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PRELIMINARY REPORT ON MITOCHONDRIAL DNA VARIATION IN *MACACA FASCICULARIS* FROM SINGAPORE

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***ABSTRACT.* –** Few studies have examined the molecular relationships of a single natural population of macaque species. This preliminary study sought to conduct a phylogenetic analysis of mitochondrial DNA (mtDNA) variation in Singapore's macaque population and to describe the geographic structure of that variation. Hair samples were obtained from wild *Macaca fascicularis fascicularis* (n=54) trapped at multiple locations in the Bukit Timah Nature Reserve (BTNR) and the Central Catchment Nature Reserve (CCNR). The mtDNA sequence data obtained from the hair samples were used to generate phylogenetic trees using the neighbor joining and maximum parsimony methods. Based on mtDNA fragments, two groupings were apparent within Singapore, one from BTNR and the other from CCNR. Neighbor joining bootstrap values between 99% (COII) and 82% (rRNA 12S) supported this finding. With the exception of the rRNA 12S fragment, similarly strong bootstrap support was observed for the maximum parsimony analysis. The apparent differentiation within Singapore might be due to some form of random drift, such as founder effect, associated with habitat fragmentation stemming from artificial modification of the landscape which has occurred since the founding of Singapore in 1819. Fragmentation of the tropical lowland rainforest, therefore, seems to have resulted in two primary sub-populations of macaques on the island.

***KEY WORDS.* –** Long-tailed macaque, phylogeny, mitochondrial DNA, Singapore.

INTRODUCTION

A number of studies have examined the molecular phylogenetic relationships among macaque species or populations across relatively large geographic areas of Southeast Asia (e.g., Blancher et al., 2008; Chakraborty et al., 2007; Chu et al., 2007; Evans et al., 1999, 2001; Hayasaka et al., 1996; Li and Zhang, 2005; Morales and Melnick, 1998; Smith et al., 2007; Tosi et al., 2000, 2003), or have used molecular phylogenies to estimate the geographic origins of non-endemic human-introduced macaque populations (Kawamoto et al., 2007; Stevison and Kohn, 2008; Tosi and Coke, 2007). Few studies, however, have focused on a single endemic population of macaques (see Hoelzer et al., 1994). In this report we examine mitochondrial DNA (mtDNA) variation in an insular population of long-tailed macaques from Singapore, a relatively small and highly urbanized island nation.

The island of Singapore would have been covered largely with lowland evergreen rain forest when it was founded in 1819 (Corlett 1992). Since then, the natural landscape has been largely modified through various land use decisions in both historical and modern times. The initial clearance of primary

forest on the island after its founding was for the planting of gambier (*Uncaria gambir*), a cash crop with medicinal properties, and also used in the leather industry (Corlett & Turner, 1997). Most of the existing forest fragments in Singapore have been isolated for some 150 years (Corlett & Turner, 1997). Much of the deforestation occurred under British rule from 1819 to 1900 (Corlett & Turner, 1997), a period which saw rapid economic development and population growth. From 1884, the majority of the larger forest patches were incorporated into forest reserves (Corlett 1992). All but the central part of Singapore's forests (totaling some 1600 ha) was eventually abandoned, and the current rain forest nature reserves retain most of the remaining primary forest patches on the island (Corlett & Turner, 1997). However, the construction of roads, reservoirs, golf courses and military facilities further fragmented the forests of the nature reserves, thereby creating barriers to primate dispersal. Currently, the two largest forested nature reserves (BTNR and CCNR) are separated by the Bukit Timah Expressway, a large 6 lane road built in 1985 which experiences a high volume of vehicular traffic.

Despite its size and urbanicity, Singapore is home to three genera of nonhuman primates (*Macaca* sp., *Presbytis* sp. and *Nycticebus* sp.), and a number of other members of the grand order Archonta, including tree shrews (Order Scandentia, Tupaiidae: *Tupaia glis*), colugos (Order Dermoptera, Cynocephalidae: *Galeopterus variegatus*), and 24 extant species of bat (Order Chiroptera).

Among the nonhuman primate taxa, the long-tailed macaque [*Macaca fascicularis* (Raffles, 1821)], is by far the most numerous. A census conducted in 2007 (Sha, 2009a) estimated there were between 1,218 and 1,454 macaques on the island (also see Agoramoorthy & Hsu, 2006). Singapore offers a unique and highly urban ecology for its macaques which consists of a patchwork of small primary and secondary forest fragments, disturbed forests, urban parks and even very small forested patches within and adjacent to residential neighborhoods. About 70% of the island's macaque population resides in Bukit Timah and Central Catchment Nature Reserves which comprise a series of forests and reservoirs located near the center of the island (Sha et al., 2009a, 2009b). As recently suggested by Sha et al. (2009), the majority of the island's macaque population is confined to these two nature reserves, likely as a result of roads and highways.

Recent research on Singapore's long-tailed macaques has focused on morphological variation (Schillaci et al., 2007), bidirectional disease transmission (Jones-Engel et al., 2006), and human-macaque interactions (Fuentes et al., 2008; Sha et al., 2009a, 2009b). Macaques from Singapore have also been included in the phylogenetic analysis of simian foamy virus (Jones-Engel et al., 2008). To date, however, there has not been an analysis of the molecular genetic variation of Singapore's macaques. The primary goal of the present research is to conduct a phylogenetic analysis of mitochondrial DNA variation of Singapore's macaque population and to examine the geographic structure of that variation.

MATERIALS AND METHODS

DNA extraction, PCR amplification and DNA sequencing. – In this study, hair samples were collected from 54 individuals of *M. fascicularis* trapped at six different locations within the Central Catchment Nature Reserve (CCNR) and two locations within the Bukit Timah Nature Reserve (BTNR) in Singapore (Fig. 1). Each location corresponds to a separate social group or troop. For the purposes of this paper we consider all macaque troops on the island to comprise a single potential breeding population. Hairs were plucked using gloved hands, placed into sterile tubes and stored in a cool, dark place. Approximately 5-6 hairs from each individual were cut with sterile scissors directly into a 1.5 ml microfuge tube; each section of cut hair was approximately 4 mm in length and did not include the root. Total genomic DNA was extracted from each sample with the following buffer: 1M Tris-HCl (pH 8.0), 0.5 M NaCl, 0.5 M EDTA, 0.2% SDS, 0.039 M DTTand 0.1 mg/mL proteinase K (Promega). The reactions were incubated overnight at 56ºC with rigorous shaking (1000 rpm). Following the addition of 0.10% sodium acetate, the DNA was precipitated with 2.5 volumes of chilled 100% ethanol and a 30 minute incubation period on ice. All the DNA samples were furthered purified using the HiYield Gel/PCR DNA Extraction Kit (Real Biotech Corp.) according to manufacturer's instructions.

Using the DNA samples, sections of five mitochondrial genes were amplified via the polymerase chain reaction (PCR). PCR was performed using primers for 12s rRNA, $tRNA^{Glu}$- cytochrome b and cytochrome oxidase I, II and III, as previously described by Li and Zhang (2005). The target sequence of each primer pair and its location relative to the reference mitochondrial gene is shown in Table 1.

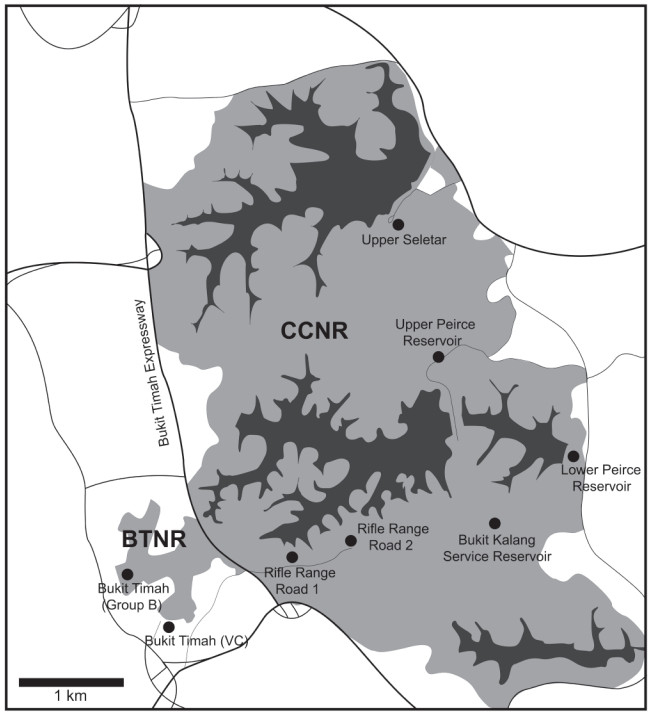

Fig. 1. Map of sample locations within the Bukit Timah (BTNR) and Central Catchment (CCNR) Nature Reserves.

Table 1. Size of the amplified sequences and their location relative to the mitochondrial gene sequence.

Mitochondrial gene	base pairs (bp)	Amplified sequence location	Amplicon size (bp)
12s rRNA	948	257–677	421
cytochrome oxidase I (COI)	1539	698–1113	416
cytochrome oxidase II (COII)	684	39–533	495
cytochrome oxidase III (COIII)	784	169–660	492
$tRNA^{Glu}$- cytochrome b (cytb)	1210	52–471	420

Table 2. Genbank DNA sequences included in phylogenetic analyses.

Taxon	Origin	GenBank accession number
Macaca fascicularis	Vietnam	AY685714–AY685717, AY685774, AY685775, AY685777, AY685796–AY685799, AY685837–AY685840, AY685878–AY685881
M. fascicularis	Not specified	M58008
M. mulatta	India	AY685718, AY685769, AY685800, AY685841
M. mulatta	Fujian, China	AY685720–AY685722, AY685802–AY685804, AY685843–AY685845, AY685884–AY685886
M. mulatta	Hainan, China	AY685787
M. mulatta	Myanmar	AY685783–AY685785, AY685809, AY685727, AY685891, AY685850
M. sylvanus	Not specified	NC_002764

Amplification of some genes was unsuccessful for some individuals.

Each PCR reaction had a total volume of 25 µl and contained 1x PCR Buffer (Invitrogen), 200 mM of each dNTP (Invitrogen), 2 mM $MgCl_2$, 2 U Platinum *Taq* DNA Polymerase (Invitrogen), 0.2 µM of each forward and reverse primer and sterile water. Approximately 1µg of DNA sample was used per reaction. The PCR cycling parameters were an initial denaturation at 94^0C for 2 minutes then 45 cycles of 94^0C for 30 seconds, 60^0C for 1 minute and 72^0C for 2 minutes. The PCR products were purified with an ethanol-sodium acetate procedure similar to the one described above.

DNA sequencing was performed using the Big Dye Terminator Cycle Sequencing (3.1) kit (Applied Biosystems) along with the same primers used in PCR. Each 10 µl sequencing reaction consisted of 3 µl of Big Dye Terminator Cycle Sequencing Ready Reaction Mix, 0.3 µM of either forward or reverse primer and approximately 1 µg of purified DNA. The sequencing cycling parameters were the same to those stated for PCR. The sequencing reactions were purified the same way the PCR reactions were prior to sequencing. The sequences were analyzed by the ABI 3100 Genetic Analyzer. The sequences were edited and aligned using BioEdit Sequence Alignment Editor. Along with the 41 *M. fascicularis* samples described here, *M. sylvanus*, *M. mulatta* and additional *M. fascicularis* sequences from the GenBank were also included in downstream analyses (Table 2).

Data analysis. **–** Although the five mtDNA sequences are inherited as effectively one locus without recombination and can be assumed to have experienced the same branching history, we were not able to combine the five gene fragments into a single analysis (see Li & Zhang 2005: 379) because we were constrained by the availability of comparative sequences from the GenBank. We used the sequence data to generate phylogenetic trees using the neighbor joining (NJ) and Maximum Parsimony (MP) methods (Saitou & Nei, 1987). Following Chu et al. (2007: 420), when constructing our NJ phylogenetic trees we used a simple p-distance model because genetic distances estimated using more sophisticated models often generate large variances which reduce the resolution of bootstrapped tree topologies based on short nucleotide sequences (see Nei & Kumar, 2000). Bootstrap support for nodes was estimated using 2000 replications (Felsenstein, 1985). For all trees *M. sylvanus* was used as the outgroup. All positions containing gaps and missing data were excluded. For the MP phylogenetic analysis each three represented a most parsimonious tree from the Close-Neighbor-Interchange algorithm (Nei & Kumar, 2000), with a search level of 2. The initial trees were obtained with the random addition of sequences with 100 replicates. The branch lengths were calculated using the average pathway method. Bootstrap values reflect the percentage of replicate trees in which the associated taxa clustered together after 100 replicates (Felsenstein, 1985).

Nucleotide diversity (π) and average within-group evolutionary divergence (d_x) were calculated as the number of base differences per site from averaging over all sequence pairs (Tamura et al., 2007). Standard errors for within-group divergence estimates were obtained by a bootstrapping procedure using 500 replications. The neutral mutation model for each gene fragment for the pooled *M. fascicularis* sample was examined using Tajima's test of neutrality (Tajima, 1989). All analyses were conducted using the MEGA4 computer software package (Tamura et al., 2007).

Table 3. Results from Tajima's neutrality tests for *Macaca fascicularis* from Singapore and Vietnam. M: number of sites; S: number of segregating sites; p_s = S/M; θ = ps/a1; π: nucleotide diversity; D: Tajima's test statistic (Tajima, 1989).

	M	S	p_s	θ	π	D
Cytb	52	14	0.03302	0.00731	0.00665	-0.26942
12s rRNA	39	11	0.02619	0.00619	0.00356	-1.30001
COI	54	14	0.03365	0.00739	0.00504	-0.95020
COII[1]	55	11	0.02222	0.00485	0.00317	-0.99637
COIII	46	26	0.05295	0.01205	0.00673	-1.46814

[1]The M58008 sequence of unknown provenance was excluded.

RESULTS AND DISCUSSION

The results from Tajima's neutrality tests indicated the neutral mutation hypothesis can explain the observed mtDNA polymorphisms for the combined grouping of *M. fascicularis* for all five gene fragments (Table 3). The NJ and MP phylogenetic analyses yielded trees with almost identical topologies (cf. Figs. 2 & 3). For each of the five mitochondrial genes amplified, the macaques from Singapore formed a monophyletic grouping relative to the small comparative sample of *M. fascicularis* from Vietnam. Bootstrap support values for the nodes forming the Singapore monophyletic groupings varied from 100–98% (cytb, COI, COII, COIII) to 68% (12s rRNA) for the NJ phylogenies, and from 89–99% (cytb, COI, COII, COIII) to 40% (12s rRNA) for the MP phylogenies. Four of five bootstrap values therefore strongly support the inference of monophyly, with values ≥ 89%. Consistency and retention indexes of greater

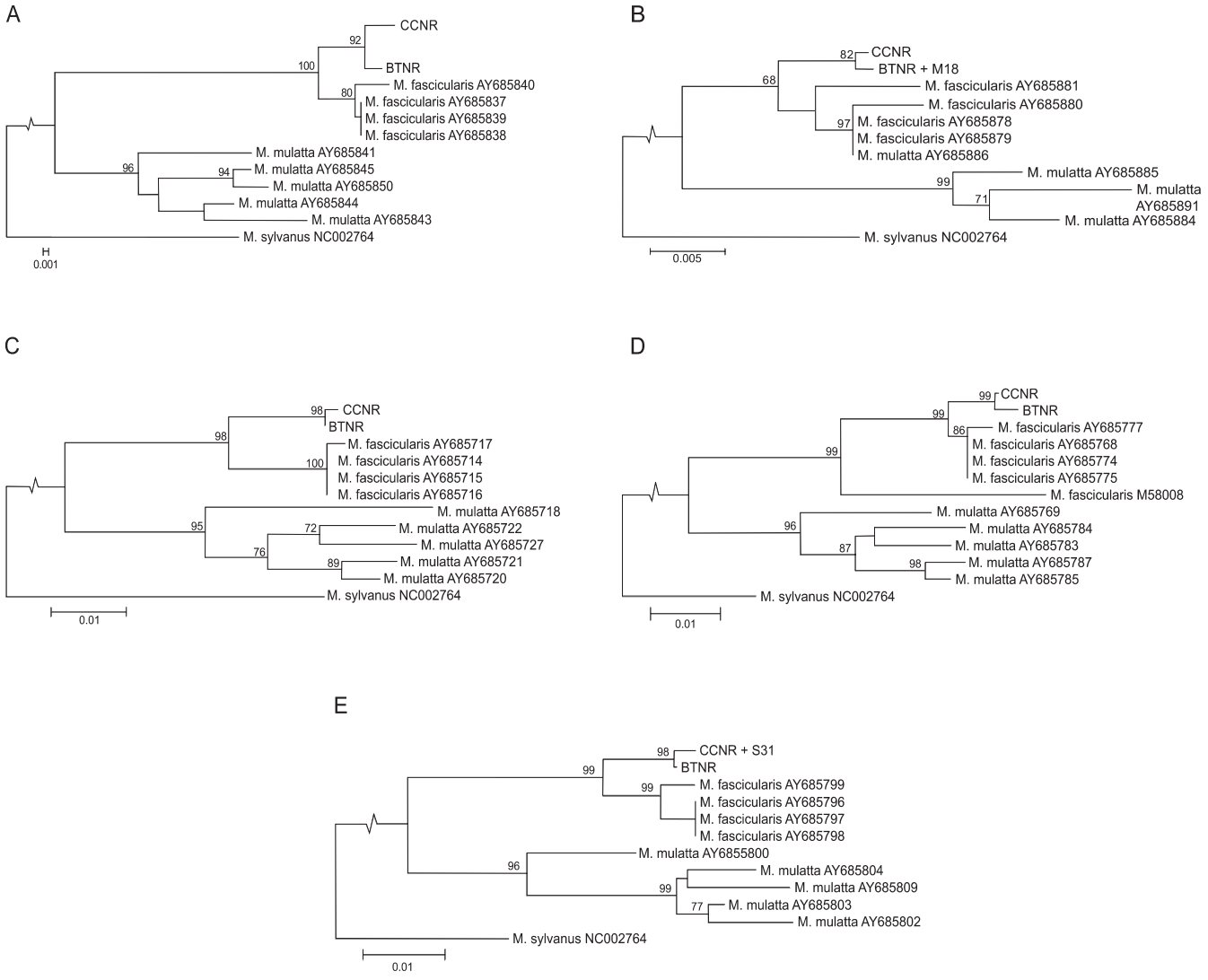

Fig. 2. Neighbor-joining trees for each of five mtDNA gene fragments including: A, cytochrome b; B, 12s rRNA; C, COI; D, COII; E, COIII. Bootstrap support values are presented at each node.

Table 4. Statistics for the most parsimonious tree used in this MP analysis based on the parsimony-informative sites.

	Number of trees evaluated	Consistency Index	Retention Index	Composite Index	Number of Positions	Parsimony Informative Positions
Cytb	6258	0.672131	0.874214	0.587586	409	40
12s rRNA	2473	0.750000	0.895522	0.671642	414	19
COI	9773	0.661538	0.862500	0.570577	410	42
COII	7719	0.636364	0.862069	0.548589	493	62
COIII	7829	0.716216	0.905405	0.648466	484	51

than 0.5 for the MP analysis indicate that the phylogenetic reconstructions are largely unaffected by recurrent mutation and parallel evolution (Feris, 1989) (Table 4). It is important to state, however, that the present study is limited by the number of comparative sequences available in the GeneBank, precluding important comparisons with macaques from the Malay Peninsula and the Indonesian archipelago.

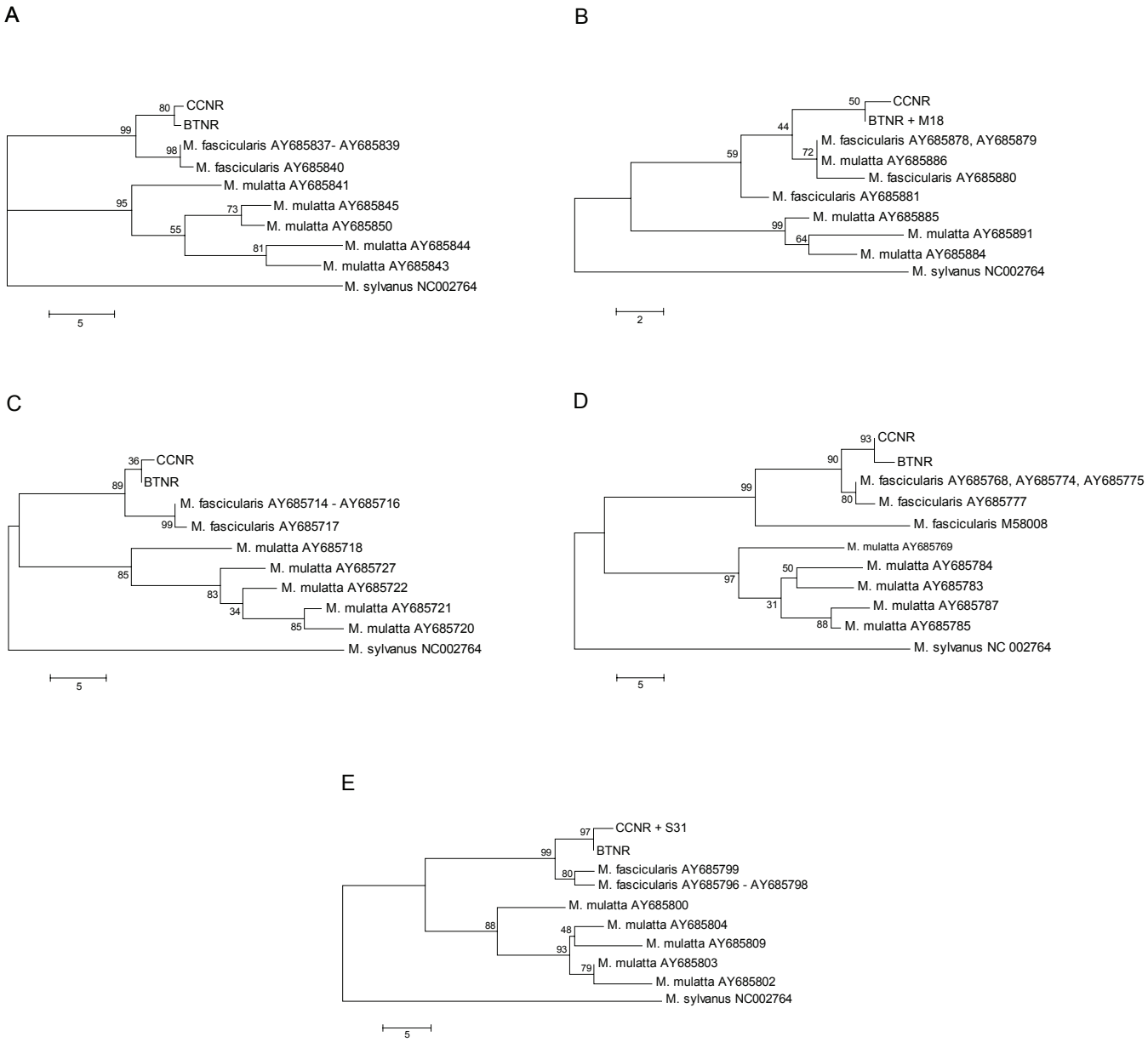

Fig. 3. Maximum parsimony trees for each of five mtDNA gene fragments including: A, cytochrome b; B, 12s rRNA; C, COI; D, COII; E, COIII. Bootstrap support values are presented at each node.

Table 5. Average evolutionary divergence (d_x) within macaque groupings for 5 mtDNA gene fragments.

	Macaca. fascicularis				*Macaca mulatta*	
	Singapore		Vietnam			
	d_x	SE	d_x	SE	d_x	SE
Cytb	0.00397	0.00165	0.00354	0.00195	0.03538	0.00556
12s rRNA	0.00114	0.00097	0.00716	0.00275	0.02546	0.00528
COI	0.00154	0.00095	0.00120	0.00115	0.03750	0.00646
COII[1]	0.00178	0.00113	0.00202	0.00135	0.03475	0.00530
COIII	0.00390	0.00127	0.00407	0.00189	0.02846	0.00473

[1]The M58008 *M. fascicularis* sequence of unknown provenance was excluded.

With the exception of the 12s rRNA gene fragment, the *M. fascicularis* grouping exhibited similar levels of within-group evolutionary divergence as the Vietnam grouping (Table 5). The observed within-group divergence estimates for both *M. fascicularis* groupings were much less than that observed for *M. mulatta*. For all mtDNA gene fragments, two groupings were apparent within Singapore, one from BTNR and the other from CCNR. With the exception of one individual for each of two gene fragments (12s rRNA, COIII) these groupings were monophyletic (Appendix). Statistical support for these groupings varied with NJ bootstrap values between 99% (COII) and 82% (12s rRNA). Lower bootstrap support values for these groupings were observed for the MP trees.

Macaque social organization is characterized by strict female philopatry with obligatory male migration at adulthood. Very large troops may eventually split into separate troops according to matrilines, occupying separate geographic home ranges. Because mtDNA is maternally inherited, macaque social organization produces geographically restricted mitochondrial DNA variation with marked intergroup heterogeneity (Melnick & Hoelzer, 1992). As discussed by Hoelzer et al. (1994: 451), mitochondrial genome divergence begins when a single mutation differentiates a copy of the genome from the ancestral sequence. This differentiated copy is a non-recombinant clone which will accumulate mutations over evolutionary time (Hoelzer et al., 1994). Hoelzer et al. (1994) point out that this accumulation of mutations can produce substantial divergence within a species, even within populations, without reproductive isolation. For the present study, it seems unlikely that the divergence observed between the two Singapore sub-populations for each of 5 mtDNA gene fragments would correspond to a geographic barrier merely by chance. It seems possible, therefore, that the smaller BTNR group (n≈125; see Appendix in Sha et al. 2009a), is a subset of the larger original CCNR sub-population (n≈902; see Appendix in Sha et al. 2009a), that may have become cut off by forest fragmentation associated with the artificial modification or remodification of the landscape surrounding the Bukit Timah area. For example, the construction of the Bukit Timah Expressway may have resulted in some form of random drift such as founder effect for the BTNR group. Under this scenario, random drift, in conjunction with isolation from any possible migrating females, resulted in two differentiated mtDNA sub-populations. It is important to note, however, that isolation and random drift could have resulted from any number of changes to the landscape associated with the historical development of Singapore.

CONCLUSION

In conclusion, though subject to important limitations stemming from a dearth of comparative sequences, the results of our preliminary study seem to suggest the possibility of monophyly for the long-tailed macaques from Singapore relative to other Southeast Asian populations for five different mitochondrial gene fragments. Additional analyses with a larger comparative sample, however, are needed to more adequately assess the possibility of monophyly. Our phylogenetic analysis also indicated there are two largely monophyletic sub-populations within Singapore corresponding to two forested nature reserves.The observed mtDNA differentiation may be attributable to a subset (i.e., BTNR) of the larger CCNR sub-population becoming isolated by the building of the Bukit Timah Expressway, or some other artificial modification of the landscape. This isolation, in conjunction with random genetic drift is likely responsible for the observed geographically mediated population structure within Singapore.

Future phylogenetic analyses of mtDNA variation in Singapore's macaques should include a larger set of comparative sequences. Specifically, sequences from *M. fascicularis* populations in Indonesia, Malaysia and Thailand, particularly from populations on the Malay Peninsula south of the Isthmus of Kra, are needed to more robustly determine the phylogenetic position of the Singapore population.

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Appendix. Sample information and NJ and MP phylogenetic group affiliation (CC=CCNR, BT=BTNR).

ID#	Sex	Age	Location (Troop)	Cytb	rRNA	COI	COII	COIII
S18	F	Juvenile	Bukit Timah (VC group)	BT	BT	BT		
S21	F	Juvenile	Riffle Range Road (Mid-way)	CC	CC			
S23	M	Juvenile	Riffle Range Road (Mid-way)	CC	CC	CC		
S24	F	Adult	Riffle Range Road (Mid-way)	CC	CC	CC		
S25	M	Adult	Riffle Range Road (Mid-way)	CC				
S26	M	Adult	Riffle Range Road (End)	CC	CC	CC		
S27	M'	Juvenile	Riffle Range Road (End)	CC	CC	CC		
S28	M	Juvenile	Riffle Range Road (End)	CC	CC	CC	CC	
S29	M	Adult	Riffle Range Road (End)	CC	CC	CC	CC	CC
S30	F	Juvenile	Riffle Range Road (End)	CC	CC	CC	CC	
S31	F	Adult	Bukit Timah (group B)	BT	BT	BT	BT	CC
S32	F	Adult	Bukit Timah (group B)	BT	BT	BT		
S33	F	Adult	Bukit Timah (group B)	BT				
S34	F'	Juvenile	Bukit Timah (group B)	BT	BT	BT	BT	BT
S35	M	Juvenile	Bukit Timah (group B)	BT	BT	BT	BT	BT
S37'	F	Juvenile	Bukit Timah (group B)	BT	BT	BT	BT	
S38	M	Subadult	Bukit Timah (group B)	BT	BT	BT		
S39			Bukit Timah (group B)	BT	BT	BT	BT	BT
M1	M'	Juvenile	Bukit Timah (VC group)	BT	BT	BT	BT	BT
M2	M	Juvenile	Bukit Timah (VC group)	BT	BT	BT	BT	
M3	F	Juvenile	Bukit Timah (VC group)	BT	BT	BT	BT	BT
M4	M	Juvenile	Bukit Timah (VC group)	BT				
M5	M	Juvenile	Bukit Timah (VC group)	BT	BT	BT	BT	BT
M6	F	Adult'	Riffle Range Road (End)	CC	CC	CC	CC	CC
M7	F	Adult	Riffle Range Road (End)	CC	CC	CC	CC	
M8	F	Juvenile	Riffle Range Road (End)	CC	CC	CC	CC	CC
M9	M	Juvenile	Riffle Range Road (End)	CC	CC	CC	CC	
M10	F	Adult	Riffle Range Road (End)	CC	CC	CC	CC	
M11	M	Adult	Upper Peirce Reservoir (Road)	CC	CC	CC	CC	CC
M12	M	Subdult	Upper Peirce Reservoir (Road)	CC	CC	CC	CC	CC
M13	M	Juvenile	Upper Peirce Reservoir (Road)	CC	CC	CC	CC	
M14	M	Adult	Lower Peirce Reservoir	CC	CC	CC	CC	CC
M16	F	Subdult	Lower Peirce Reservoir	CC	CC	CC	CC	CC
M17	M	Infant	Lower Peirce Reservoir	CC	CC	CC	CC	CC
M18	M	Juvenile	Lower Peirce Reservoir	CC	BT	CC	CC	CC
M19	M	Adult	Bukit Kalang Service Reservoir	CC	CC	CC	CC	
M20	F	Adult	Bukit Kalang Service Reservoir	CC	CC	CC	CC	CC
M21	M	Juvenile	Bukit Kalang Service Reservoir	CC	CC	CC		
M22	F	Juvenile	Bukit Kalang Service Reservoir	CC	CC	CC	CC	CC
M23	M	Adult	Upper Seletar	CC	CC	CC	CC	CC
M24	F	Juvenile	Upper Seletar	CC	CC	CC	CC	CC
M25	M	Juvenile	Upper Seletar	CC	CC	CC	CC	CC
M26	M	Adult	Upper Seletar	CC	CC	CC	CC	CC
M27	M	Juvenile	Upper Seletar	CC	CC	CC	CC	
M28	M	Juvenile	Upper Seletar	CC	CC	CC	CC	CC
M29	M	Juvenile	Upper Seletar	CC	CC	CC	CC	CC
M30	F	Juvenile	Upper Seletar	CC	CC	CC	CC	CC
M31	F	Juvenile	Upper Seletar	CC	CC	CC	CC	
M32	M	Juvenile	Upper Seletar	CC	CC	CC	CC	CC
M33	M	Adult	Lower Peirce Reservoir	CC	CC	CC	CC	
M34	F	Subdult	Lower Peirce Reservoir	CC	CC	CC	CC	CC
M35	F	Juvenile	Lower Peirce Reservoir	CC	CC	CC	CC	CC
M36	M	Juvenile	Lower Peirce Reservoir	CC	CC	CC	CC	CC
M37	M	Juvenile	Lower Peirce Reservoir	CC	CC	CC	CC	