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The local distribution of highly divergent mitochondrial DNA haplotypes in toque macaques *Macaca sinica* at Polonnaruwa, Sri Lanka

G. A. HOELZER,* W. P. J. DITTUS,† M. V. ASHLEY‡ and D. J. MELNICK§
*Departments of Biology and Environmental Resource Sciences, University of Nevada Reno, Reno, NV 89557, †National Zoological Park, Smithsonian Institution, Washington, DC 20008, USA and Institute of Fundamental Studies, Kandy, Sri Lanka, ‡Department of Biological Sciences (M/C 066), University of Illinois at Chicago, Box 4348, Chicago, IL 60608, USA §Genetics Laboratory, Department of Anthropology, Columbia University, New York, NY 10027, USA

Abstract

Surveys of mitochondrial DNA (mtDNA) variation in macaque monkeys have revealed extremely high levels of intraspecific divergence among haplotypes. One consistent pattern that has emerged from these studies is that divergent haplotypes are geographically segregated so that sampling a few matrilines from a given region shows them to be identical, or a closely related subset of haplotypes. Geographically structured mtDNA variation has also been commonly observed in other taxa. In this study, haplotype variation and distribution are studied in detail within a local population of toque macaques. The results show that highly divergent haplotypes, differing by 3.1% in their nucleotide sequences, coexist in this population and that they may be spatially segregated even on this micro-geographic scale. Furthermore, these differences are maintained between social groups that exchange male migrants, and thus nuclear genes, frequently.

Keywords: biogeography, dispersal, genetic divergence, *Macaca sinica*, mitochondrial DNA, social structure

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Introduction

DNA sequences diverge from one another due to the process of mutation in combination with natural selection and/or genetic drift (Li & Graur 1991; Nei 1987). The net rate of divergence among genomes is thought to be directly proportional to the mutation rate, which should be generally equivalent among closely related taxa and stable over time, because selection is expected to be relatively rare at the molecular level (Kimura 1983). In the case of the vertebrate mitochondrial genome (mtDNA), divergence begins when a single mutation differentiates a copy of the genome from the ancestral sequence. Due to strict maternal inheritance (Case & Wallace 1981; Lansman *et al.* 1983; Gyllensten *et al.* 1985) and the lack of recombination (DeFrancesco *et al.* 1980; Solus & Eisenstadt 1984; Hayashi *et al.* 1985), such differentiated mitochondrial genomes are essentially independent clones that will continue to accumulate divergent sets of mutations over evolutionary time. Consequently, it is theoretically possible for mitochondrial genomes to diverge substantially within a species, or even within a population, without reproductive isolation. Despite this possibility, divergence between mitochondrial genomes sampled from different regions of a taxon's geographic range is often taken as evidence of genetic isolation.

The extent of intrapopulational diversity among mitochondrial genomes is determined by the rates of extinction of some matrilines and the proliferation of others. This dynamic process can proceed by either random lineage sorting, whereby the probability of a matrilineage surviving over any given period of time is random with respect to the composition of its mtDNA genome (Avise *et al.* 1984; Hoelzer *et al.*, unpublished) or it can be punctuated by occasional selective sweeps (Maruyama & Birky 1991), which can reset the diversity of mitochondrial genomes within a population to zero. Generally, if

*Correspondence: G. A. Hoelzer. Fax 702 784 4860.

mitochondrial haplotypes are lost frequently, compared to the rate of origination of new haplotypes, then intrapopulation diversity will be low. Conversely, if the rate differential favours haplotype origin, diversity will be high. Furthermore, the degree of sequence divergence between any two haplotypes within a population depends on the length of time both are retained. Our current understanding of the factors affecting the processes of lineage sorting and selective sweeps is limited (but see Avise *et al.* 1984; Takahata & Palumbi 1985; Tajima 1990; Maruyama & Birky 1991), so it is unclear, a priori, where we should expect to find high or low levels of intraspecific diversity and divergence in mtDNA sequences. Similarly, it is unclear how we should interpret observations of intraspecific homogeneity or heterogeneity in the mitochondrial genome.

Nevertheless, a substantial empirical data base has developed because intraspecific variation in mtDNA is an increasingly important source of data for investigations into the genetic structure and historical biogeography of populations. Indeed, Avise and colleagues' (1987) have coined the term 'phylogeography' to describe the use of mtDNA to map the geographic distributions of populations onto a phylogenetic tree of the haplotypes found in those populations. Such phylogeographic studies have examined species of invertebrates (e.g. Saunders *et al.* 1986; Reeb & Avise 1990), fish (e.g. Bermingham & Avise 1986; Avise *et al.* 1987; Bernatchez & Dodson 1990; González-Villaseñor & Powers 1990), reptiles (e.g. Hedges *et al.* 1991), birds (e.g. Avise & Nelson 1989), and mammals (e.g. Johnson *et al.* 1983; Cann *et al.* 1987; Plante *et al.* 1989; Baker *et al.* 1990; Riddle & Honeycutt 1990; Vigilant *et al.* 1991; Melnick *et al.* 1993).

Avise *et al.* (1987) outlined five major phylogeographic categories that include all likely patterns of mtDNA haplotype distribution. Of these, only the occurrence of category II, now or in the past, poses a potential obstacle to the study of phylogeography. This category includes cases in which large mtDNA differences (i.e. greater than 1% sequence divergence) co-occur at a given geographic site. This pattern could result from secondary contact between previously isolated lineages (Avise *et al.* 1987; Taberlet *et al.* 1992). However, if such divergence can develop between haplotypes without barriers to migration, then differences between sites cannot be used to infer the time since isolation of the populations. Indeed, the historical pattern of colonization could also be obscured if the divergent haplotypes were not spatially segregated in the ancestral population.

Macaque monkeys offer an excellent opportunity to examine the possible co-occurrence of different haplotypes because of the high levels of mtDNA sequence divergence found among geographically distant populations in several species (Hoelzer *et al.* 1992; Melnick & Hoelzer 1992; Melnick *et al.* 1993; see review in Melnick, Hoelzer & Honeycutt 1992). Factors that may contribute to this pattern include the following:

1 Some macaque species exhibit insular distributions, so that gene flow between populations is largely or completely restricted.

2 Female macaques are strongly philopatric, with most remaining in their natal social group for life (Sade 1972; Dittus 1975). On the rare occasion when females transfer between groups, either as individual migrants or by group fusion (Dittus 1986, 1987), they generally enter the new social group with low dominance rank status and exhibit low fitness (Dittus 1986). Therefore, mtDNA gene flow is severely limited, even without geographic barriers.

3 Dispersal of female macaques occurs most commonly by group fission, where females from low ranking matrilines split-off to form a new group (Dittus 1988).

Toque monkeys (*Macaca sinica*), which are endemic to the island of Sri Lanka, were chosen for this study because of the long-term information available on the social structure, demography, and maternity in a population in Polonnaruwa (Dittus 1977a,b, 1985). The study site is situated in a natural dry evergreen forest in the Polonnaruwa Nature Sanctuary and Archaeological Reserve (Fig. 1). The study area can be considered a peninsula of natural forest partly surrounded by active and abandoned cultivation, scrub forest, an irrigation channel and a lake. It is continuous with more extensive areas of natural forest to the west.

The demographic history of the Polonnaruwa population has been monitored since 1968 (Dittus 1988) and it presently comprises about 600 individuals distributed among 27 social groups. Four of these groups were formed through fission in 1979–80, and another formed through fusion in 1986. In addition, three groups became extinct, or nearly so, between 1968 and 1988 (Dittus 1977a, 1986). During that time two females emigrated from other natal groups into Groups CF and 22N, with a possible third female migration into Group H (see below).

Materials and methods

All macaques in the study area (approximately 600) have been individually identified as described in Dittus & Thorington (1981). The chronological ages of all macaques born after 1968 are known; those of individuals born earlier were estimated retrospectively according to known correlations between age and morphological development. All groups in the population were censused at least once monthly, but pregnant females were checked once every few days to record neonatal birth dates with accuracy. All matrilineal kinship relationships used here

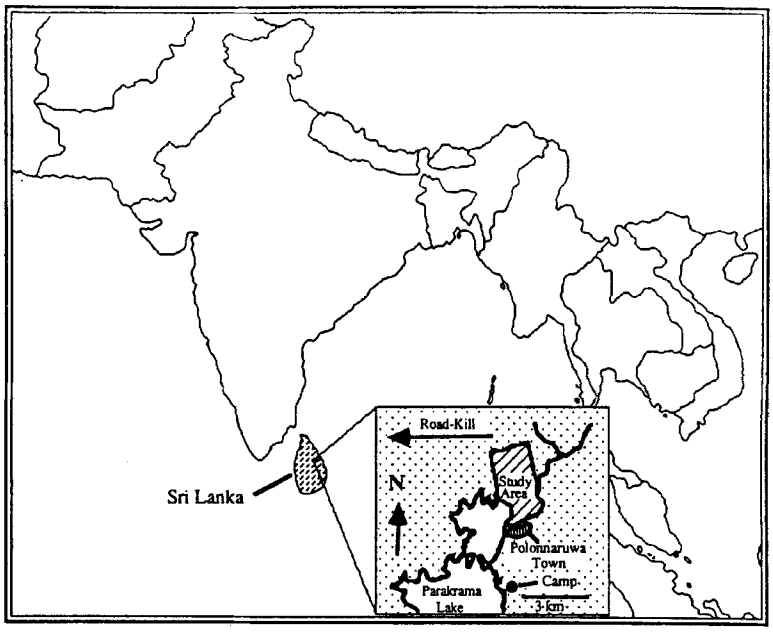

Fig. 1 Map showing the location of the island of Sri Lanka and the study area in Polonnaruwa. The position of the camp, where additional samples were taken, and the direction of the road-kill are also indicated.

were based on known births. The home ranges of all social groups were charted regularly over many years.

Genetic analysis of the Polonnaruwa toque macaques was achieved by obtaining blood samples from members of the study population. Monkeys were trapped during the 1986 and 1987 field seasons using a self-tripping box trap, the details of which can be found in Melnick (1981). The primary modifications to its prior use (Melnick 1984) were a reduction of the habituation period to 36–48 h, 'remote' tripping of the trap doors, and on-site processing of each animal. These changes were made to reduce the overall period of contact with each group, thus reducing the disturbance to behavioural data collection and the likelihood of heat stress, while maintaining the likelihood of trapping every group member.

Once trapped, each animal was coaxed into a squeeze cage, restrained, and tranquilized with ketamine hydrochloride. Tranquilized animals were subjected to several procedures including the collection of blood, saliva, faeces, milk (for adult females), body and head measurements, and other biomedical and epidemiological data (e.g. Cheverud & Dittus 1992; Peiris *et al.* 1993). Upon completion of this work at a mobile processing station within the social group's home range, all members of the group were released simultaneously. Occasionally, a single animal was released early if confinement proved unusually stressful. Finally, infants were monitored closely and rehydrated orally with an intravenous-grade saline solution towards the end of recovery from the tranquilizer. There were no serious injuries to any of the monkeys, nor did any of them succumb to the process.

In total, 13 social groups of toque macaques in the Polonnaruwa population were trapped in 1987. All animals in each group were caught within 24 hours of initiating the trapping. Hence, each trapped group was completely ascertained, and a total of 271 monkeys were sampled for blood. Among these individuals are numerous adult males that had immigrated into these groups. Finally, additional samples were obtained from toque monkeys living near the field camp and one 'road-kill' recovered approximately 20 km to the west of Polonnaruwa.

Using detailed demographic records on the population (Dittus, unpublished.), a total of 46 monkeys were chosen (Table 1) to represent: (1) all 13 groups trapped; (2) most of the matrilines in each of these groups (30 of 38, in total); (3) several individuals from each of a small subset of matrilines to check for homogeneity within matrilines; (4) eight other study groups not trapped – by using males natal to those groups who immigrated into the groups we trapped; and (5) four immigrant males from outside the study area. In total, we were able to characterize the mtDNA genomes of some portion of 19 extant (in 1987) and two extinct social groups, which comprise all of the groups in the southern three-quarters of the study site (Figs 1 and 3).

A sample of one to 14 mL of whole blood, depending upon the weight of the monkey, was collected from the femoral vein into sodium heparin. Blood was kept chilled in the field and was separated into its constituents (plasma, platelets, leukocytes, and erythrocytes) at our permanent field station laboratory some 4 km south of

the study site. Once separated and washed, each blood component was placed in cryogenic tubes and submerged in liquid nitrogen for long-term storage and shipment to the Genetics Laboratory at Columbia University.

Total genomic DNA was isolated by standard phenol, phenol/chloroform and chloroform extractions followed by ethanol precipitation (Sambrook, Fritsch & Maniatis 1989). DNA pellets were dried and dissolved in 1 × TE to provide a final concentration of 500–1500 μg/mL. Approximately 1.5 μg of DNA was used in each restriction enzyme reaction. The DNA samples were cut with a battery of 16 restriction enzymes including *Ava*I, *Bam*HI, *Bcl*I, *Bgl*II, *Bste*II, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*II, *Hinc*II, *Hind*III, *Kpn*I, *Pst*I, *Sst*I and *Xba*I. The products of these reactions were separated according to size by agarose gel electrophoresis in 1 × TBE buffer. The DNA was then transferred to a nylon membrane and fixed by UV cross-linking. The membrane was probed with purified macaque mtDNA labelled with digoxygenin and all labelling, hybridization, washing and visualization procedures were carried out as recommended in the Genius Kit (Boehringer Mannheim). This non-radioactive technique results in a golden-brown precipitate forming directly on the membrane where the probe has bound. The positions of restriction sites for each enzyme and each haplotype were mapped by the double-digestion method (Dowling *et al.* 1990).

A mtDNA haplotype consisted of a unique map of restriction sites revealed by the 16 restriction enzymes listed above. Genetic distances between different haplotypes were calculated based on the maximum likelihood methods of Nei & Tajima (1981, 1983) for restriction site data using the computer algorithm MAXLIKE (courtesy of M. Nei & L. Jin).

Results

Restriction enzyme analysis revealed a total of 58 restriction sites, encompassing 348 nucleotide bases or about 2.1% of the mitochondrial genome. Only two haplotypes were found among 42 individuals born in 21 different social groups and four males of unknown group origin (Table 1, Fig. 2). These two haplotypes differed by an estimated 3.1% of their nucleotide sequence. Haplotype A, which occurs in relatively few groups in the south-west corner of the study site, probably has a wider distribution to the south as we reconstructed that haplotype for the extinct group SG, whose range overlapped the southernmost part of the home ranges of groups A and 22. We also found the same haplotype in an adult female (#003) in a group that frequented our field camp (Fig. 1). Genetic analysis of the 'road-kill' showed that this individual's mtDNA matched haplotype B, suggesting a more extensive distribution of this haplotype to the west.

Table 1 A list of social groups that were sampled for this study*. Group characteristics and mtDNA haplotypes are listed. All individuals sampled were females known to represent different matrilines unless otherwise indicated

Group ID	Date sampled	mtDNA haplotype	Group size‡	No. of matrilines	No. of matrilines sampled
A	7/13/87	A	10	3	2
B†		B	22	5	1
BQ1†		B	47	8	1
BQ2	8/22/87	B	4	1	1
CF	7/27/87	B	9	4	3
Ch	8/18/87	B	11	5	2
D1	7/20/87	A	16	4	4
D2	9/20/87	A	9	1	1
D3	7/6/87	A	13	3	3
F†		B	20	5	1
G	5/13/87	B	21	3	3
H1	8/5/87	A	3	2	2
H2†		B	5	1	1
I	8/3/87	B	9	2	2
J	9/26/87	B	8	2	2
M†		B	23	6	1
O†		B	18	11	1
P†		B	(extinct)	1	1
SG†		B	(extinct)	1	1
22D	8/25/87	B	12	4	2
22N	9/1/87	B	21	4	4

* Four males of unknown origin that had migrated into groups A, Ch, D1 and 22N were also sampled. All of these males exhibited haplotype B.

† Males from known matrilines.

‡ Group size indicates the number of females in the social group at the time of sampling.

Three critical features of the data conform to our expectations based on the maternal inheritance of mtDNA.

1 Females known to belong to the same matriline (i.e. daughter–mother–grandmother; aunt–niece) consistently shared the same mtDNA haplotype.

2 As expected from the patterns of female philopatry and lineage sorting, all comparisons of natal females within groups, regardless of their matrilineal membership, showed them to be identical with regard to their mtDNA haplotypes (n = 11; Table 1).

3 The distribution of haplotypes A and B among social groups is consistent with the known history of group fission in this population (Fig. 3).

Discussion

Despite the geographically limited species range of toque macaques, mtDNA haplotypes differing by 3.1% of their nucleotides coexist. The current distribution of these highly divergent haplotypes is not coincident with any

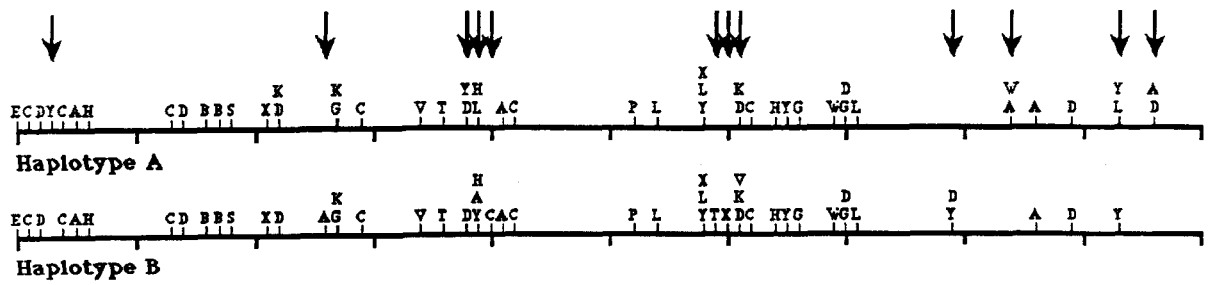

Fig. 2 Restriction site maps for haplotypes A and B found in *M. sinica*. The origin of each mtDNA map coincides with a highly conserved *Eco*RI restriction site found in all macaque species. Restriction endonucleases are denoted as: *Ava*I = A; *Bam*HI = B; *Bcl*I = L; *Bgl*II = G; *Bst*EII = T; *Cla*I = W; *Dra*I = D; *Eco*RI = E; *Eco*RV = V; *Hae*II = Y; *Hin*cII = C; *Hin*dIII = H; *Kpn*I = K; *Pst*I = P; *Sst*I = S; *Xba*I = X.

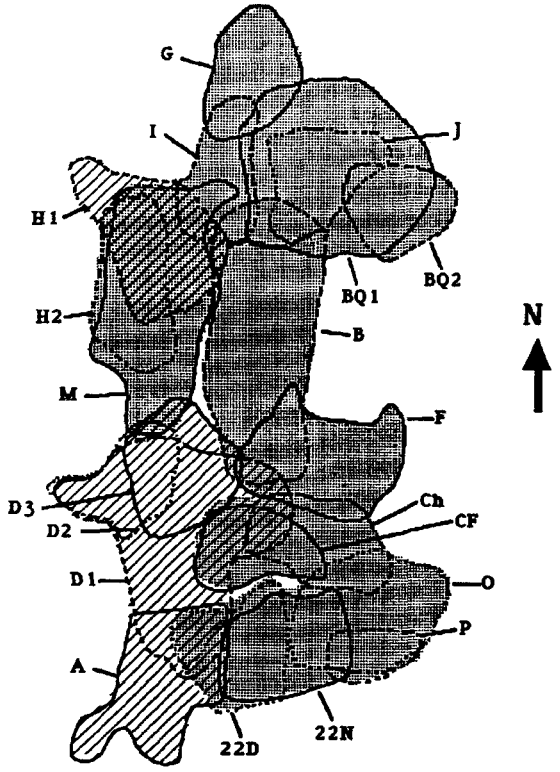

Fig. 3 Map of the home ranges of *M. sinica* social groups in Polonnaruwa. Groups are labelled as in Dittus (1988). Groups with the same prefix (e.g. D1, D2, and D3) are fission products of the same, once larger 'parent' group. The mtDNA haplotypes of females in each group are indicated by the presence of cross-hatching (haplotype A) or stippling (haplotype B).

known geographical barrier to dispersal. Indeed, the two haplotypes are found in neighbouring social groups in Polonnaruwa. These groups are known to exchange males, and therefore nuclear genes, on a regular basis. However, because new groups form primarily through group-fission and female macaques are so highly philopatric, mtDNA haplotypes generally do not vary within social groups or flow between them.

The only social group to include matrilines with both haplotypes A and B was group H, based on our historical reconstruction. However, this may be the exception that proves the rule because the history of group H was unusual in several ways (Dittus 1988). Unlike our records for other groups, the matrilineal history of group H (first censused in 1971) was poorly understood prior to 1975, because it was peripheral to the study area at that time. Group H fissioned in 1976 to form groups H1 (haplotype A) and H2 (haplotype B) (Dittus 1988). This was notable because Group H did not conform to the characteristics of the other groups that were observed to fission. The home range of Group H consisted primarily of marginal habitat for toque monkeys, including scrub forest and abandoned areas of cultivation. The group was smaller than the other fissioning groups and its group size was constant or declining at the time of fission. Thus, group H may have been inherently unstable while other fission groups grew unstable with increasingly large group size. The subordinate fission product, group H1, consisted of just a few individuals that had apparently ranked very low in the dominance hierarchy of group H. Therefore, it is possible that group H1 consisted of two matrilines that had temporarily transferred into group H from outside our study area prior to 1975.

This survey also revealed that social groups containing the same haplotype are clustered in space, so an irregular geographic boundary can be drawn between an area occupied by haplotype A and another area occupied by haplotype B. This is clearly a result of the known history of group fission. More extensive sampling of toque macaques throughout their entire range would be required to determine whether this boundary divides larger regions dominated by these two haplotypes, and whether any additional haplotypes exist in *M. sinica*. However, the haplotype of the road-kill discovered 20 km to the west of Polonnaruwa suggests that the geographic distribution of haplotype B extends well beyond the local population surveyed in this study.

Group-fission limits the dispersal of mtDNA haplotypes in macaques because new groups generally occupy

available habitat nearby to the source group's home range (see Fig. 3; Dittus 1988). This feature of macaque social behaviour promotes the development of regional boundaries to mtDNA lineage sorting in arbitrary places. In other words, when female dispersal distance is severely limited relative to the species' range, lineage sorting is ineffective over large areas due to isolation by distance. A computer simulation study of mtDNA evolution that incorporated many aspects of macaque social structure yielded similar results (Hoelzer *et al.*, unpublished), with different regions occupied by increasingly divergent haplotypes and arbitrary boundaries where the ranges of these different haplotypes abutted.

We do not have sufficient historical information to suggest whether haplotypes A and B developed in sympatry or allopatry, but there is currently a free flow of nuclear genes between groups containing either haplotype (Melnick, unpublished) and across the whole island (Melnick 1988; Shotake *et al.* 1991; Melnick & Hoelzer 1993). Therefore, this study constitutes a direct observation of extensive mtDNA divergence without nuclear genetic isolation (Avise *et al.* 1987 – Category II). This is consistent with findings in other macaque species where the mtDNA genomes in different populations can be quite different even when nuclear genetic variation is approximately evenly distributed among populations (Melnick & Hoelzer 1992, in press; Hoelzer *et al.* 1992; Melnick *et al.* 1993). Although examples of Category II mtDNA distributions are rare, it has been observed in a population of bluegill sunfish (*Lepomis macrochirus*; Avise *et al.* 1984), a population of East African black-backed jackals (Wayne *et al.* 1990) and a population of blue tits (*Parus caeruleus*; Taberlet *et al.* 1992). In these cases, the authors attributed the current situation to secondary contact between previously allopatric populations. However, while allopatry remains one possible explanation for our findings, there is no specific biogeographic or demographic evidence to support the existence of allopatric toque monkey populations in the past. Thus, the possibility of sympatric or parapatric mtDNA divergence remains a plausible hypothesis in this case.

Allowing for a large error associated with an estimation of divergence time (Hillis & Moritz 1990), haplotypes A and B appear to have been diverging over the past 800 000 to 1 600 000 years based on the standard molecular clock rate for primate mtDNA (2–4% per million years: Brown *et al.* 1979, 1982). Indeed, this polymorphism may be as old as the origin of the *M. assamensis/M. thibetana* clade that shared a recent common ancestor with *M. sinica* (Hoelzer & Melnick, in press; Hoelzer *et al.* 1992). If these haplotypes were discovered from individuals living on opposite sides of Sri Lanka, in the absence of data from nuclear markers, current practices might lead the investigators to conclude that the two populations are currently isolated and had been isolated for a very long time. Some might even suggest that such a large difference between mtDNA haplotypes is evidence of two separate species. This would clearly be erroneous, as no reproductive isolation exists at all. It is only the mtDNA, not the nuclear genomes, that has been diverging over this period due to mutation and lineage sorting. Therefore, in a species like the toque monkey the coexistence of divergent mtDNA haplotypes tells us virtually nothing about the isolation of populations, past or present. Similarly, if residents from different locations exhibit highly divergent mtDNA haplotypes, consideration must be given to the possibility that there may be an intermediate location where both haplotypes coexist and nuclear genes flow freely, as illustrated by the toque macaques in Polonnaruwa.

Conclusions

The work presented here represents the first detailed study of the population genetic structure of mtDNA variation in a local population of primates. At least three important features of that structure emerge.

1 Mitochondrial DNA divergence within a population can be quite high, even in the face of extensive nuclear gene flow and broad nuclear genetic homogeneity.

2 The distribution of mtDNA haplotypes is clumped, reflecting the effects of female philopatry and matrilineal social group fission.

3 The level of mtDNA divergence cannot be assumed to reflect the length of time two populations have been separated, because large differences can coexist within a single breeding population. Hence the behaviourally conditioned dynamics of mtDNA gene flow and their resulting effects on mtDNA population structure in macaque monkeys are very different from what one finds in the nuclear genome. These striking differences suggest that one must be cautious in using mtDNA data to estimate time of population divergence and levels of overall genetic difference.

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This research began as a collaboration between Don Melnick, a population geneticist who has worked extensively with mitochondrial DNA variation in macaques, and Wolfgang Dittus, who has been studying the behavioural ecology and population biology of the toque macaque in Sri Lanka for over two decades. Guy Hoelzer and Mary Ashley each conducted postdoctoral research in Melnick's lab and have since established their own research laboratories.
