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INTERSPECIES HYBRIDIZATION AND THE STRATIFICATION OF NUCLEAR GENETIC VARIATION OF RHESUS (*MACACA MULATTA*) AND LONG-TAILED MACAQUES (*MACACA FASCICULARIS*)

Sree Kanthaswamy^{1,2,*}, Jessica Satkoski¹, Debra George¹, Alexander Kou¹, Bethany Joy-Alise Erickson, and David Glenn Smith^{1,2}

¹Department of Anthropology, University of California–Davis, Davis, California

²California National Primate Research Center, University of California–Davis, Davis, California

Abstract

Genotypes for 13 short tandem repeats (STRs) were used to assess the genetic diversity within and differentiation among populations of rhesus macaques (*Macaca mulatta*) from mainland Asia and long-tailed macaques (*M. fascicularis*) from mainland and insular Southeast Asia. These animals were either recently captured in the wild or derived from wild-caught founders maintained in captivity for biomedical research.

A large number of alleles is shared between the two macaque species but a significant genetic division between them persists. This distinction is more clear-cut among populations that are not, or are unlikely to have recently been, geographically contiguous. Our results suggest there has been significant interspecies nuclear gene flow between rhesus macaques and long-tailed macaques on the mainland. Comparisons of mainland and island populations of long-tailed macaques reflect marked genetic subdivisions due to barriers to migration. Geographic isolation has restricted gene flow, allowing island populations to become subdivided and genetically differentiated. Indonesian long-tailed macaques show evidence of long-term separation and genetic isolation from the mainland populations, while long-tailed macaques from the Philippines and Mauritius both display evidence of founder effects and subsequent isolation, with the impact from genetic drift being more profound in the latter.

Keywords

Introgression; hybridization; microsatellites; gene flow; genetic structure

Introduction

Rhesus macaques (*Macaca mulatta*) and long-tailed macaques (*Macaca fascicularis*, also known as crab-eating or cynomolgous macaques) represent, respectively, the most and the second-most used non-human primate models for biomedical research in the US. Each species' suitability as a model in biomedical experimentation is influenced by its ancestry whether this genetic history is natural or anthropogenic (Kanthaswamy *et al.* 2006, Satkoski *et al.* 2007).

*Correspondence to: Sree Kanthaswamy, PhD, Department of Anthropology, Young Hall, University of California, Davis, CA 95616, USA, Phone: 530-752-6665, Fax: 530-752-8885, Email: skanthaswamy@ucdavis.edu.

Population level genetic differences are the major source of variation among research subjects, which influences the repeatability of experimental results. The reduction of genetic variation permits the use of fewer animals and cost-efficient research programs. For these reasons, Smith and McDonough (2005), Satkoski *et al.* (2007) and Kanthaswamy *et al.* (submitted) have all argued that the significant genetic homogeneity of the Indian rhesus macaques makes them more appealing for types of research for which the Chinese origin animals are not considered more appropriate for other reasons (Joag *et al.* 1994, Cohen 2000, Ling *et al.* 2002). Similarly, long-tailed macaques from Mauritius have been recommended for use as animal models for infectious diseases (Krebs *et al.*, 2005) because they exhibit an extreme level of genetic homogeneity of certain class I MHC genes that influence immune response. However, few studies have been conducted of levels of genetic diversity in regional populations of long-tailed macaques. If captive groups of long-tailed macaque exhibit as much intra- and inter-population genetic heterogeneity as captive Indian and Chinese rhesus macaques, their genetic background, as reflected by their ancestry, may also affect their suitability for some types of research. In addition, such studies inform the evolutionary history of the species.

The distribution of the long-tailed macaque is primarily confined to the regions south of the subtropical and temperate geographic range of the rhesus macaque, although a small region of parapatry occurs in northern Indochina (Fittinghoff and Lindberg 1980, Fooden 1995, 1997 Groves 2001; see Fig. 1). Fooden (1995) and Groves (2001) used morphometric measurements to delineate the inter-specific boundaries of rhesus and long-tailed macaques in the South and Southeast Indo-Malayan regions. The wide assortment of morphometric differences coupled with the broad geographic distribution of rhesus and long-tailed macaques fosters an expectation of a high level of genetic diversity within and between these species.

Investigations by Andrade *et al.* (2004), Doxiadis *et al.* (2003, 2006), Ferguson *et al.* (2007), Hernandez *et al.* (2007), Kanthaswamy and Smith (1998, 2004), Kanthaswamy *et al.* (2006), Malhi *et al.* (2007), Morin *et al.* (1997), Penedo *et al.* (2005), Satkoski *et al.* (2007), Smith *et al.* (2006) and Viray *et al.* (2001) have all characterized the genetic composition of rhesus macaque populations in the wild and in captivity. Most of these studies have reported discrete genetic differences between Indian and Chinese rhesus macaques and much greater genetic heterogeneity and substructuring in the latter than in the former. Though some, but not all, of the taxonomy based on morphology is reflected in these genetic studies, caution should be exercised in drawing inferences about the evolution of genus *Macaca* from morphology alone. Furthermore, while rhesus macaque population genetics has been extensively described, much less is known about the genetic differentiation of the long-tailed macaques or how this diversity is stratified within the species.

The macaques originated in northeastern Africa some 7 million years ago (mya) and had spread through most of continental Asia by 5.5 mya. They are subdivided into four groups of species (Fooden 1976, Delson 1980) to one of which, the *fascicularis* group, both rhesus (*M. mulatta*) and long-tailed (*M. fascicularis*) macaques belong. The earliest split in genus *Macaca* was probably between an ancestor of the *silenus*-group of macaques [which include lion-tailed (*M. silenus*) and pig-tailed (*M. nemestrina*) macaques] and a *fascicularis*-like ancestor, some 4–5 mya, from which all non-*silenus* species evolved later. The species of the *fascicularis*-group share a common ancestor that lived about 2.5 mya. Several researchers agree that long-tailed macaques are the most plesiomorphic taxon within the *fascicularis*-clade (Delson 1980, Zhang and Shi 1993, Melnick *et al.* 1993, Hayasaka *et al.* 1996, Morales and Melnick 1998, Tosi *et al.* 2000, 2002 and 2003). Therefore, it is argued that rhesus macaques probably emerged from a *fascicularis*-like ancestor that reached the mainland from its homeland in Indonesia approximately 1.0 mya (Tosi *et al.* 2003).

While natural populations of long-tailed macaques were widely distributed throughout insular Southeast Asia by early Pleistocene times, the distribution of rhesus macaques in the Indian subcontinent stemmed from a more recent east to west colonization from a Pleistocene refugium after the species' spread eastwardly into China and mainland Southeast Asia (Fooden 1995, Smith and McDonough 2005). This divergence between the western (i.e., Indian) and eastern (i.e., Chinese) rhesus macaques has been estimated to have occurred approximately 170,000 years ago (Hernandez *et al.* 2007) during the penultimate glacial maximum when sea levels were near their lowest point.

The rhesus and long-tailed macaques form paraphyletic taxonomic groups with the other species of the *fascicularis*-group of macaque species. For example, the mtDNA of Chinese rhesus macaques more closely resembles that of *M. cyclopis* and *M. fuscata* than that of the Indian rhesus macaque with which it is conspecific (Zhang and Shi 1993, Melnick *et al.* 1993, Morales and Melnick 1998, Tosi *et al.* 2003, Smith *et al.* 2007), suggesting that species distinctions currently made within the *mulatta* group of macaques may not completely reflect the close relationships between taxa. Moreover, Marmi *et al.* (2004) proposed that the taxonomy of the *fascicularis*-group of macaque species be revised to include one subclade comprising Chinese rhesus macaques, Japanese macaques and Taiwanese macaques and a second subclade including only Indian rhesus macaques.

Natural hybridization has been reported among *Macaca* species (Fooden 1964, 2000, Bernstein 1966, Supriatna *et al.* 1992, Froehlich *et al.* 1996, Bynum *et al.* 1997, Evans *et al.* 2001; Fooden 1964, 2000; Froehlich *et al.* 1996; Supriatna *et al.* 1992; Tosi *et al.* 2002, 2003). Sympatric hybridization occurs where the geographic ranges of *M. nigra*, *M. brunnescentis*, *M. hecki*, *M. maura*, *M. nigrescens*, *M. ochreata* and *M. tokeana* overlap on the Indonesian island of Sulawesi (Ackermann *et al.* 2006). Parapatric interbreeding between rhesus and long-tailed macaque troops in Indochina where they co-occur has been suspected (Fooden 2000, Tosi *et al.* 2002, Hamada *et al.* 2004, 2006, Malaivijitnond *et al.* 2008). Based on relative tail-length, Fooden (2000) reported a probable rhesus-long-tailed macaque hybrid zone in northern Thailand between latitudes 15–20° N. Tosi *et al.* (2002) hypothesized that during the Pleistocene, long-tailed macaques in Indochina experienced male-mediated gene flow from rhesus macaque groups. Sequences from non-recombining fragments on the primate Y chromosome also have shown that long-tailed macaque populations in northern Thailand form a monophyletic group with rhesus macaques from India, China and Burma (Tosi *et al.* 2002). This is supported by Hamada *et al.*'s (2004, 2006) observation that northern Thai rhesus macaques have tail length, body size and mass measurements and a pelage coloring intermediate between those of Indian and Chinese rhesus macaques and those of parapatric long-tailed macaques. The greater similarity of Thai rhesus macaque blood group profiles and mtDNA haplotypes to those of parapatric long-tailed macaque than to those of Indian or Chinese rhesus macaques, however, suggests that Indochinese rhesus macaques are in fact products of hybridization events between adjoining populations of rhesus and long-tailed macaques in that region (Hamada *et al.* 2004, Malaivijitnond *et al.* 2008). Street *et al.* (2007) have suggested that the evolutionary divergence of the rhesus and long-tailed macaques may be even more recent than previously deduced by morphological and mtDNA studies because of the sharing of over half the SNPs in the *Macaca* nuclear coding regions.

Intentional admixing of regional populations bred in captivity has been reported at least in rhesus macaques. Satkoski *et al.* (2007) showed that the mitochondrial genome of free-ranging rhesus macaques in China were genetically subdivided with an east/west cline but the nuclear and mitochondrial genomes of captive Chinese animals bred in geographically dispersed Chinese breeding centers exhibited no clinal distribution. Kanthaswamy *et al.* (in press) reported the development of Chinese-Indian rhesus hybrids at the California National Primate Research Center in Davis, CA. Thus far, there are no reports of production of intentional rhesus-

long-tailed hybrid animals in the US. Whether hybridization occurs naturally or is mediated by human activity, the tasks of (1) quantifying this phenomenon between genetically and geographically distinct congeneric and conspecific populations, (2) developing species-diagnostic markers permitting rapid identification of inter-specific hybrids, (3) assessing the genetic properties of hybrid populations, and (4) developing genetic methods to conclusively identify admixed animals in captive colonies will become crucial future objectives in the field of biomedical research.

By ascertaining the magnitude and spatial distribution of genetic diversity, our study aims to identify genetically distinct and adjoining populations of rhesus and long-tailed macaques in the wild, and to detect evidence of historical or artificial interbreeding between regional populations. We used nuclear data from 13 highly polymorphic short tandem repeat (STR) loci to compare and apportion genetic diversity among a sample of rhesus and long-tailed macaques derived from a broad sampling of isolated and contiguous geographic populations, and to estimate past admixture among subpopulations from the Indochinese hybrid zones.

Methods and Materials

We analyzed DNA samples from 414 rhesus and long-tailed macaques. These animals originated in eight distinct subpopulations across mainland Asia and insular Southeast Asia. All were purchased from various commercial suppliers and/or bred in captivity and descend from wild-caught founders. Proveniences represented by these samples, which for some long-tailed macaque samples are known only to the level of country of origin, are described in more detail in Smith and MacDonough (2005) and Smith et al. (2006, 2007) and tabulated here in Table I. A complete description of the DNA extraction and PCR protocols for the 13 STRs (D1s548, D3s1768, D4s1626, D51457, D6s501, D8s1106, D8s1466, D9s921, D9s934, D10s1432, D13s765, D13s318 and AGAT007) employed here are provided in the citations listed above. The ancestry of the Philippine and Indochinese long-tailed macaques sampled is from Mindanao and Vietnam, respectively, but the precise origin of the Indonesian samples is not known with certainty. Nevertheless, the samples are widely distributed throughout the natural range of the species.

We used the genotypic data collected from the 277 rhesus and 137 long-tailed macaques to compute observed and expected heterozygosities, hierarchical F-statistics (Wright 1951 and 1978) and pairwise F_{st} (Weir and Cockerham 1984) using the GENEPOP (version 3.4, Raymond and Rousset 1995) and the PopGene (version 1.32, Yeh and Boyle 1997) software. Nei's (1987) genetic identity (I) measurements were used to estimate pairwise genetic distances, where Nei's standard distance, $D = -\ln I$.

We used the GENEPOP program to test for the presence of linkage disequilibrium (LD, or the non-random association of genotypes occurring at different loci) among the 13 loci, using the exact probability test. The null hypothesis is that genotypes at one locus segregate independently of genotypes at the other unlinked locus. We created unbiased estimates by randomization (1,000 iterations) and used the Markov-chain method to construct a contingency table representing random association of genotypes at all possible pairs of loci. We used this table to test for significant association at the 0.01 level of probability.

As depicted in Table I, the number of animals sampled from each population was highly variable, ranging from 13 to 177. Because differences in sample size can affect allele representation and estimates of genic variation (particularly due to the presence or absence of rare alleles), population genetic parameters were also recalculated using up to 20 randomly selected animals from each population (Table I).

We computed the effective number of migrants (N_m) between sampling sites using the non-hierarchical rare allele method (Slatkin 1985) implemented in the GENEPOP program (Raymond and Rousset 1995), which assumes migration-drift equilibrium. Here, estimates of 0.25, 0.5, 1 and 2 reflect one migrant every fourth generation, one migrant every second generation, one migrant per generation and two migrants per generation, respectively. Estimates of gene flow between pairs of mainland and insular groups were also calculated from the F_{st} values according to Wright's (1978) hierarchical methods. Both Wright's (1978) and Slatkin's (1985) applications can be used to detect subtle differences in population structure and to yield reasonably accurate estimates of gene flow under different conditions (Slatkin and Barton 1989). Gene flow among populations was estimated for the entire sample, separately for each species of macaques and also for all paired combinations of populations. Of course, such estimates are artificial in that true gene flow is seldom uniform over time or space and are used here only to capture the accumulated influences of gene flow for comparison among populations sampled.

Based on the allele frequencies, we used the program STRUCTURE 2.1 (Prichard *et al.* 2000) to characterize the genetic structure among the eight rhesus and long-tailed macaque populations. We conducted this analysis at sweeps of 10^3 reiterations after a burn-in period of 10^3 with and without *a priori* population information. It was assumed that allele frequencies among regional populations are correlated. When the assignment of individual animals was conducted with prior population information it was assumed that although an animal had a high probability of belonging to its assigned population, it could have ancestors in other populations. To avoid biases from the difference in sample sizes, we ran the STRUCTURE analysis with equal numbers (up to 20) of animals selected randomly from each population. Similarly, we performed the principal component analyses (PCAs) on a data set containing the 20 random samples using the adegenet 1.1 package for R (Jombart 2008).

Results

None of the loci assayed, including the syntenic ones D8s1106; D8s1466 (on rhesus Chr 8), D9s921; D9s934 (Chr 15) and D13s765; D13s318 (Chr 17), showed any statistically significant linkage at the $p < 0.01$ level.

Considerable genetic diversity in these populations was suggested by values of observed (H_o) and expected (H_e) heterozygosity ranging from 0.59 to 0.78 and 0.64 to 0.83, respectively (Table I). The actual (n_a) and effective (n_e) numbers of alleles ranged from 4.6 and 3.0 (Mauritian long-tailed macaques) to 10 and 6.2 (Chinese rhesus macaques; Table I). The rhesus-wide values of n_a and n_e were 12.2 and 6.2 while the long-tailed macaques cumulatively exhibited 17.1 and 7.4 actual and effective alleles, respectively. Mainland populations of rhesus and long-tailed macaques exhibited numbers of actual and effective alleles (13.4 and 7.0, respectively) comparable to those for the insular populations of long-tailed macaques (14.2 and 7.0, respectively).

Pairwise within-species F_{st} values ranged from 0.234 (between the Mauritian and Indonesia long-tailed macaques) to 0.014 (between the Chinese and Burmese rhesus macaques (Table I). Between-species pairwise F_{st} estimates ranged from 0.03 for Chinese rhesus macaques and Vietnamese long-tailed macaques, (lower than for all within-species F_{st} values except that between Chinese and Burmese rhesus macaques), to 0.216 for Nepalese rhesus macaques and Indonesian long-tailed macaques. Unbiased pairwise genetic identity (I , Nei 1987) between populations closely concurred with, and was inversely proportional to, the pairwise F_{st} estimates (Table I). Population-specific mean F_{st} , i.e., the average coefficient of coancestry among individuals within a population, given in Table II, corroborated the results based on heterozygosity estimates. The F_{is} , F_{it} and F_{st} estimates among the rhesus macaque populations

were 0.04, 0.110 and 0.069 and for the long-tailed macaques were 0.037, 0.171 and 0.140, respectively.

Estimates of F_{is} , F_{it} and F_{st} among all mainland macaques regardless of species affiliation were 0.039, 0.109 and 0.073 while values of 0.041, 0.188 and 0.153 were obtained when only the insular long-tailed populations were considered. Hierarchical differentiation among all macaque populations was $F_{is} = 0.040$, $F_{it} = 0.165$ and $F_{st} = 0.130$, respectively.

Using Slatkin's (1985) method, a migration rate of 1 migrant per generation was estimated among all populations of macaques studied here (Table I). Among rhesus macaques a migration rate of 7.2 was estimated and among long-tailed macaques a 10-fold lower rate of 0.7 was estimated. A mainland gene flow estimate of 6.9 among rhesus and long-tailed populations was obtained compared to an estimate of 0.6 migrations among the insular populations of long-tailed macaques. This is consistent with the greater differentiation suggested by the higher F_{st} estimates for insular long-tailed macaque populations.

With Wright's (1978) method, comparable trends in migration patterns were obtained but the number of migrants were different. Differences in estimates using the two methods have been described by Wolf and Soltis (1992). Among all mainland macaque populations a value of 3.4 was estimated when the Vietnamese animals were included, compared to 3.2 migrants per generation when they were excluded. These mainland figures were only twice as great as the number of animals moving among insular populations of long-tailed macaques with (1.5) and without (1.7) the inclusion of the Vietnamese long-tailed macaques.

The STRUCTURE analysis shows that the 13 cross-specific STR loci used here detect with equal robustness Chinese and Indian rhesus macaques of mixed ancestry that Satkoski *et al.* (2007) identified with a much larger panel of markers. Clustering patterns indicate that the Chinese and Burmese rhesus macaques vary more in their assignment probabilities than any of the other populations of macaques, regardless of species (Table II, Fig. 2 and Fig. 3). To avoid sample size biases, assignments involving randomly selected animals (Fig. 3) confirmed the genetic structure inferred from the entire sample set (Fig. 2). Patterns of well-differentiated Chinese and Indian rhesus macaques, as well as the suspected migrants and admixed animals in the Chinese-Burmese-Vietnamese cluster of animals observed in Fig. 2 are reproduced in Fig. 3.

The PCA (Fig. 4) indicates a clinal distribution of variation among the mainland and insular macaques from India, Nepal, China, Burma and Vietnam along PC1, while PC2 demonstrates the substantial differentiation of the Indonesian animals.

Discussion

The long-tailed macaques from Mauritius and the Philippines are more genetically homogenous than, and differentiated from, other groups. Nearly half the Philippine long-tailed macaque matriline studied by Smith *et al.* (2007) belonged to a single haplogroup, implying not only a low number of founders but also low levels of genetic variation introduced into the archipelago by founding animals. The Philippine animals may represent a remnant of ancient dispersals from continental Asia that is now confined to the outer limits of the species' natural geographic range. The Mauritian animals, purportedly introduced from Java as recently as the seventeenth or eighteenth centuries (Sussman and Tattersall 1981, 1986), reflect strong founder effects by exhibiting approximately one fourth of all the alleles found in long-tailed macaques and in both macaque species, respectively. The low Mauritian diversity at STR (as observed in this study as well as that by Bonhomme *et al.* 2008), MHC (Kawamoto *et al.* 2008) and mtDNA (Smith *et al.* 2007) loci and the absence of Y chromosomal variation (Tosi and Coke 2007, Kawamoto *et al.* 2008) have been attributed to small founder representation and

subsequent rapid population expansion (Smith *et al.* 2007, Kawamoto *et al.* 2008, Bonhomme *et al.* 2008). In contrast to the findings here that the Mauritian and Indonesian animals are indeed genetically distinct, Bonhomme *et al.* (2008) and Kawamoto *et al.* (2008) reported a higher degree of similarity between the Mauritian and Javan animals suggesting that the founders of the former population actually descend from Javanese ancestors. An Indonesian homeland for the Mauritian long-tailed macaques is also supported by the presence of the Indonesian MHC class I Mafa-B*4501 and Mafa-B*5101 alleles in these animals (Weisman *et al.* 2007). However, Tosi and Coke (2007) suggest Sumatra as an alternate source for Mauritian long-tailed macaques because these macaques exhibit the otherwise uniquely Sumatran combination of mitochondrial DNA that is shared with insular populations and Y-chromosome DNA shared with mainland populations. The study of additional genetic polymorphisms, such as SNPs, will be required to conclusively identify the true origin of Mauritius long-tailed macaques.

Estimates of gene flow and genetic subdivision suggest the extreme mutual genetic isolation among the insular (long-tailed macaque) populations and imply that the occurrence of inter-specific gene flow among the mainland macaque populations cannot be discounted. Inter-population migration estimates inferred from these values are much higher for the mainland than for the insular locations. While gene flow undoubtedly varied considerably through time as changes in sea levels and other barriers occurred, this asymmetry in gene flow between mainland and insular populations is probably predominantly a consequence of differences in the topography of both locations between the last glacial maxima, approximately 20 kya, and the post glacial high-stand about 7 kya. As a result, the distribution of genetic diversity of the mainland macaques is more geographically complex than the insular macaques where the diversity is apportioned according to the naturally discontinuous terrain.

Long-tailed macaque populations, especially those in insular Southeast Asia, exhibit greater levels of genetic differentiation than rhesus macaque populations. Random genetic drift among geographically isolated long-tailed populations is the most likely cause of the differentiation among the mainland, Philippine and the Indonesian populations. Within Indonesia, Kawamoto *et al.* (1984) has attributed the elevated differentiation between, and reduced variation within, island populations to heterogeneity decay resulting from the absence of gene flow. Our gene flow estimate of 0.6 migrants per generation among all insular populations of long-tailed macaques is consistent with their conclusion.

Differentiation among the semi-isolated populations of rhesus macaques may also be attributed to the weakened effect of drift due to migration ($F_{st} = 0.07$). Slatkin (1981) considers values of Nm of less than one to be low because significant population differentiation can still occur through drift, but Allendorf (1983) argues that even at migration rates as low as 2 migrants per generation a considerable scope for drift-driven genetic divergence still exists. Approximately seven migrants per generation among rhesus macaques (7.2) and among all mainland populations (6.9), respectively, should be easily sufficient to prevent dramatic differentiation via drift, but estimates of 0.7 among all long-tailed macaques may not match drift's force to differentiate populations. Therefore, a combination of distance-limited dispersal in the mainland regions and geographic barriers to migration in the insular regions is probably responsible for the genetic structure of the rhesus and long-tailed macaque populations.

Although we observed high levels of nucleotide diversity in Indonesian and Vietnamese long-tail macaques (as did Street *et al.* 2007), different causes may be responsible. Smith *et al.* (2007) observed a much higher level of mtDNA variation in Indonesian than in Vietnamese animals. The Indonesian long-tailed macaque population also appears in a well-defined cluster that is genetically quite distinct from all the other populations. These observations are

compatible with an insular Southeast Asian origin of long-tailed macaques and the subsequent spread throughout the region.

The idea of the long-tailed macaque's emergence out of Indonesia, and as Delson (1980) claimed, specifically from the island of Java, is concordant with the occurrence of 10 macaque species within Indonesia (Fooden 1995, Groves 2001, Abegg and Thierry 2002), although most of these species inhabit Sulawesi and belong to the *silenus* group of macaque species. The gradual loss of genetic diversity might have occurred as new areas on the mainland were colonized by the species. However, Tosi *et al.*'s data (2003), along with that of Street *et al.* (2007) and Bonhomme *et al.* (2008) imply that the long-tailed macaques in Indochina represent a very old population because of the large number of private alleles it retains, supporting the alternative hypothesis that the long-tailed macaques originated instead in Indochina. The fractured landscape of the Indonesian archipelago, rife with significant physical barriers that isolate wildlife populations, might then have caused the Indonesian macaques to diverge into new subspecies. Therefore, based on this logic, Indonesia is not the source of the *fascicularis* diaspora.

Our Vietnamese long-tailed macaque sample contains individuals that were identified as partially Chinese rhesus macaque and partially Vietnamese long-tailed macaque, representing potential inter-specific hybridization. It is unlikely that this results from intentional inter-species cross-breeding in captivity, because phenotypic effects of such necessarily recent inter-species cross-breeding are visible and there is no commercial market for such animals. Natural introgressive hybridization with rhesus macaques could have inflated local genetic diversity of one or both mainland populations, in which case inferring a mainland homeland for long-tailed macaques or hypothesizing demographic events to explain the higher genetic diversity in Chinese than in Indian rhesus macaques based on their high level of genetic diversity might be unwarranted. The high proportion of allele sharing between the Vietnamese long-tailed and Chinese rhesus macaques could either reflect recent gene flow or the retention of variants shared before the genetic separation of both species. In the present study, Vietnamese long-tailed macaques more closely resembled Chinese and Burmese rhesus macaques than their own conspecifics in insular Southeast Asia.

Street *et al.* (2007) also noted that the long-tailed macaques shared a greater number of SNPs with the Chinese rhesus macaques than with Indian rhesus macaques suggesting that introgression occurred after the divergence of Indian and Chinese rhesus macaques, or after approximately 170,000 years ago (Hernandez *et al.*, 2007). Street *et al.*'s (2007) finding that >50% of the SNPs observed in long-tailed macaques are also found in rhesus macaques but that only a third of the rhesus SNPs are shared between the Indian and Chinese rhesus macaque populations is congruent with unrestricted gene flow between both species of macaques. The estimate of gene flow among populations of macaques from China, Burma and Vietnam was more than twice that obtained for macaques from India, Nepal and Vietnam. Therefore, results from this study suggest that there has been natural or human-assisted hybridization between long-tailed macaques and rhesus macaques. Studies of Y-chromosome haplotypes (Tosi *et al.*, 2002) and STR loci (Bonhomme *et al.*, 2008) provide evidence that inter-species gene flow was unidirectional, with introgression of rhesus macaque DNA into the long-tailed macaque genome. Thus, the high level of genetic heterogeneity in mainland long-tailed macaques might not reflect a mainland origin for the species. In addition, a population bottleneck in Indian rhesus macaques and a population expansion in Chinese rhesus macaques probably explains the higher level of genetic diversity in the latter than the former population, as hypothesized by Hernandez *et al.* (2007).

Because suitable rhesus macaque habitats in India and China are not geographically contiguous, gene flow between Chinese and Indian populations at an estimated rate of three

animals per generation might be artificially high due to anthropogenic factors. Burma, however, might represent a natural route for gene flow between Chinese and Indian rhesus macaques. Smith and McDonough (2005) have argued that the Indian mtDNA haplogroup IND2, which comprises between 5% and 10% of mtDNA genomes of Indian rhesus macaques, is closely related to the Burmese mtDNA haplogroup BURM, suggesting gene flow from Burma to India. The fact that the observed and expected heterozygosity values estimated here significantly differed in this population also support the idea that this region may represent a conduit for gene flow between India and China. The heterozygote deficit within Burma may also be indicative of the presence of local inbreeding and geographical population structuring (Weir 1996). As for the Chinese and Burmese rhesus macaques, the Indian and Nepalese rhesus macaques form a unique clade, suggesting that genetic exchange occurs between these populations. Kyes *et al.* (2006) also showed a close relationship between the Nepalese and Indian populations but noted that Nepalese animals were highly homogeneous as observed in the present study.

Given the degree of inter-species gene flow estimated here, hybrid individuals have probably been introduced into the captive gene pools in the US. Being of intermediate genetic composition, admixed animals may respond very differently to experimentation involving traits which have a phenotypic variance that is lower than either unmixed parent population, making the inter-specific hybrids either a boon as an untapped biomedical research stock as we have previously argued for Chinese-Indian rhesus macaque hybrids (Kanthaswamy *et al.*, in press) or a bane if animals differ substantially in terms of their ancestry and their genetic composition cannot be characterized. Attention should be given to comparing results of experiments using Indochinese and non-Indochinese long-tailed macaques as subjects to assess possible difference in their phenotypic responses.

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Fig. 1. Geographic range of rhesus (dark grey) and long-tailed (light grey) macaques based on Groves (2001).

Fig. 2.

Assignment results of the STRUCTURE analysis based on the entire sample. (The putative countries of origin: 1 – India, 2 – Nepal, 3 – China, 4 – Burma, 5 – Vietnam, 6 – Mauritius, 7 – Philippines, 8 – Indonesia).

Fig. 3. Assignment results of the STRUCTURE analysis based on up to 20 random samples from each population. (The putative countries of origin: 1 – India, 2 – Nepal, 3 – China, 4 – Burma, 5 – Vietnam, 6 – Mauritius, 7 – Philippines, 8 – Indonesia).

Fig. 4. Results from the PCA analyses (The percentage of variation explained by PC 1 and 2. The corresponding first and second eigenvalues are 0.466 and 0.404, respectively).

Table 1

Estimates of actual and effective allele numbers (na and ne based on up to 20 random animals), observed (Ho) and expected (He) heterozygosity are in the top 4 rows. Number of migrants per generation are above diagonal (where [migration rate, $m = (1-F_{st})/2F_{st}$]) and below diagonal are Nei's (1987) unbiased genetic identity values and pairwise F_{st} (estimated as $F_{st}/[1-F_{st}]$ is underlined).

Indian rhesus macaque (N = 69)	Nepalese rhesus macaque (N = 18)		Chinese rhesus macaque (N = 177)		Burmese rhesus macaque (N = 13)		Vietnamese long-tailed macaque (N = 40)		Mauritian long-tailed macaque (N = 26)		Philippine long-tailed macaque (N = 37)		Indonesian long-tailed macaque (N = 34)		
	na	ne	na	ne	na	ne	na	ne	na	ne	na	ne	na	ne	
7.2	4.3	5.1	10.0	6.2	7.2	4.8	8.7	5.4	4.6	3.0	5.8	3.6	8.2	4.7	
Ho	He	Ho	He	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	
0.74	0.78	0.75	0.70	0.83	0.60	0.76	0.77	0.80	0.59	0.64	0.66	0.70	0.77	0.80	
-	1.588		3.329		1.217		1.097		0.419		0.553		0.417		Indian rhesus macaque
0.7682	<u>0.0697</u>	-	1.635		0.802		1.004		0.267		0.362		0.429		Nepalese rhesus macaque
0.7668	<u>0.0528</u>	0.6885	0.0823		2.162		2.801		1.114		1.654		0.356		Chinese rhesus macaque
0.6840	<u>0.0758</u>	0.6654	<u>0.0992</u>	0.8987	0.0139		1.221		0.581		1.004		0.610		Burmese rhesus macaque
0.6450	<u>0.0857</u>	0.6250	0.1067	0.8516	0.0297	<u>0.0491</u>	-		1.331		1.439		0.492		Vietnamese long-tailed macaque
0.4768	<u>0.1748</u>	0.5009	<u>0.1971</u>	0.5723	<u>0.1310</u>	<u>0.1487</u>	0.5830	<u>0.1432</u>	-		0.473		0.349		Mauritian long-tailed macaque
0.5282	<u>0.1382</u>	0.5036	<u>0.1701</u>	0.7041	<u>0.0804</u>	<u>0.0831</u>	0.7527	<u>0.0831</u>	0.5924	<u>0.1644</u>	-		0.432		Philippine long-tailed macaque
0.1388	<u>0.1881</u>	0.1500	<u>0.2155</u>	0.1696	<u>0.1541</u>	<u>0.1808</u>	0.2226	<u>0.1808</u>	0.2246	<u>0.2336</u>	0.2427	<u>0.1976</u>	-		Indonesian long-tailed macaque

Table II

relations based on the STRUCTURE analysis and their respective mean Fst estimates. The

Species	Chinese rhesus macaque	Burmese rhesus macaque	Vietnamese long-tailed macaque	Mauritian long-tailed macaque	Philippine long-tailed macaque	Indonesian long-tailed macaque	Mean Fst values
	0.004	0.002	0.002	0	0.001	0	0.1149
	0.002	0.001	0.001	0	0.001	0	0.2406
	0.973	0.006	0.002	0	0.001	0	0.0272
	0.153	0.827	0.004	0	0.001	0	0.0737
	0.002	0.002	0.992	0.001	0.001	0	0.0741
	0	0.001	0	0.998	0.001	0	0.3114
	0	0.001	0.001	0.003	0.993	0	0.2068
	0	0	0	0	0	0.999	0.2242

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