RESEARCH ARTICLE

Quaternary Climate Change and Social Behavior Shaped the Genetic Differentiation of an Endangered Montane Primate From the Southern Edge of the Tibetan Plateau

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Multiple factors, including climate change, dispersal barriers, and social behavior influence the genetic structure of natural populations. While the effects of extrinsic factors such as historical climatic change and habitat topography have been well studied, mostly in temperate habitats, the simultaneous effects of intrinsic factors such as social behavior on genetic structure have rarely been explored. Such simultaneous effect, however, may particularly be common in social mammals such as many primates. Consequently, we studied the population structure of a rare and endangered social primate, the Arunachal macaque *Macaca munzala*, endemic to the northeastern Indian state of Arunachal Pradesh, located on the subtropical southern edge of the Tibetan Plateau and forming part of the Eastern Himalayan biodiversity hotspot. We studied a 534 bp-long mitochondrial DNA sequence and 22 autosomal microsatellite loci in individuals from three populations, Tawang, Upper Subansiri, and West Siang. The mtDNA data revealed three major divergence events: that between the Arunachal and bonnet macaques (ca. 1.61 mya), the founding of the West Siang population and the ancestral population of the present-day bonnet macaques (ca. 1.32 mya), and the divergence between the Tawang and Upper Subansiri populations (ca. 0.80 mya) that coincided with the major glacial events in the region. Comparing mitochondrial DNA with autosomal microsatellites, we also found evidence for female philopatry and male-driven long-distance gene flow. Arunachal macaques thus appear to be characterized by groups of philopatric females separated by geographical barriers and harsh climate but with dispersing males exerting a homogenizing effect on the nuclear gene pool. Given that severe population differentiation is of major concern in species conservation, we suggest that our study populations represent significant conservation units of this rare, endangered primate but, more importantly, emphasize the complex interplay of extrinsic and intrinsic factors in shaping the population structure of a social mammalian species. Am. J. Primatol.

Key words: Arunachal macaque; phylogeography; Pleistocene climate change; female philopatry; conservation unit; Eastern Himalaya

INTRODUCTION

The population genetic structure of most natural populations is shaped by a number of factors, several of which are extrinsic in nature. The effects of such extrinsic factors on population structure have largely been studied using tools of statistical phylogeography and landscape genetics, particularly in temperate regions [Hewitt, 2000, 2004]. Pleistocene glacial fluctuations and/or specific physical barriers, for example, have been described as important forces driving the population genetic structure of many social primates such as apes [Anthony et al., 2007; Arora et al., 2010; Eriksson et al., 2004; Nater et al., 2013] and a few Old World monkeys [Belay & Mori, 2006; Marmi et al., 2004; Modolo et al., 2005; Shimada, 2000; Smith & McDonough, 2005]. Moreover, the population structure of gregarious species, where individuals form social groups, may be

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strongly influenced by intrinsic biological factors such as social behavior and these effects become particularly pronounced in the absence of obvious extrinsic influences [Di Fiore, 2012; Ross, 2001].

Although most studies exploring the population genetic structure of different taxa have examined the effects of extrinsic and intrinsic factors independently, it is obvious that the structure of these populations have clearly been shaped by both of these factors at various levels of organization. Only a few studies have, however, adopted this holistic approach [Arora et al., 2010; Eriksson et al., 2004; Nater et al., 2013]. The study by Arora et al. [2010], for example, established the existence of strong population genetic structure in Bornean orang-utans *Pongo pygmaeus*, driven, on one hand, by rivers acting as geographical barriers and female philopatry, on the other, serving as a behavioral barrier to dispersal.

The discovery of such combined effects in fashioning genetic structure has strong implications for biological conservation. Extrinsic and intrinsic factors, for example, can together accentuate the isolation of local gene pools within a large population. Genetically, this translates into smaller subpopulations that form fragments of the larger, undifferentiated one [Charlesworth, 2009]. Due to their small size, these isolated subpopulations often eventually end up with lower genetic diversity, higher number of deleterious mutations and increased level of inbreeding depression [Hedrick & Kalinowski, 2000]. These subpopulations may then become more vulnerable to random genetic drift, which is particularly a major concern for large-bodied animals with small population sizes, slow life histories and low rates of reproduction [Hedrick & Kalinowski, 2000]. Consequently, it is imperative that we better understand the various extrinsic and intrinsic factors that have together influenced the genetic structure of populations as we know them today—from the perspective of both a fundamental understanding of evolutionary processes as well as the conservation management of many of these endangered populations.

One such mammalian species is the Arunachal macaque *Macaca munzala*, described relatively recently and endemic to the state of Arunachal Pradesh, northeastern India [Sinha et al., 2005; but see Biswas et al., 2011]. Our knowledge of its biology is fragmentary although it is possibly one of the most endangered of all Indian primates [IUCN, 2012]. It belongs to the *sinica* species-group of macaques and its ancestors possibly originated east of the river Brahmaputra approximately 3.2 million years ago or mya [Chakraborty et al., 2007]. Although this species is evolutionarily closely related to the bonnet macaque *M. radiata* from the plains of peninsular India, the factors that have led to their separation and current distributions remains completely unknown [Sinha et al., 2013].

The Arunachal macaque is widely distributed in the western-most districts of Tawang and West Kameng in Arunachal Pradesh, bordering Bhutan and Tibet [Sinha et al., 2013]. It is thus likely that the species may also occur in those two regions, although these regions are yet to be surveyed [but see Choudhury, 2008; Kawamoto et al., 2006]. The eastern-most distribution of the species, on the other hand, is likely to extend till the river Brahmaputra, a natural barrier that presumably separates populations of this species from those of the Eastern Assamese macaque *M. assamensis* [Sinha et al., 2013].

In addition, the species occurs on the southern edge of the Tibetan Plateau, a geologically significant subtropical region with a complex climatic history, bounded to the south by the Himalayan mountain range. Although poorly investigated, Pleistocene glacial fluctuations must have had an important effect on the biota of the plateau, especially as this region forms part of eastern Eurasia [Fan et al., 2011; Qu et al., 2010; Shi, 2002; Yang et al., 2009; Zhan et al., 2011; Zhao et al., 2012; Zheng et al., 2002; Zhou et al., 2006]. In Europe, which marks the western extent of the Eurasian landmass, for example, Pleistocene ice sheets covered the northern part of the continent during the major glaciations events [Taberlet et al., 2002]. As a result, many species either went extinct, shifted their distribution ranges or moved further south to warmer climatic refugia in the Balkans and the Italian and Spanish peninsulas, and following subsequent warmer inter-glaciations, expanded northwards again, reoccupying their old habitats [Hewitt, 2000].

In the subtropical Tibetan Plateau, however, there were no large-scale continental glaciations during the Quaternary period [Royden et al., 2008; Xia, 1997; Zhou et al., 2006]. Instead, this region is composed of multiple, historically dynamic, tectonic areas that experienced considerable geological changes before and during the Pleistocene epoch [Royden et al., 2008]. This, in effect, created a region with complex topography, consisting of a network of mountains as high as 6,000 m and deep valleys, and, as a consequence, led to the formation of relatively small alpine glaciers, about 2,000 m above sea level, on the plateau and along its edges [Xia, 1997; Zhou et al., 2006]. Consequently, the population genetic structure of several taxa on the Tibetan Plateau has been influenced by more complex geo-climatic effects than has those of temperate biota, which have largely been shaped by historical glaciations alone [Yang et al., 2009; Zhao et al., 2012]. This may be expected of the genetic structure of the Arunachal macaque populations as well.

Being a social primate, the Arunachal macaque could exhibit social behavioral patterns that could potentially significantly influence its population structure. The species typically exhibits a matrifocal society with tolerant social relationships among the adult males and females within a troop [Sinha et al., 2013]. Although we currently lack detailed

knowledge of the dispersal behavior of the species, it is likely that they exhibit female philopatry and male-biased gene flow, as is typical of Old World primates, including the *sinica* species-group of macaques [Chikhi & Bruford, 2005].

In this article, we attempt to examine the effects of sex-biased dispersal, if any, on the population structure of the Arunachal macaque, especially against the background of large-scale influences, wrought by historical climate change and regional topography. Being an "edge" species of the Tibetan Plateau, we investigate how the phylogeographic patterns displayed by this species compare to those recently reported in a few other non-primate taxa that occur on the Plateau [Fan et al., 2011; Qu et al., 2010; Yang et al., 2009; Zhao et al., 2012].

It is noteworthy that our study is also of special relevance from a conservation perspective. The rich biodiversity of the Eastern Himalayas [Mittermeier et al., 1997; Olson & Dinerstein, 1998], which includes the Arunachal macaque and several other newly discovered plant and animal taxa [Gillison, 2004; Thompson, 2009], appears to be distributed across a complex combination of specialized ecological niches and glacial refugia, which may have faced long periods of isolation. Unfortunately, however, the Eastern Himalayas now face unprecedented ongoing habitat destruction and wildlife hunting [Aiyadurai et al., 2010; Cincotta et al., 2000]. It is, thus, crucial, that we investigate the various factors that have shaped the populations of these unique, endemic species; such an understanding is essential to develop suitable management plans for these taxa if we are to conserve them in an uncertain future.

METHODS

Ethics Statement

This study was conducted in accordance with all relevant Indian laws, with due permits from the State Forest Department of Arunachal Pradesh. Additionally, this research adhered to the principles for the ethical treatment of primates of the American Society of Primatologists. We, however, did not handle any live or dead animals for this study. We collected only small pieces of dried skin samples from local communities without any kind of payment. As these were from old hunting trophies and no fresh hunting was reported from our study sites, we are confident that our sample collection did not encourage killing of the species in any way. Additionally, over the last 7 years we have led a conservation program for the species in this region.

Study Area and Sampling

We obtained 26 dried skin samples of Arunachal macaque individuals, killed and preserved as hunting trophies, from 14 villages across the districts of Tawang, Upper Subansiri and West Siang in the state of Arunachal Pradesh (Fig. 1). The samples were collected between June 19, 2005 and June 19, 2006. While Tawang forms the western edge of the state, both Upper Subansiri and West Siang are located in remote central Arunachal Pradesh. These samples were preserved in 95% ethanol at ambient temperature till they were transported to the laboratory at the National Centre for Biological Sciences, Bangalore, India. A single blood sample was also obtained from a captive individual in Typee, Tawang District. We considered the samples to belong to three tentative populations—Tawang ($N = 5$), Upper Subansiri ($N = 12$) and West Siang ($N = 9$) on the basis of their geographic origins (Table I).

For the purpose of phylogenetic comparisons, we collected six samples (one feces from Chamundi hills and five blood) of individual bonnet macaques *M. radiata* from wild or wild-caught, captive individuals of known origin maintained in government-regulated animal facilities in southern India (Table I).

DNA Extraction and PCR Amplification

We extracted genomic DNA from the skin samples of Arunachal macaque and the blood samples of bonnet macaque using the QIAGEN DNeasy Blood & Tissue Kit (Qiagen GMBH, Hamburg, Germany), following the manufacturer's protocols. For the bonnet macaque fecal sample, we used QIAamp DNA Stool Mini Kit (Qiagen GMBH) and followed the manufacturer's protocol with a slight modification; we doubled the time for all incubation and elution steps. We used extraction blanks as negative controls in downstream polymerase chain reaction (PCR) amplifications.

In order to sample a non-coding region of the Arunachal macaque mitochondrial genome, we amplified a 534 bp-long D-loop (hyper-variable segment 1) region using the primer set from Li and Zhang [2005]. We conducted standard 35-cycle PCR to amplify the target regions following Chakraborty et al. [2007].

We amplified 22 fluorescently labeled microsatellite loci (DXS571, DXS6810, DXS8043, DXS6799, D20S171, D4S243, D12S372, D8S1466, D9S934, D7S794, D10S611, D8S1106, D15S823, D19S255, D2S146, D17S791, D18S869, D18S537, D6S2419, D16S403, D5S1457, D10S179, D11S2002), already established for other macaque species [Kanthaswamy et al., 2006; Rogers et al., 2005]. PCR amplification of 35 cycles was conducted for up to five loci simultaneously with combinations selected on the basis of fragment size, annealing temperature, and the fluorescent dye set DS—33 components used (dy6FAM, VIC, PET, or NED).

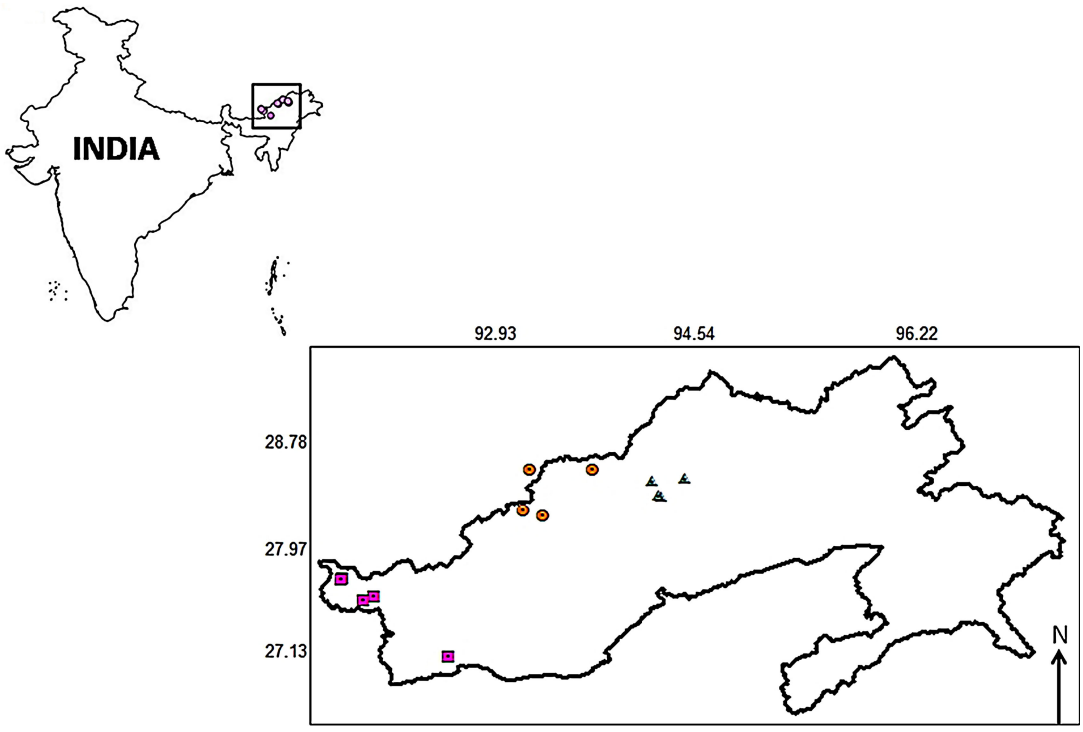

Fig. 1. Map of Arunachal Pradesh with locations of the sampling sites. The triangles correspond to sampling sites in West Siang, circles to those in Upper Subansiri and rectangles to those in Tawang. Please note that one of the villages in Upper Subansiri, located along the Indo-Tibet border, has been erroneously projected in Tibet possibly due to technical limitations of the GPS used. Inset: Location of the study site at the edge of Tibetan Plateau.

MtDNA Sequencing and Microsatellite Genotyping

Before sequencing mtDNA, we purified amplification products of excess nucleotides, primers, enzymes, and other leftover PCR reagents using the ExoSAP-IT protocol (Affymetrix, Santa Clara, CA). We cycle-sequenced PCR products with ABI BigDye Terminator Ready Reactions kits and electrophoresed our samples on an ABI 3130xl DNA Analysis System. We carried out base calling using Sequencing Analysis v 5.2 (ABI), verifying by eye, and assembled sequences using the software MEGA 4 [Tamura et al., 2007]. We aligned sequences with CLUSTAL W [Higgins et al., 1994] and manually deleted locations with gaps in the alignment. We only included sequences verified with triple or quadruple coverage in both directions in our analyses. We could successfully sequence all of the individual samples except one (S14). The accession numbers of all the 25 sequences have been given in Table I. We also downloaded D-loop sequences of Arunachal, bonnet, Assamese, Tibetan, stump-tailed macaques and baboons from Genbank and used them in reconstructing phylogenetic tree. The Genbank accession numbers of these sequences are also mentioned in Table I.

We conducted electrophoreses of microsatellite PCR products on an ABI 3130xl DNA Analysis System with the GENESCAN^{TM} 500 LIZ^{TM} size standard and called genotypes using GeneMapper software (version 4.0; Applied Biosystems, Foster City, CA, USA). Finally, we could generate clear genotypes of 24 individuals, all but two (S22 and S40) collected, for all the 22 microsatellite markers (Table II).

Phylogenetic Reconstruction

We conducted model selection tests on jModelTest 0.1 [Posada, 2008], using the Akaike information criterion to choose the most suitable substitution model for our data. These analyses revealed HKY + Γ + I to be the most appropriate evolutionary model for our mtDNA data. Phylogenetic analyses were conducted using the maximum likelihood (ML) and Bayesian inference (BI) methods in PAUP* [Swofford, 2002] and MrBayes, version 3.2 [Ronquist et al., 2012] respectively. The ML trees were reconstructed with 1,000 bootstrap replicates. The BI analyses were run for 10^7 generations for the mitochondrial data to ensure convergence. Samples were collected every 1,000 generations and 4 chains (1 cold and 3 heated) were used for the Markov chain Monte Carlo (MCMC) procedure in all cases. The first 25% of the collected posterior data were discarded to allow "burn-in" [Ronquist et al., 2012].

TABLE I. Individual mtDNA Sequences Used in the Study, Sites of Origin of the Source Samples and Accession Numbers

Species, population	Location	Sample	Latitude	Longitude	Accession numbers
Arunachal macaque, Tawang	Jang	S1	27.58	91.98	KC844118
	Gronkhar	S2	27.55	91.90	KC844119
	Zemithang	S3	27.72	91.73	KC844120
	Typee	S6	27.11	92.57	KC844122
	Lomphu	S7	27.71	91.72	KC844123
Arunachal macaque, Upper Subansiri	Taksing	S4	28.58	93.22	KC844121
	Orak	S8	28.35	93.49	KC844124
	Ketenallah	S9	28.21	93.32	KC844125
	Yeaza	S10	28.26	93.16	KC844126
	Taksing	S11	28.58	93.72	KC844127
		S12	28.58	93.72	KC844128
		S13	28.58	93.72	KC844129
		S14	28.58	93.72	NA
		S15	28.58	93.72	KC844130
		S16	28.58	93.72	KC844131
	TCC Camp	S17	28.58	93.72	KC844132
		S18	28.58	93.72	KC844133
Arunachal macaque, West Siang	Lungte	S19	28.36	94.24	KC844134
		S20	28.36	94.24	KC844135
		S21	28.36	94.24	KC844136
	Peidi	S22	28.37	94.21	KC844137
	Papikurung	S23	28.48	94.16	KC844138
		S24	28.48	94.16	KC844139
		S25	28.48	94.16	KC844140
		S26	28.48	94.16	KC844141
	Tato Gitu	S40	28.51	94.42	KC844142
Bonnet macaque	Yehalanka	607	13.13	77.59	DQ859973[a]
	Nelamangala	326	13.50	77.23	KC844112
	Tumkur	30	13.34	77.10	DQ859974[a]
	Aurangabad	Au1	19.89	75.32	KC844113
	Chamundi Hills	BS1	12.30	76.65	KC844114
	Pimpri	Pi3	18.62	73.80	KC844115
	Surat	Su1	21.20	72.82	KC844116
	Thrissur	TCR3	10.52	76.22	KC844117
Baboon	NA	NA	NA	NA	Y1800[a]
	NA	NA	NA	NA	AY682605[a]
Stump-tailed macaque	Yunnan, China	Yunnan1	NA	NA	AY682588[a]
	South of China	South of China	NA	NA	AY682590[a]
	Vietnam	Vietnam1	NA	NA	AY682591[a]
Tibetan macaque	Sichuan, China	Sichuan1	NA	NA	AY682607[a]
	Sichuan, China	Sichuan3	NA	NA	AY682610[a]
Assamese macaque	Vietnam	Vietnam1	NA	NA	AY682611[a]
	Yunnan, China	Yunnan4	NA	NA	AY682612[a]
	Vietnam	Vietnam2	NA	NA	AY682613[a]
	Yunnan, China	Yunnan2	NA	NA	AY682615[a]
	Yunnan, China	Yunnan3	NA	NA	AY682616[a]
	South of China	South of China	NA	NA	AY682618[a]
	Myanmar	Myanmar2	NA	NA	AY682621[a]
	Myanmar	Myanmar4	NA	NA	AY682622[a]

NA, not available.
[a]From GENBANK.

To infer the coalescence date for Arunachal macaque mtDNA haplotypes, we used a Bayesian Markov chain Monte Carlo analysis as implemented in BEAST 1.7.2 [Drummond et al., 2012]. Based on the Akaike information criterion from jModelTest 0.1 [Posada, 2008], we selected the $HKY+\Gamma+I$ model. We used an uncorrelated relaxed log-normal clock [Drummond et al., 2006], specifying a normal

TABLE II. Microsatellite Loci Amplified in Arunachal Macaque Samples

Loci	Repeat type	No. of alleles	Size range	H_o
DXS571	Di	7	125–141	0.25
DXS6810	Tetra	4	177–189	0.17
DXS8043	Di	10	185–205	0.96
DXS6799	Tetra	7	195–247	0.17
D20S171	Di	11	112–140	0.67
D4S243	Tetra	14	158–230	0.54
D8S1466	Tetra	7	276–308	0.75
D9S934	Tetra	6	187–207	0.5
D7S794	Tetra	8	117–157	0.71
D10S611	Tetra	8	166–202	0.42
D8S1106	Tetra	7	124–152	0.58
D15S823	Tetra	14	309–397	0.46
D19S255	Tetra	8	113–153	0.58
D2S146	Di	9	181–211	0.92
D17S791	Di	9	162–182	0.83
D18S869	Tetra	10	176–216	0.88
D18S537	Tetra	5	164–180	0.74
D6S2419	Tetra	20	150–238	0.96
D16S403	Di	10	128–158	0.42
D5S1457	Tetra	8	103–139	0.71
D10S179	Tetra	8	102–142	0.26
D11S2002	Tetra	7	253–277	0.79

distribution with a mean HVS1 substitution rate of 0.1643 substitutions per nucleotide per million year (Myr) for the mean rate prior. We chose this corrected HVS1 estimate [Soares et al., 2009] because it takes into account the effects of purifying selection on the entire mtDNA molecule as well as saturation factors affecting the molecular rate decay described in many studies [Endicott et al., 2009; Ho et al., 2005]; it is therefore appropriate for population-level analyses [Ho et al., 2008]. The 95% confidence interval for the normal distribution spanned HVS1 substitution rates obtained in other studies, from 0.07 to 0.25 substitutions/site/Myr [Santos et al., 2005]. Additionally, we used three divergence estimates as priors. The three calibration points were the baboon-macaque divergence, approximately between 8.6 and 10.9 million years ago (mya) based on whole mtDNA [Raaum et al., 2005], the *sinica-fascicularis* species-group divergence, at approximately 3.2 (SD 0.3) mya [Tosi et al., 2003] and the last common ancestor of the *sinica* species-group, at approximately 1.7 mya [Tosi et al., 2003]. For the baboon-macaque divergence, we used a normal mean of 9.75 and SD of 0.42, thereby obtaining a broad distribution with a 95% interval from 8.6 to 10.9 mya. For the *sinica-fascicularis* species-group calibration, we used a normal mean of 3.2 and SD of 0.3, spanning a 95% interval from 2.3 to 3.9 mya. Finally, we used a normal mean of 1.7 and SD of 0.4 for the last calibration point, thus spanning a wide timescale between 1.04 and 2.36 mya.

Using the birth–death prior for tree-branching rates, we carried out three runs for 10^6 generations with parameter sampling every 1,000 generations. Tracer 1.4.1 [Rambaut and Drummond, 2005] was then used to examine whether the 10% burn-in period and effective sample sizes were adequate. All the runs were combined in LogCombiner 1.4.8 [Drummond et al., 2012], and the resulting tree visualized and edited using FigTree 1.2 [Rambaut, 2008].

Genetic Structure Analysis

The clean and repeatedly generated genotypes were alone accepted for our genetic structure analysis. We nevertheless used the program Micro-Checker, version 2.2.3 [Van Oosterhout et al., 2004] to check the data for null alleles. We further tested each population, locus by locus, for any significant deviation from the Hardy–Weinberg equilibrium that may have been caused by the presence of null alleles in the data or due to the occurrence of inbreeding in the populations. We employed the exact test, implemented in Arlequin, version 3.5 [Excoffier et al., 2005] for this purpose. Finally, we used the program, ML-Relate [Kalinowski et al., 2006] to calculate maximum likelihood estimates of pair-wise relatedness and relationship categories between the individuals from the genotypic data.

We first calculated modified moment estimates of *F*-statistics using an analysis-of-molecular-variance (AMOVA) approach with Arlequin, version 3.5 [Excoffier et al., 2005] to investigate the genetic differentiation between the three study sites for both the data sets.

For haplotypic data (mtDNA), Arlequin estimates Φ_{ST} using information from both the allelic content and frequency of haplotypes [Excoffier et al., 1992]. For genotypic data (microsatellites), with an unknown gametic phase, as is the case for most natural populations, the AMOVA is based on *F*-statistics. The algorithm partitions the total genetic variance into covariance components, due to inter-individual and inter-population differences, following a hierarchical analysis of variance [Weir, 1996]. The covariance components were then used to compute fixation indices [Wright, 1965]. The values span between 0 and 1. Values in the range of 0–0.05 indicate "little" genetic differentiation; 0.05–0.15, "moderate" differentiation; 0.15–0.25, "great" differentiation; and values above 0.25, "very great" genetic differentiation [Hartl & Clark, 1997; Wright, 1978]. The significance of the fixation indices was tested using a non-parametric permutation test [Excoffier et al., 1992] with 10,000 permutations. The tested populations were considered to be genetically differentiated if inter-population genetic variation was found to be higher than that within the populations.

Geographic patterns of genetic structure can often entail complex combinations of clines, clusters

and patterns of isolation by distance [François & Durand, 2010], and multiple analysis methods can provide complimentary information regarding such patterns [Balkenhol et al., 2009]. Thus, as another means of examining patterns of genetic structure, we employed a spatial Bayesian clustering method using the program BAPS 5 [Corander et al., 2008]. For mtDNA data, we performed a mixture analysis using the "clustering of linked molecular data" method. The analysis consisted of five iterations of each value of K_{max} (the maximum number of populations) for the range $K_{max} = 1–20$. This step determines the optimum number of genetic clusters in the sample based on the partition with the maximum likelihood [$L(K)$] and highest posterior probability (P), and then assigns each individual to a cluster. For microsatellite data, we first performed a mixture analysis using the "clustering of groups of individuals" model and with similar number of iterations as above. The model showed limited power to differentiate the populations for microsatellite data.

To further test the population structure with more complex models, we employed the model-based algorithm in the program Structure, version 2.3.3 [Falush et al., 2003, 2007; Pritchard et al., 2000] which, given the number of clusters (K), estimates allele frequencies in each cluster and the population membership of each individual. We initially tested two-ancestry models with both "no-admixture" and "admixture" scenarios to estimate admixture proportions for every individual. As these models showed limited assignment power, the Locprior model, which allows for the use of sample group information in the clustering process and which is capable of detecting structures at lower levels of divergence or with less data, was then implemented [Hubisz et al., 2009]. It is noteworthy that this model does not find structure when none is present and is able to ignore the sample group information when the ancestries of individuals do not correlate with their sampling locations [Arora et al., 2010; Hubisz et al., 2009]. Each model considered K between one and six and all the models were tested with 100000 Markov Chain Monte Carlo (MCMC) simulations (20000 burn-in) with each simulation repeated ten times. We used the online version 6.0.1 of Structure Harvester [Earl, 2011] to plot the maximal values of ln $P(D)$, the posterior probability of the data for a given K and ΔK based on the rate of change in the log probability of data between successive K values [Evanno et al., 2005], and identify K.

Gene Diversity in Arunachal Macaque Populations

We calculated gene diversity, the expected heterozygosity under random mating, for both the datasets. For microsatellite data, gene diversity can be defined as the probability that two randomly chosen haplotypes are different in the sample. It is basically equivalent to the average of expected heterozygosities over all the loci. For mtDNA, gene diversity, also known as nucleotide diversity for sequence data, is the probability that two randomly chosen homologous nucleotide sites are different. It is a good measure of genetic diversity and is relatively little affected by sample size [Chikhi & Bruford, 2005]. For microsatellites, gene diversity can vary enormously even for large sample sizes, but this effect can be reduced when many loci are used such as in this study (22 loci), or when the two alleles have similar frequencies [Chikhi & Bruford, 2005].

Gene Flow Between Arunachal Macaque Populations

We estimated population pair-wise estimates of F_{ST}, which provide some insight into the degree to which the populations are historically connected [Holsinger & Mason-Gamer, 1996; Slatkin, 1991]. They, by themselves, however, do not allow us to determine whether the connections between populations are actually results of long-term gene flow between them or rather that of recent common ancestry [Nielsen & Wakeley, 2001]. But we tried to differentiate between these two scenarios with the help of phylogenetic reconstruction of the studied populations. We employed Arlequin, version 3.5 [Excoffier et al., 2005] to compute pairwise F_{ST} for all pairs of populations.

The null distribution of pairwise F_{ST} values under the hypothesis of no difference between the populations was obtained by permuting haplotypes between them. The P-value of the test was the proportion of permutations leading to a F_{ST} value larger or equal to the observed one.

RESULTS

Phylogenetic Reconstruction and Divergence Times

We recorded a total of 24 mitochondrial DNA haplotypes with only the individuals S11 and S18 of the Upper Subansiri population sharing a haplotype between them. We examined the phylogenetic relationships between these haplotypes by generating both the maximum likelihood (ML) and Bayesian inference (BI) phylogenetic trees. We only show the BI cladogram here as both of them are well supported and show similar topologies (Fig. 2). Most of the nodes have bootstrap values more than 80% and none lower than 50%. The eastern Assamese and Tibetan macaque individuals form a monophyletic out-group to the Arunachal and bonnet macaque clades, as has been demonstrated earlier [Chakraborty et al., 2007]. The Arunachal macaque individuals, however, show extensive diversification across sampling sites.

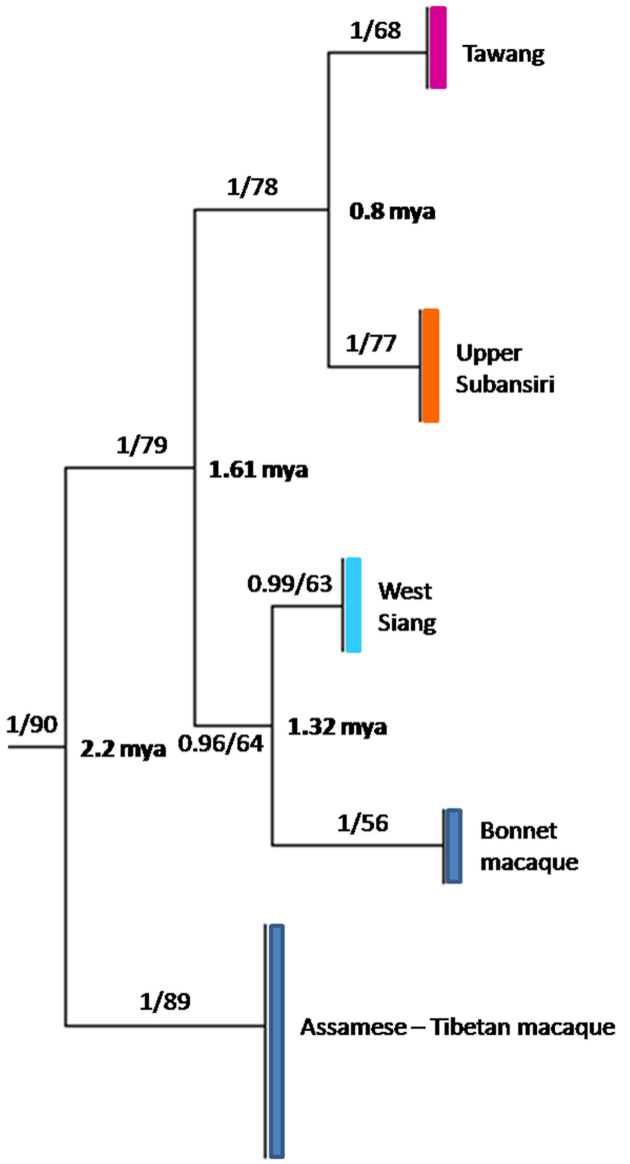

Fig. 2. Bayesian phylogenetic tree of the Arunachal macaque mtDNA haplotypes. The posterior probabilities and maximum likelihood values are above or below the tree branches. The differently colored bars at the branch tips correspond to the species and population locations in Figure 1.

Individuals across the three sampling locations form three distinct monophyletic clades according to their geographic origins.

Surprisingly though, while the Tawang and Upper Subansiri groups share a most recent common ancestor, the West Siang group seemed to have diverged from a common ancestor with the bonnet macaque, which itself is monophyletic in nature. None of these groups, however, are reciprocally monophyletic, thus attesting to an intriguing phylogeographic pattern and a resulting complex phylogenetic relationship between the two species.

According to our estimates, the putative ancestor of the *sinica* species-group began to diversify during the transition period between the Pliocene and Pleistocene epochs, approximately 2.2 (95% Highest Posterior Density or HPD 1.65–2.75) mya. Next, the diversification of the Arunachal macaque-bonnet macaque ancestor into two stocks appears to have occurred during the early Pleistocene, approximately 1.61 (95% HPD 1.14–2.12) mya. It was soon followed by the separation of the ancestors of the West Siang group from that of the bonnet macaque, approximately 1.32 (95% HPD 0.87–1.81) mya. Finally, the ancestors of the present-day Tawang and Upper Subansiri populations diverged approximately 0.80 (95% HPD 0.5–1.16) mya, during the middle Pleistocene period.

Population Genetic Structure

We did not find any locus in our analysis that significantly deviated from the Hardy–Weinberg equilibrium or that exhibited null alleles. There was also no significant linkage disequilibrium after the Bonferroni correction was applied to the data. Only two pairs of individuals appeared to be half-siblings across two different populations while the rest were unrelated. Consequently, we analyzed the population genetic structure with and without these individuals but both analyses yielded the same result.

The AMOVA yielded results that were clear but contradictory in nature for both the mtDNA and microsatellite markers. A considerable 64.09% of the total genetic variation in mtDNA was partitioned among the studied populations than within (35.91%) each of them. As a result, the fixation index ($\Phi_{ST} = 0.64$, $P \ll 0.05$) was quite high. Thus, the mtDNA analysis exhibited a signature of population differentiation between the three Arunachal macaque populations (Table III).

In contrast, microsatellite data analysis revealed an immense 95.98% of the total genetic variation to be partitioned within the study sites in comparison to a mere 4.02% amongst them. Consequently, the

TABLE III. Analysis of Molecular Variance (AMOVA) of the Arunachal Macaque Populations Showing Strong Population Differentiation for Mitochondrial DNA but a Lack of Differentiation for Nuclear DNA

Source of variation	Percentage of variation (mtDNA)[a]	Percentage of variation (microsatellites)[b]
Among populations	64.09	2.56
Within populations	35.91	97.45
Fixation index	0.64	0.03

Both the fixation indices are significant ($P \ll 0.05$).
[a]Populations: Tawang, Upper Subansiri, and West Siang.
[b]Populations: Tawang-West Siang and Upper Subansiri.

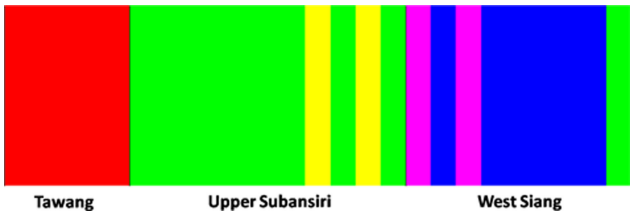

Fig. 3. Genetic structure of the Arunachal macaque populations ($N = 25$) based on mitochondrial DNA. A Bayesian clustering method (BAPS 5) was used to infer the clusters.

fixation index was low ($F_{ST} = 0.04$, $P \ll 0.05$), suggesting that 4% of the observed genetic diversity alone was accounted for by genetic differences among the three tentative geographical populations; these populations, thus, appear to be more genetically similar to one another than different, at least, for the nuclear markers.

The mixture model for the linked sequence data, analyzed by the program BAPS 5 (see MtDNA Sequencing and Microsatellite Genotyping Section), predicted population structuring within the Arunachal macaque samples (Fig. 3). The three sampling locations largely exhibited their distinctness with the formation of three separate clusters—Tawang (Fig. 3; red bars), Upper Subansiri (green bars) and West Siang (blue bars). However, while all the individuals from Tawang clearly exhibited geographic fidelity, this was not true for a few individuals from the other two locations. For example, individuals S19, S21, and S40 were sampled from West Siang but according to their genetic assignment, were associated with the Upper Subansiri (S40) and an unknown (S19 and S21; pink bars) population. Similarly, two individuals (S15 and S17; yellow bars), sampled from Upper Subansiri, were found to be of unknown origin. Four of these "migrant" individuals were males (S15, S19, S21, and S40) while only one was female (S17), according to a molecular sexing exercise that we independently carried out (data not shown).

Our analysis of the microsatellite data with the program BAPS 5 failed to predict any population structure, which may have been the result of extremely low level of population differentiation. Similarly, in Structure too, the admixture model did not yield a clear genetic population structure for the samples, providing further support to a lack of strong population structure in the sampled Arunachal macaque populations. The Locprior model, however, predicted a putative two-population ($K = 2$) genetic structure, though the signal continued to be weak (log likelihood, $\ln'(K) = -94.93$). In accordance with this prediction, individuals from the Tawang and West Siang populations clustered together as one population while individuals from the Upper Subansiri population maintained their distinctness (Fig. 4).

At this stage, following the Structure results, we reconsidered our AMOVA for the microsatellite data and classified the Tawang and West Siang individuals together. As a result, the percentage of total genetic variation between the tested populations further reduced to a scant 2.56% (Table III).

Gene Diversity

The values of the mtDNA nucleotide diversity of the Arunachal macaque populations seemed to vary from moderate to low, with values of 0.06 (West Siang), 0.03 (Upper Subansiri), and 0.01 (Tawang). The average microsatellite gene diversity over the 22 loci, however, was higher than those of mtDNA, with values of 0.81 (Tawang—West Siang) and 0.80 (Upper Subansiri).

Gene Flow

Finally, the population pairwise F_{ST} for mtDNA showed significant ($P \ll 0.05$) differentiation between the three sampling sites (Table IVA). It is noteworthy that the Tawang and Upper Subansiri groups, which share the most recent common ancestor, also showed the highest F_{ST} value (0.7, $P = 0.001$). In contrast, the West Siang population, which is phylogenetically paraphyletic to both the other groups, shared the same F_{ST} values with both of them (0.62, $P \ll 0.05$).

The Bayesian clustering analysis of the microsatellite data (Table IV) revealed a significant F_{ST} value ($P = 0.04$) only when the Tawang—West Siang group was compared to the Upper Subansiri group (population pairwise $F_{ST} = 0.03$; Table IVB).

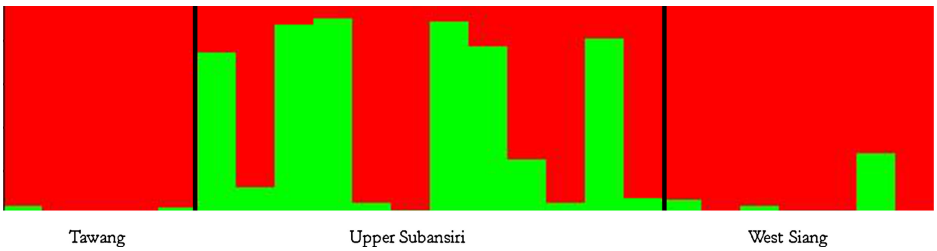

Fig. 4. Genetic structure of Arunachal macaque populations ($N = 24$) based on nuclear (microsatellite) markers. A Locprior model (Structure 2.3.3) was used to infer the population structure.

TABLE IV. Population Pairwise F_{ST} Values Between the Arunachal Macaque Populations

Pairwise F_{ST}	Tawang	Upper Subansiri	West Siang
A			
Tawang			
Upper Subansiri	0.70 ($P \ll 0.05$)		
West Siang	0.62 ($P \ll 0.05$)	0.62 ($P \ll 0.05$)	
Pairwise F_{ST}	Tawang-West Siang		Upper Subansiri
B			
Tawang-West Siang			
Upper Subansiri	0.03 ($P = 0.04$)		

(A) Pairwise values for mitochondrial DNA, (B) pairwise values for nuclear markers.

DISCUSSION

Like several other species from the Tibetan plateau that have recently been studied [Fan et al., 2011; Qu et al., 2010; Yang et al., 2009; Zhao et al., 2012], the Arunachal macaque exhibits significant population structure across its distribution range. But, unlike most of the other species investigated, the population structure of this social primate, when analyzed using both mitochondrial and nuclear markers, appears to be shaped by a complex interplay of multiple forces that have resulted in complex phylogeographic patterns.

Pleistocene Glaciations and Population Divergence

The Tibetan Plateau is recognized to have experienced at least four major glaciation events during the Quaternary period—the Xixiabangma (Early Pleistocene), Naynayxungla (Middle Pleistocene), Guxiang (late Middle Pleistocene) and the last glaciation, including two glacial stages, the latter of which corresponds to the last glacial maximum (LGM), ca. 20,000 years ago [Zheng et al., 2002]. Molecular dating analysis suggests that the common ancestor of the extant populations of Arunachal and bonnet macaques harks back to the Early Pleistocene period (1.61 mya). This date, more or less, overlaps with "the most recent common ancestor" (TMRCA) of other species of birds and mammals from the Tibetan Plateau [Qu et al., 2010; Yang et al., 2009]. This time period is marked, geologically, as the beginning of the Quaternary glacial period in China and adjacent areas such as the Tibetan Plateau [Zhan et al., 2011].

The next divergence event that followed (1.32 mya) appears to have resulted in the founding of the West Siang population of the Arunachal macaque and the ancestors of the present-day bonnet macaque. This date coincides with another cold stage in the Xixiabangma glaciations [Zheng et al., 2002]; it was one of coldest recent climatic periods in the region, possibly creating many refugia in the southern and eastern boundaries of the Tibetan Plateau [Zhan et al., 2011]. In the Eastern Himalayas, however, glaciations were restricted to the relatively high altitudes and did not affect the lower slopes or valleys in these ranges [Zhou et al., 2006]. Palaeo-climatic and palynological studies from this region reveal a shift of vegetation over the Pleistocene glacial cycles [Kou et al., 2006; Yu et al., 2007]; during the glacial periods, cool-temperate vegetation, such as shrublands, expanded to the lower elevations and contracted to the high elevations during the warmer and wetter interglacial periods. Such an elevation-dependent niche separation may have been responsible for the population diversification of the species that inhabit this region [Qu et al., 2011]. In accordance with such a pattern, we postulate that a lower-elevation group of macaques, adapted to a grassland habitat, may have found itself isolated from its high-altitude relatives during these climatic and resultant vegetation shifts, and may have eventually served as the ancestors of present-day bonnet macaques.

We could date the last intraspecific diversification event to the Middle Pleistocene (0.80 mya). Expectedly, this period too recorded another glaciations event, the Naynayxungla glaciation between 0.78 and 0.5 mya [Zheng et al., 2002]. This was considered to be the most extensive glaciation event with many large ice caps, glacier complexes and great valley glaciers, covering a total area more than 500,000 km^2 across the Tibetan Plateau.

Historical Factors: Effect of Pleistocene Climate Change and Topography

It is now well known from many recent studies that the Quaternary glaciations fragmented large populations in the Tibetan Plateau to many small refugial relict populations over the long term, leading to distinct population structures [Qu et al., 2010, 2011; Zhan et al., 2011; Zhao et al., 2012]. The Arunachal macaque populations too show a considerable amount of structure for mitochondrial DNA

with the Tawang, Upper Subansiri and West Siang populations being largely isolated from one another. An intriguing feature that the macaque populations display, however, is a moderate to low nucleotide diversity that reduces from the east to the west, unlike the other species in this region, virtually all of which have fairly low nucleotide diversities [Qu et al., 2010, 2011; Zhan et al., 2011]. Such low nucleotide diversity is also characteristic of other macaque species known to be affected by Pleistocene glaciations across the world [Chikhi & Bruford, 2005; Modolo et al., 2005], with rhesus macaques being an exception, displaying a gradient similar to that of the Arunachal macaque, but across south Asia [Smith & McDonough, 2005]. The directional decrease in nucleotide diversity shown by the Arunachal macaque supports the hypothesis of the origin of the species in the east of the Indian subcontinent, as was suggested by the phylogenetic analysis of Chakraborty et al. [2007]. In this scenario, as in the case of the eastward dispersal of modern humans from Africa [Cann et al., 1987; Vigilant et al., 1991], founder effects would be expected to lead to reduced genetic heterogeneity in the Tawang group relative to that in West Siang, exactly as we find in our study. In other words, the ancestors of the Tawang-Upper Subansiri population may have originated in the Upper Subansiri region approximately 1.6 mya and later diversified to give rise to the Tawang population about 0.80 mya.

A second implication of our analysis is that while most of the species that have low nucleotide diversity are distributed further north in the Tibetan Plateau, sometimes extending into the northern hemisphere [Qu et al., 2010, 2011; Zhan et al., 2011], the Arunachal macaque is restricted to the southern edge of the Tibetan Plateau, the southernmost border of the Pleistocene glaciers. The eastern and southeastern parts of the Tibetan Plateau have long been considered to harbor several glacial refugia during the Pleistocene [Zhan et al., 2011]. It is thus expected that the Arunachal macaque would be least affected by the Quaternary climatic shifts, as compared to other, more temperate, species. The Pleistocene glaciations on the Tibetan Plateau are also known to be punctuated by four to five inter-stadials or interglaciations [Zheng et al., 2002]. We thus hypothesize that although the ancestral Arunachal macaque populations became periodically isolated and small in size during the glaciation events, they may have intermittently increased in size during the interstadials when the climate became warmer and wetter, and consequently, more conducive to population expansion. The only other species from the Tibetan Plateau that shows a similar level of gene diversity is a small passerine bird, the black redstart *Phoenicurus ochruros*, with an elevation range from 2,000 to 3,500 m on the southeast and northwest edges of the plateau [Qu et al., 2010].

An examination of the pairwise F_{ST} values of the Arunachal macaque populations for mitochondrial DNA also makes evident that among the three studied populations, the Upper Subansiri population is the most isolated one. Geographically, the Upper Subansiri and West Siang populations are closer to one another than is West Siang to Tawang. The West Siang population, nevertheless, displays comparative F_{ST} values with both the other populations. The Upper Subansiri population, in turn, reveals a higher pairwise F_{ST} value with the Tawang population, with which it shares common ancestry. These patterns suggest the possible existence of a corridor between West Siang and Tawang while there may be a physical barrier that segregates the Upper Subansiri population from the other two and which may have arisen after the Tawang and Upper Subansiri populations separated from one another, approximately 0.8 mya. The present-day Arunachal macaque populations are mostly distributed in the high-altitude areas of the Eastern Himalayas, with lofty ranges separated by deep valleys. Such a rugged topography may make migration difficult, as is also evident from the deep isolation shown by the local human populations of the region from populations across the rest of India [Cordaux et al., 2004]. The typical topography of the region may have thus also served as a barrier for the macaque populations in recent times.

These patterns are further reinforced by our analysis of the biparental microsatellite markers. Both AMOVA and Bayesian clustering analyses suggest the Tawang and West Siang groups to genetically belong to a single population while the Upper Subansiri population appears to be distinct. Its relatively low pairwise F_{ST} value with the other conjoined population, as revealed by the microsatellite analysis in contrast to that with the mitochondrial DNA, nevertheless, hints at a low level of genetic connectivity between them.

Socio-Behavioral Factors: Effects of Female Philopatry and Male Migration

Despite the geographical proximity of the study populations, we could not detect a single mitochondrial haplotype that was shared among them, a pattern typical of other cercopithecine primates as well [reviewed in Chikhi & Bruford, 2005]. This, however, contrasts with the situation presented by patrilocal chimpanzees and bonobos [Eriksson et al., 2006; Langergraber et al., 2007] in which mtDNA sharing is extensive, such gene flow being promoted by dispersing females typical of the species. It is noteworthy that the Bayesian cluster analysis of the mtDNA revealed the presence of five Arunachal macaque individuals, two sampled from Upper Subansiri and three from West Siang, which were genetically dissimilar from their parent populations.

While four of these individuals, including a female, were assigned to an unknown population, which was not sampled, the fifth individual, a male, was a potential migrant from Upper Subansiri to West Siang. The geographic fidelity of the maternally inherited mitochondrial DNA, the majority of the potential migrants being male, and the high nuclear DNA nucleotide diversity accompanied by a reduction in population structure for nuclear markers suggest female philopatry and male-mediated gene flow in this species, features again characteristic of cercopithecine primates in general [Chikhi & Bruford, 2005]. Along with these four lines of evidence, the relatively higher F_{ST} values for the mtDNA data than that reflected in the microsatellite analysis further confirms the importance of the typical cercopithecine social structure in determining the patterns of genetic diversity in the study species. The typical female philopatry observed in cercopithecine primates including the Arunachal macaque is expected to generate higher F_{ST} values in mtDNA because firstly, mtDNA has a lower effective population size, which increases the chances of random fixation of alleles and secondly, female philopatry effectively reduces mtDNA gene flow between populations [Chikhi & Bruford, 2005].

In conclusion, the distribution patterns and population structure of the Arunachal macaque appear to have been uniquely influenced by multiple factors and their interactions, which include the location of the species on the southern edge of the Tibetan Plateau, the extremely rugged topography of the region, Pleistocene climatic oscillations and by the socio-behavioral patterns of male dispersal and female philopatry, typical of cercopithecine primates. Our results also support an early to middle Pleistocene radiation of the species to the southern edge of the Tibetan Plateau, characterized by groups of females strongly separated by harsh climate, geographical barriers and phylogenetically constrained socio-behavioral patterns. These influences led to highly differentiated populations of the species but with dispersing males exerting a homogenizing effect on the nuclear gene pool. There is, however, a need to independently analyze the demographic history of the populations to test if there were indeed changes in historical population size correlated with Pleistocene climate change, as postulated above. Independent of such investigations, however, there is also an urgent necessity to sample the species more intensively in order to determine precise locations of the corridors and barriers between the study populations, and to detect other, yet unknown, populations of the species, all of which have been predicted by the genetic analyses described in this study. Such discoveries are likely to be of utmost importance while planning the management of this endangered, endemic montane primate in the years to come.

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