibit MHC haplotypes that are common in Chinese, but not Indian rhesus macaques. The results of the study reported here are consistent with the conclusion that the mtDNA of animals 51 and 61 is of Chinese origin. For them, the average pairwise differences from Chinese sequences (16.0 and 17.6, respectively) were lower than those for Indian sequences (23.2 and 21.3, respectively). The mean pairwise difference between sequences form animal M9, suspected of having originated in Thailand, and the Indian, Burmese and Chinese sequences is 0.72, 15.0, and 20.3, respectively, has led to the conclusion that this animal and the remnant breeding colony of which it is a member originated in India rather than Thailand, notwithstanding the absence of comparative data on rhesus known to have originated in Thailand. This underscores the need to securely document the country of origin and/or region of rhesus macaques to be used in biomedical studies if the influence of genetic variance on traits under study is to be minimized.

Mitochondrial DNA allows ready distinction between admixed rhesus of Indian and Chinese ancestry so that genetic heterogeneity among subjects of biomedical research can be minimized. Because colony managers in China are said to select their breeding stock from throughout the country rather than solely from nearby their respective breeding centers, undoubtedly with variable success, the geographic locations of these centers from which a rhesus macaque is purchased may not be the actual provenience of most, and certainly not all, of the animals bred there (19, 57). Notwithstanding the close clustering of the Shanghai haplotypes, which suggests persistence of regional genetic subdivision, animals exported from China may have diverse and unpredictable multi-regional origins. Therefore, genetic testing might be desirable if an animal's region of origin is deemed important to research protocols in which it is used as a study subject.

The matrilineally inherited haplotypes studied here provided an additional suite of genetic markers to gauge levels of taxonomic representation, genetic subdivision, and kinship among

captive rhesus macaques. The more rapidly evolving control region of the rhesus mtDNA, now under study in our laboratories, should provide even more detailed information from which local origin of rhesus macaques within China and India can be easily documented. Biogeographic affiliations of captive rhesus macaques provide an excellent opportunity to examine the distribution of populations and correlates of behavioral and physiologic diversity. Further comparisons involving more animals from additional colonies may justify the maintenance of distinct mtDNA matrilines between the various geographic units as separate models for specific biomedical objectives.

Acknowledgments

This study was supported by the National Institutes of Health grants RR016023 (awarded to J. A. Roberts at the California National Primate Research Center), RR00169 and RR05090 (awarded to DGS) and a pilot project grant (awarded to SK) from the California National Primate Research Center.

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