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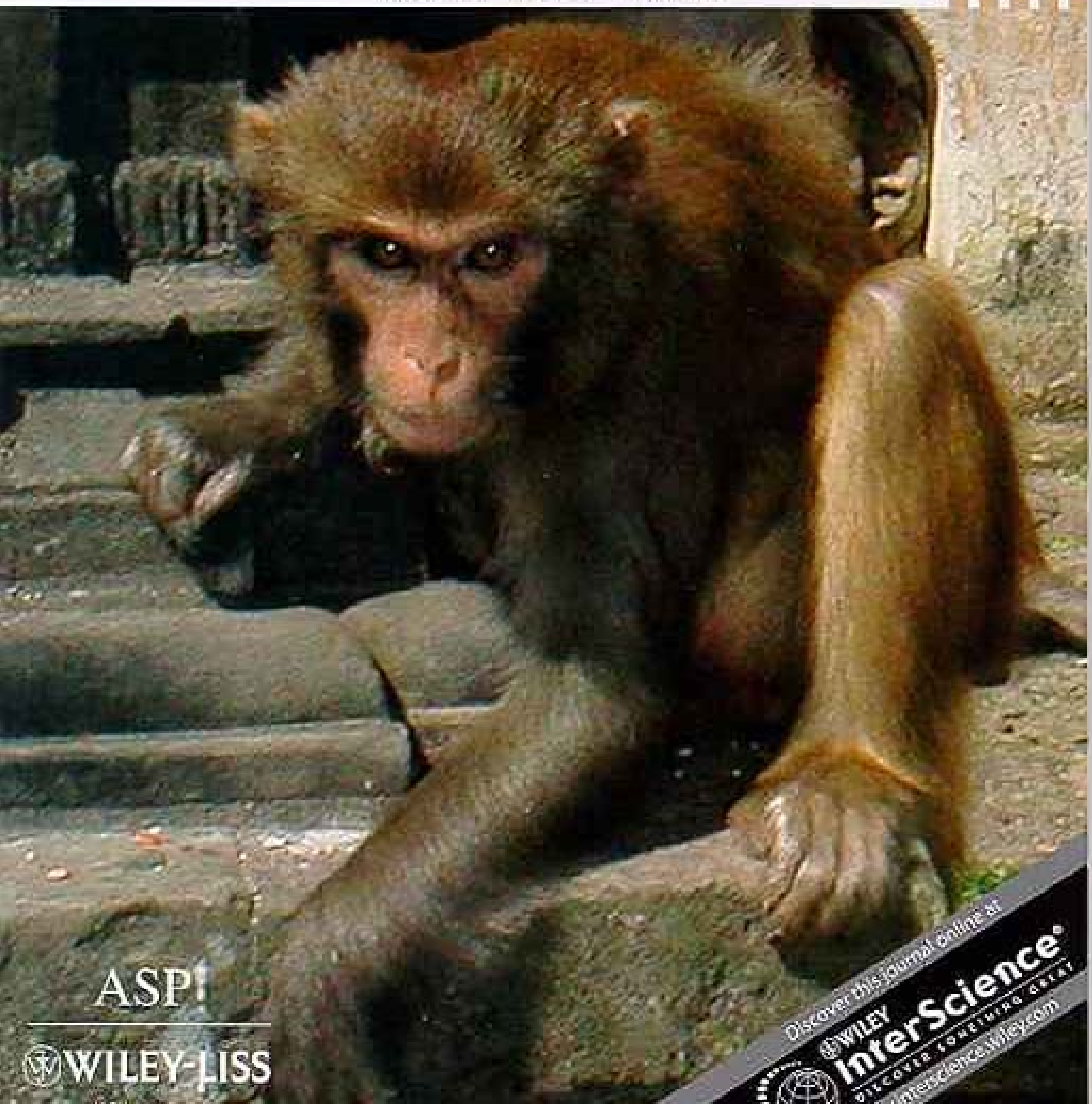

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RESEARCH ARTICLE

Genetic Characterization of Rhesus Macaques (*Macaca mulatta*) in Nepal

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Indian-origin rhesus macaques (*Macaca mulatta*) have long served as an animal model for the study of human disease and behavior. Given the current shortage of Indian-origin rhesus, many researchers have turned to rhesus macaques from China as a substitute. However, a number of studies have identified marked genetic differences between the Chinese and Indian animals. We investigated the genetic characteristics of a third rhesus population, the rhesus macaques of Nepal. Twenty-one rhesus macaques at the Swoyambhu Temple in Kathmandu, Nepal, were compared with more than 300 Indian- and Chinese-origin rhesus macaques. The sequence analyses of two mitochondrial DNA (mtDNA) loci, from the HVS I and 12 S rRNA regions, showed that the Nepali animals were more similar to Indian-origin than to Chinese-origin animals. The distribution of alleles at 24 short tandem repeat (STR) loci distributed across 17 chromosomes also showed greater similarity between the Nepali and Indian-origin animals. Finally, an analysis of seven major histocompatibility complex (MHC) alleles showed that the Nepali animals expressed Class I alleles that are common to Indian-origin animals, including Mamu-A*01. All of these analyses also revealed a low level of genetic diversity within this Nepali rhesus sample. We conclude that the rhesus macaques of Nepal more closely resemble rhesus macaques of Indian origin than those of Chinese origin. As such, the

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Key words: rhesus macaque; *Macaca mulatta*; Nepal; genetics; Indian-origin; animal model; AIDS

INTRODUCTION

For more than a half century the rhesus macaque (*Macaca mulatta*) has served an important role in the study of human disease. The rhesus macaque contributed to some of our early vaccine discoveries for viruses such as polio and yellow fever, and remains the primate model of choice for much of biomedical research–most recently as a model for human AIDS [Feinberg & Moore, 2002]. Historically, much of the groundbreaking work on AIDS has been done with rhesus macaques of Indian origin. There is, however, increasing concern over the critical shortage of Indian-origin rhesus macaques available for biomedical research [Cohen, 2000; Roberts et al., 2000]. This shortage has serious implications for the future of vaccine development and studies of disease pathogenesis, and as such has received considerable attention at the highest levels of government both in the United States and abroad [RRIC, 2003].

Although many researchers contend that rhesus macaques of Indian origin are better suited (genetically) for the study of certain viruses and diseases, including HIV/AIDS, there is increasing debate about the preferred status of the Indian- vs. Chinese-origin rhesus macaque as a model for AIDS. This issue is undoubtedly closely linked to the declining availability of Indian-origin animals [Ling et al., 2002]. In the wake of India's 1978 ban on the export of primates, China has emerged as the primary source of captive-bred rhesus macaques for biomedical research. Nevertheless, the demand for Indian-origin rhesus continues.

In an effort to extend the genetic characterization of rhesus macaques throughout their natural range and to identify additional resource options, we conducted a genetic study on a sample of rhesus macaques of Nepal. To our knowledge, this paper is the first to report on the genetic characterization of Nepali rhesus macaques and to assess their relatedness to Indian- and Chinese-origin rhesus.

MATERIALS AND METHODS

Study Site and Sample Collection

During May 2003 we collected blood samples from 39 rhesus macaques living at the Swoyambhu temple site in Kathmandu, Nepal (Fig. 1). The samples were obtained as part of a comprehensive health screening effort conducted at the request of the Federation of Swoyambhu Management and Conservation Committee. The Swoyambhu temple is located atop a hill in the western part of the city. The site covers an area of approximately 2.5 sq. km and supports seven to eight social groups of rhesus macaques, with an estimated population of 400 animals [Chalise & Ghimire, 1998]. For the past 40 years the site has remained effectively isolated as a small forest fragment, surrounded by the city and cut off from any significant animal migration. Prior to that time, there remained a substantial forest corridor that provided the macaques access to the surrounding

Fig. 1. Location of the Swoyambhu temple site in Kathmandu, Nepal, and approximate origin of the Indian and Chinese rhesus macaques. The Indian rhesus (or their ancestors) originated in or near the cities of Jammu (Kashmir region) and Lucknow, as well as in several other locations in north-central (N-C) India that are geographically intermediate between those two cities. The Chinese rhesus (or their ancestors) originated in primate breeding facilities in the western (Sichuan), southern (Guangxi and Guangdong), and eastern (Jiangsu) provinces of China.

forests of the Himalayan foothills. Today, the only routes of travel for monkeys that venture out of the Swoyambhu temple site are through the urban areas.

The monkeys sampled represented all age/sex classes (except infants) and included members from three social groups. The animals were trapped/darted and sampled following a protocol approved by the University of Washington Institutional Animal Care and Use Committee (3143-03). The monkeys were sedated with 3 mg/kg of Telazol® (tiletamine HCI/zolazepam HCL). Using universal precautions, approximately 7–10 ml of blood were drawn from the femoral vein of each animal. The macaques were monitored closely during anesthesia and recovery, and then returned to their groups. Approximately 7 ml of blood were placed in a separator tube and centrifuged in the field to extract the serum. The remaining whole blood was placed in an EDTA tube. Both whole

blood and serum samples were frozen and stored at −20°C prior to shipment to the WaNPRC. Following the issuance of all required international and government permits (i.e., from CITES and the CDC), samples were sent to the WaNPRC for further testing. A portion of the samples from 21 animals was then sent to the Molecular Anthropology Laboratory of the University of California–Davis for additional analysis.

To genetically characterize the rhesus macaques of Nepal and assess their phylogenetic relationship to rhesus of neighboring regions, we analyzed 21 of the Nepali rhesus for which we had adequate amounts of DNA. This subset represented each of the three social groups sampled. We then compared them with DNA from rhesus macaques of Indian and Chinese origin. The Indian and Chinese samples were acquired from primate centers in the United States and Europe, but the animals or their ancestors originated from several regions across India and China [Smith & McDonough, 2005] (see Fig. 1).

DNA Isolation and mtDNA Analysis

The DNA from the 21 Nepali rhesus was isolated from EDTA whole blood using a QIAmp Blood Mini Kit (Qiagen, Valencia, CA). As an initial assessment of genetic diversity within and between the rhesus macaque populations, mitochondrial DNA (mtDNA) sequences were PCR amplified, sequenced, and compared. Determining sequence diversity in mtDNA has been shown to be an efficient and effective means of distinguishing interpopulational differences among rhesus macaques [Kanthaswamy & Smith, 2004; Melnick et al., 1993; Smith & McDonough, 2005]. An 835 basepair (bp) fragment of mtDNA that includes hypervariable segment I (HVS I) was amplified using the following primers: forward 5′ CCGCCCACTCAGCCAATTCCTGTTCT 3′, and reverse 5′CCCGTGATCCATCGAGATGTCTT 3′. The thermocycler protocol was 94°C for 3 min, then 50 cycles at 94°C for 10 sec, 62°C (–0.1°C/cycle) for 20 sec, and 72°C for 20 sec, followed by a final extension at 72°C for 5 min. The PCR amplified products were sequenced in both directions on an ABI 3730 sequencer using the Big Dye Terminator Cycle Sequencing kit (version 3.1; Applied Biosystems, Foster City, CA). The 21 Nepali rhesus sequences were compared with 128 Indian and 158 Chinese rhesus sequences, most of which have already been described [Smith & McDonough, 2005]. The DNA sequences were aligned using Sequencher (Gene Codes Corp., Ann Arbor, MI). The mean numbers of (uncorrected) pairwise nucleotide differences within and between groups of samples were calculated using the ARLEQUIN package (version 2.001 [Schneider et al., 2000]). Transitions, transversions, and gaps associated with single-base indels were treated equally, except that all gaps in the poly C regions 15739–15741 and 15719–15721.1 were treated as missing data. Genetic diversity was expressed as nucleotide diversity (π), the average proportion of the 835 nucleotides studied that differed between paired samples. The GenBank accession numbers are as follows: HVSI Nepal 1-21 AY823276-96, 12S rRNA Indian1 AY794047, 12S rRNA Nepal1 AY794048, and 12S rRNA Chinese 1,2,3,5 AY794049-52.

To verify the initial mtDNA sequence diversity results, we also compared the mtDNA sequences at the 12S rRNA locus, using the same 21 Nepali rhesus and 12 additional rhesus macaques originating from India and China. These 12 animals (six unrelated Indian and six unrelated Chinese rhesus macaques) were housed at the Oregon National Primate Research Center. A 390 bp fragment of the 12S rRNA locus was amplified using the following primers: forward 5′- CAAACTGG-

GATTAGATACCCCACTAT- 3′, and reverse 5′-AGGGTGACGGGCGGTGTGT-3′. The reactions underwent 30 cycles at 94° for 45 sec, 55° for 45 sec, and then 72° for 1 min. The amplification products were purified from 2% agarose (QIAquick Gel Extraction Kit; Qiagen, Valencia, CA) and were then sequenced and aligned as described above.

STR Analysis

Next we compared the chromosomal DNA similarity among the Nepali, Indian, and Chinese populations by determining the genotypes and gene diversity (H) of short tandem repeat (STR) loci distributed across 17 chromosomes. The 21 Nepali, 185 Indian, and 205 Chinese samples were genotyped for 24 microsatellite (STR) loci, some of which have been previously reported [Kanthaswamy & Smith, 1997; Morin et al., 1997; Schneider et al., 2000; Smith et al., 2000]. The STR loci were as follows: D1s548, D2s1333, AGAT001, D3s1768, D4s1626, D5s1457, D6s501, D7s794, D7s1826, D8s1106, D8s1466, D9s921, D9s934, D10s1432, 271j8, D11s2002, AGAT007, D13s318, D13s765, D15s644, 270e8, D18s537, D18s861, and DXs2506. The touch-down PCR conditions for each STR locus are available upon request. Amplification products were run on an ABI 310 sequencer following the manufacturer's recommendations, and the Liz 500 (Applied Biosystems, Foster City, CA) size standards were included to establish allele size. The size, number, and frequency of alleles at each locus were compared within and between populations.

MHC Analysis

As a final test of genetic similarity, we analyzed the major histocompatibility complex (MHC) alleles in the 21 Nepali macaque samples. There is a growing body of literature showing that Indian and Chinese rhesus macaques differ in their distributions of Class I and Class II MHC alleles [Doxiadis et al., 2003; Muhl et al., 2002; Viray et al., 2001; Vogel et al., 1995]. In this study we sought to determine whether the Nepali rhesus macaques expressed any of the Class I alleles that are commonly found in Indian rhesus macaques. Of particular interest was Mamu-A*01, which has been found exclusively in Indian rhesus macaques and is associated with resistance to simian immunodeficiency virus (SIV) [Evans et al., 1999; Mothe et al., 2003; Pal et al., 2002]. The MHC alleles were amplified using site-specific primer (SSP) PCR conditions (W.M. Rehrauer, personal communication). The primer sequences were as follows: Mamu-A*01 5′-GACAGCGACGCCGCGAGCCAA-3′ and 5′- GCTGCAGCGTCTCCTTCCCC-3; Mamu-A*08 5′- TTGGGACCGGAACACACGGATCTA-3′ and 5′- TGCGCTGG GTGTTCTGAGCA-3′; Mamu-A*11 5′- CGGGGAGCCCCGCTTCTTCA- 3′ and 5′- CTCGCCCTCCAGGTAGGT- 3′; Mamu-B*01 5′- CAGCGACGCCGAGA GTCG- 3′ and 5′-CCGCGGCGGTCCAGGAGT-3′; Mamu-B*17 5′-GCCGGCTC GCACTCCATGAA-3′ and 5′-GCGCGCTGCAGCGTCTCC-3′; Mamu B*29 5′-GGCCGGAGTATTGGGAAGAGGAA-3′ and 5′-GGAGCGCAGGTCCTCGTT CAC-3′. The thermal cycling conditions were as follows: 1 min at 96°C, then five cycles at 96°C for 25 sec, 70°C for 50 sec, and 72°C for 45 sec; then 21 cycles at 96°C for 25 sec, 66°C for 50 sec, and 72°C for 45 sec; and finally four cycles at 96°C for 25 sec, 55°C for 60 sec, and 72°C for 60 sec. PCR products were separated on 2% agarose and monitored for the presence of the allele-specific product. The amplified products were isolated and sequenced as described above.

RESULTS

mtDNA

The sequences of the 835 bp mtDNA fragment containing the HVS I region were identical in all 21 Nepali animals. By comparison, the Indian rhesus sequences included 50 haplotypes that divided into two groups of closely related haplotypes, or haplogroups: Ind1 and Ind2. Almost 95% of the Indian rhesus macaques were classified as members of Ind1. There were 114 unique haplotypes among the Chinese rhesus that sorted approximately evenly into two haplogroups: ChiE and ChiW [Smith & McDonough, 2005]. A comparison of all of the animals analyzed showed that the Nepali sequence clustered within the middle of haplogroup Ind1. As a measure of sequence divergence, we calculated the average values of nucleotide diversity (π) using one representative of each unique haplotype in the four haplogroups. The degrees of divergence of the Nepali sequence from the Ind1, Ind2, ChiE, and ChiW sequences were 0.004, 0.087, 0.069, and 0.085, respectively. In contrast, the values of π between pairs within the Indian and Chinese haplogroups were 0.005, 0.007, 0.031, and 0.036, respectively. Thus, on average the Nepali mtDNA sequence was as similar to that of a randomly selected rhesus from haplogroup Ind1 as were two randomly selected Indian sequences within haplogroup Ind1. Figure 2 displays the results

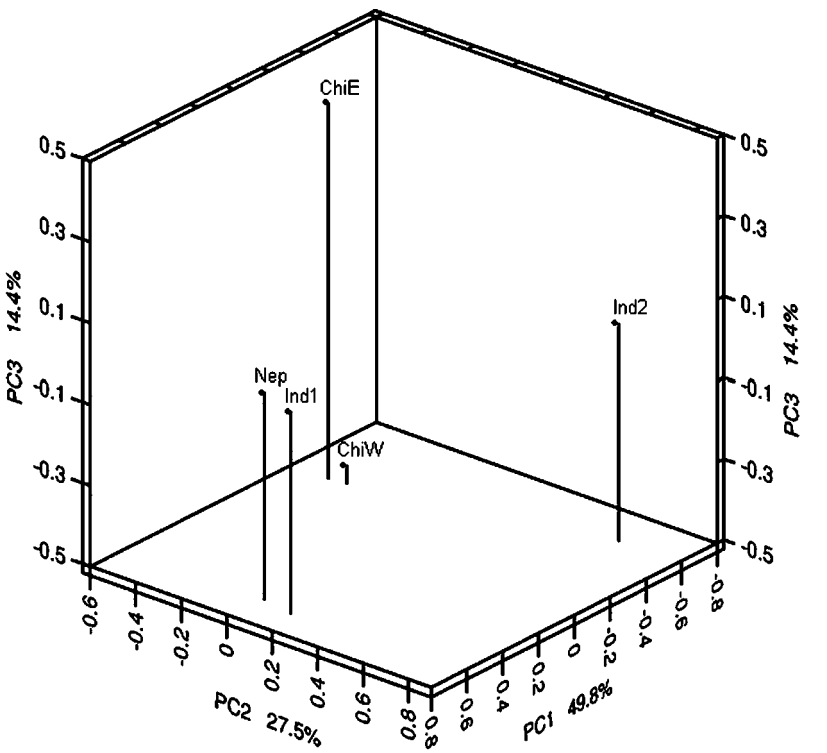

Fig. 2. PCA based on an 835-bp mtDNA sequence containing HVS I. The plot illustrates the similarity of the Nepali rhesus macaque sequence to sequences representing each of the four major haplogroups in Indian (Ind1 and Ind2) and Chinese (ChiE and ChiW) rhesus macaques. The first, second, and third coordinates explain 49.8%, 27.5%, and 14.4%, respectively, of the sequence variance.

Fig. 3. The 12S rRNA sequences for Indian (I1-6), Nepali (N1-21), and Chinese (C1-6) rhesus macaques. Base positions that differ from Nepali sequences are indicated in bold.

of a principal coordinates analysis (PCA) based on the genetic distance (F_{ST}) matrix compiled using these sequences in ARLEQUIN [Schneider et al., 2000]. The first, second, and third principal coordinates accounted for 49.8%, 27.5%, and 14.4%, respectively, of the genetic variance, and illustrate the close similarity between the Nepali and Indian (Ind1) sequences.

In the analysis of the 12S rRNA sequences, the Nepali and Indian sequences were found to be identical, while there were 10–12 bp differences between the Chinese and Nepali sequences (Fig. 3). The finding that both the HVS I and 12S rRNA sequences were identical among all 21 Nepali animals suggests a very low level of genetic diversity among these Nepali animals. These results also show that Nepali and Indian animals are more similar to each other than either is to Chinese rhesus macaques. Finally, the amount of sequence variation within the Chinese group is consistent with previous studies that reported greater subspecies diversity in Chinese rhesus macaques than in Indian rhesus macaques [Kanthaswamy & Smith, 2004; Smith & McDonough, 2005; Viray et al., 2001].

STR

The analysis of 24 polymorphic STR loci in the Nepali, Indian, and Chinese populations showed that the Nepali macaques exhibited the fewest number of alleles per locus (5.1 ± 2.0), and the lowest level of gene diversity ($H = 0.65 \pm 0.12$) of the three groups, perhaps due in part to the small sample size. The Chinese rhesus had the largest number of alleles per locus (14.8 ± 8.1) and the highest

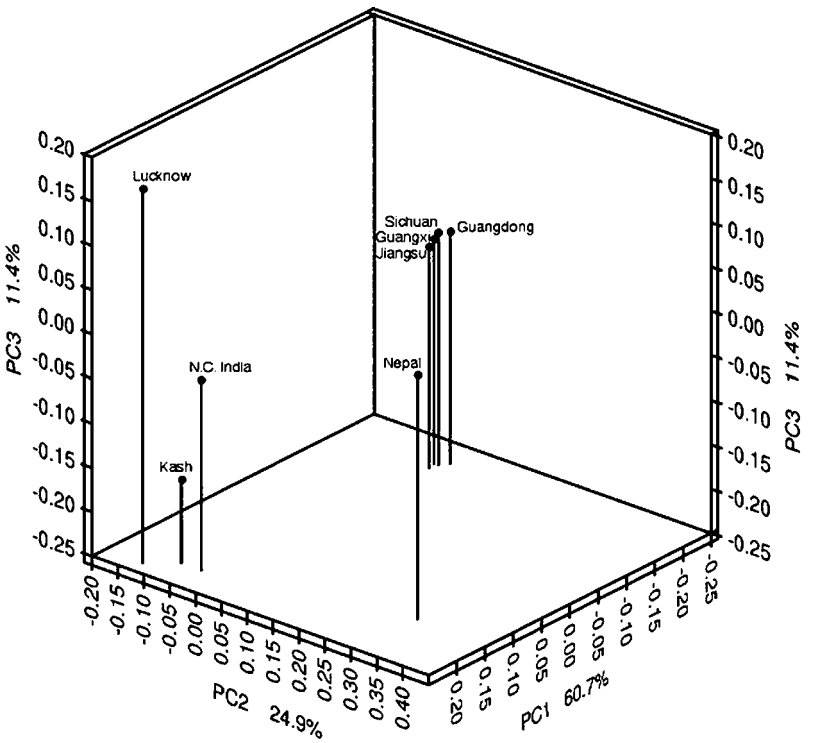

Fig. 4. PCA based on 24 nuclear STR loci. The plot illustrates the similarities between the gene frequencies of the Nepali rhesus macaques and those of rhesus macaques (or their ancestors) originating from three different regions of India and from breeding centers in four different provinces of China. The Kashmir and Lucknow samples, respectively, represent the westernmost and easternmost of the three Indian samples (N-C: north-central), while the Sichuan and Jiangsu samples respectively represent the westernmost and easternmost of the Chinese rhesus samples. The first, second, and third coordinates explain 60.7%, 24.9%, and 11.4%, respectively, of the genetic variance.

level of gene diversity ($H = 0.80 \pm 0.13$), with the Indian rhesus exhibiting a moderate level of both (8.3 ± 3.6; $H = 0.73 \pm 0.15$). Figure 4 illustrates a PCA of the F_{ST} distance matrix, also compiled in ARLEQUIN, based on genotypes for the 24 STR loci. The first, second, and third principal coordinates accounted for 60.7%, 24.9%, and 11.4%, respectively, of the genetic variance. As with the mtDNA data, the Nepali samples more closely resemble samples from India than those from China. While the first and most influential coordinate of the PCA differentiates the Chinese from other rhesus, the Nepali rhesus diverge from the Indian rhesus on the second coordinate. This result suggests that Chinese rhesus exhibit some alleles that may be absent in both Indian and Nepali rhesus, and that the frequencies of many alleles shared by Indian and Nepali rhesus differ.

MHC

Some MHC alleles have been found more often in Indian than in Chinese rhesus macaques, or exclusively in Indian macaques. For example, the Mamu-A*01 allele has been detected in 14–46% of Indian-origin rhesus macaques [Muhl et al., 2002; Vogel et al., 1995], but has been shown by multiple methods to be

TABLE 1. MHC Analysis in Nepali Rhesus Macaques

MHC allele	# Of animals (percentage) expressing allele
Mamu-A*01	7/21 (33%)
Mamu-A*02	ND
Mamu-A*08	7/20 (35%)[a]
Mamu-A*11	4/20 (20%)[a]
Mamu-B*01	6/20 (30%)[a]
Mamu-B*17	ND
Mamu-B*29	1/21 (5%)

[a]One animal was not analyzed due to insufficient DNA.
ND, not detected.

absent in Chinese rhesus [Muhl et al., 2002; Vogel et al., 1995]. In this study seven of the 21 Nepali rhesus, including animals from all three social groups, expressed Mamu-A*01 (Table 1). In addition, four other alleles that are commonly found in Indian rhesus (Mamu A*08, Mamu A*11, Mamu B*01, and Mamu B*29) were detected in the Nepali animals, while two others (Mamu-A*02 and Mamu-B*17) were not (Table 1). The total allele distribution among the Nepali animals did not show any common haplotypes. The presence of five MHC alleles that are typical of Indian rhesus further supports the proposition that Nepali- and Indian-origin rhesus macaques are genetically similar. The inability to detect the Mamu-A*02 and Mamu-B*17 alleles may reflect the small size of the Nepali sample, or it may be a consequence of the PCR methodology used. Strict sequence conservation in flanking primers is required for discrimination among the MHC alleles. Any divergence in the Nepali primer target-sequences of an MHC allele would dramatically decrease the annealing of PCR primers to the target-sequences of that allele, even if it were in fact present in the genome. Alternatively, a complete absence of the two alleles in other Nepali populations would distinguish Indian from Nepali rhesus macaques. To determine whether the Mamu-A*02 and -B*17 alleles are truly absent throughout the Nepali population, both a larger number of animals and the development of allele detection methods that do not require strict conservation of flanking sequences would be needed.

DISCUSSION

Based on a comparison of mtDNA sequences, genomic STR, and MHC allele frequencies, we conclude that the Nepali rhesus macaques included in this study are genetically similar to Indian-origin rhesus macaques. Although the Swoyambhu population represents a small, isolated sample, its geographic location within the country and previous opportunities for migration (gene flow) to and from surrounding areas suggest that this population may be a representative sample of Nepali rhesus macaques. As has been speculated, the differences between the Chinese-origin and Nepali/Indian-origin rhesus macaques are likely due to a geographic barrier (e.g., the Himalayas) inhibiting gene flow between the populations. However, the exact barrier is unclear [Smith & McDonough, 2005]. Plans are currently under way to extend genetic assessment of the rhesus population throughout Nepal.

The implications of these findings are of special significance to the biomedical research community, where the shortage of Indian-origin rhesus macaques is well documented [Cohen, 2000; RRIC, 2003; Roberts et al., 2000]. As indicated by the

results of this study, the rhesus macaques of Nepal may offer an additional resource option for researchers who wish to maintain research protocols with animals that possess key genetic features characteristic of Indian-origin rhesus macaques.

To ensure the conservation of Nepal's naturally occurring rhesus population, in 2003 the Nepali government enacted the Wildlife Farming, Breeding and Research Working Policy, which stipulates that only captive-bred animals may be used for scientific research. The Department of National Parks and Wildlife Conservation in Nepal also has suggested that rhesus macaques used to establish a captive breeding colony should be obtained from areas where there is ongoing conflict between the animals and the people (e.g., rural farming communities that frequently appeal to the government for assistance in relieving problems associated with the monkeys, such as crop raiding).

As part of a collaborative international program between the Nepal Biodiversity Research Society and the Washington National Primate Research Center, efforts are now under way to establish Nepal's first primate research center [Kyes & Chalise, 2002]. The center will be based on the principles of sustainable use outlined by the World Conservation Union [IUCN, 2000] and the national primate program concept initiated by the World Health Organization in 1981 [Kyes, 1993; Kyes et al., 1995].

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