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Mitochondrial DNA variation in Tibetan macaque (*Macaca thibetana*)

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Abstract. Tibetan macaques (*Macaca thibetana*) are a threatened primate species endemic to China. The current taxonomy of the species is based on external morphological and anatomical variations. To further understand the intraspecific variation and relationships among populations, we analyzed 44 mitochondrial DNA control region sequences (475 bp fragment) from individuals across the species range. Results revealed 11 major haplotypes with a high nucleotide diversity (0.792), but nucleotide diversity within haplotype lineages was only 0.042. Neighbor-joining phylogenetic analyses indicated support for four distinct haplotype clades corresponding to regional groups consistent with the recognized subspecies *M .t. thibetana, M. t. guizhonensis*, *M. t. huangshanensis* and *M. t. pullus*. As a result of regional geographic variation and genetic differences, we recommend the four subspecies should be considered different management units for conservation efforts.

Key words: phylogeny, subspecies, taxonomy

Introduction

Tibetan macaque (*Macaca thibetana*), also known as the Chinese stump-tailed macaque or Milne-Edwards' macaque, occurs in east-central China. It is found in China from 25–33° N, 102°30'–119°30' E; the range extends south into Guangxi at 23°48' N, about 110° E, and as far west as the Yangtze Gorge in western and northwestern Sichuan (Groves 2001). It is commonly considered as an endemic monkey species in China and occupies subtropical, deciduous and broadleaf evergreen forests, between 800 and 2500 m above sea level (Jiang et al. 1996, Wang 1998).

Its historic range has retreated in response farmland development and associated pressures resulting in drastically decreased population abundance and local extirpation (Wang 2000). The most serious threat to the Tibetan macaque comes from humans, including habitat destruction, herbicide and pesticide poisoning, human-transmitted diseases and illegal poaching. Tibetan macaques are protected by Chinese wildlife law, and listed on Appendix II of the Conventionon International Trade in Endangered Species.

The genus *Macaca* includes 19 different species, which have been divided into four species groups according to morphological data (Fooden 1976, 1983). Tibetan macaques are classified within the *sinica* group, and closely related to the Assamese macaque (*M. assamensis*) (Eudey 1980, Deinard & Smith 2001). *M. thibetana* is divided into four subspecies (*M. t. thibetana, M. t. pullus, M. t. huangshanensis*, and *M. t. guizhouensis*) based on morphological trait analysis (Jiang et al. 1996, Groves 2005) (Table 1). In order to provide effective management for this endangered species, it is necessary to understand its genetic diversity. Previous studies reported on genetic variation of *M. t. huangshanensis* (Liu et al. 2006) and *M. t. thibetana* (Li et al. 2008), however, there are no molecular data to test the genetic variation and the specific phylogenetic relationships among all the four subspecies.

To get further understanding of genetic variation and phylogeny of *M. thibetana*, we analysed 44 mitochondrial DNA control region sequences which represent all taxa of the macaques. Objectives in this study will be: (1) to test the genetic variation and phylogeny relations of *M. thibetana*, (2) to define

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Table 1. *The subspecies and distribution of* Macaca thibetana *taxonomy after Jiang et al. (1996).*

Subspecies	Abbreviation	Distribution
Macaca thibetana thibetana (Milne-Edwards, 1870)	*M. t. t.*	Northern and western Sichuan, southwestern Shanxi
Macaca thibetana pullus (Howell, 1928)	*M. t. p.*	Northern and eastern Fujian, eastern Jiangxi, southern Zhejiang, northern Guangdong, Zhangyi of Hunan
Macaca thibetana guizhouensis (Jiang et al., 1996)	*M. t. g.*	Southern and eastern Guizhou, northeastern Yunnan, and West Hunan
Macaca thibetana huangshanensis (Jiang et al., 1996)	*M. t. h.*	Southern Anhui

Table 2. *Subspecies, samples origin, samples type, GenBank accession numbers (NO.).*

Subspecies	Samples origin	NO.	Samples type	References
M. t. t.	Mt. Emei, Sichuan	HM754574–HM754580	Feces (7)	This study
	Tangjiahe, Sichuan	HM754581, HM754582	Feces (2)	This study
	Mabian, Sichuan	EU687449, EU687448	Sequence(2)	Li et al. (2008)
M. t. p.	Mt. Wuyi, Fujian	HM754595–HM754603	Feces (9)	This study
M. t. h.	Mt. Hangshan, Anhui	HM754583–HM754594	Feces (12)	This study
	Mt. Hangshan, Anhui	DQ335818, DQ335817 DQ335816	Sequence(3)	Liu et al. (2006)
M. t. g.	Jiangkou,Guizhou	HM754604–HM754612	Feces (9)	This study

which groups should be protected as independent management units.

Study Area

The sampling sites and the sites information were presented in Table 2 and Fig. 1. Mt. Emei is located in the west of Sichuan province (103.3° E, 29.5° N), Tangjiahe National Nature Reserve is located in the north of Sichuan province (104.7° E, 32.5° N), Mt. Wuyi is located in the north of Fujian province (117.6° E, 27.8° N), Mt. Huangshan is located in the south of Anhui province (118.2° E, 30.5° N), and the county named Jiangkou is located in the north of Guizhou province (108.7° E, 27.6° N).

Materials

This species is almost never seen in captivity outside of Asia, and it is quite rare in zoos even there. It is also difficult to collect fecal samples in the wild. We collected feces from 39 individuals, representing individuals across their range, and 5 sequences were retrieved from GenBank. Two of the five GenBank samples belong to an *M. t. thibetana* population (Li et al. 2008), and the other three belong to *M. t. huangshanensis* population (Liu et al. 2006). The fresh fecal samples were gathered in the wild and preserved in 80% or absolute ethanol, then stored at -20°C.

Methods

DNA extraction

Total genomic DNA was extracted from feces using the standard phenol/chloroform protocols (Zhao & Li 2005). The products were inspected on 1.2% agarose gel.

PCR reactions and sequencing

The mtDNA control region was amplified using

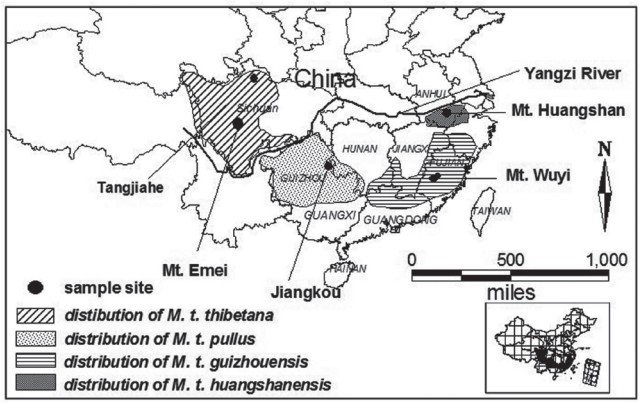

Fig. 1. *The distribution of* Macaca thibetana *in China and our sampling sites.*

the PCR primers LqqF (TCCTAGGGCAATCA GAAAGAAAG) and TDKD (CCTGAAGTAGGA ACCAGATG) (Li & Zhang 2004). Each mixture included at least 25ng DNA template, 5ul 10X reaction buffer, 1 pmol/L forward and reverse primer, 2 mmol/L mixed dNTPs, 3 mmol/L $MgCl_2$, 0.25 unit of Taq polymerase, adding sterile distilled water to 50ul. Amplification was performed on a RoboCycler Gradient 40 (Stratagene) thermal cycler. The PCR procedure began with an initial 2 min denaturation at 95°C; followed by 35 cycles of 1 min denaturation at 94°C, 1 min reannealing at 60°C and 1 min extension at 72°C; a 5 min final extension at 72°C. The PCR products were tested using agarose gel electrophoresis and purified with the Gel Extraction Mini Kit (Warson Co, Shanghai, China) following the manufacturer's instructions. Purified PCR products were labeled using the BigDyeTM Terminator cycle sequencing kits (PE Biosystems, Foster City, CA) with an ABI377 DNA automatic sequencer using the same primers as used in the PCR reactions. Cycle sequencing reactions were performed following the instructions provided by the manufacturer.

DNA analysis

44 mitochondrial DNA control region sequences were evaluated. GenBank accession numbers are reported in Table 2. Sequences were aligned using the program Clustal X (Thompson et al. 1997). Genetic diversity was estimated using haplotype diversity (Hd) and nucleotide diversity (Pi), genetic diversity and the genotype were calculated using DnaSP Version 4.10 (Rozas et al. 2003). Genetic distances were calculated via Kimura's 2-parameter method (Kimura 1980) in MEGA3.1. Phylogenetic tree was conducted via Neighbor-Joining (NJ) according to mitochondrial DNA sequences in MEGA3.1. The NJ trees were reconstructed using the Kimura's 2-parameter evolutionary models and tested with 1000 bootstrap replicates (Kumar et al. 2004). *M. assamensis* was the outgroup in the phylogenetic analysis. Analysis of molecular variance (AMOVA) was carried out to assess differentiation between geographical units using Arlequin 3.1.

Results

For all individuals, 475-bp of the mitochondrial DNA control region sequences were examined. The overall base composition of the sequences were A, 30.9%; G, 14.0%; T, 26.6% and C, 28.5%. On the basis of genetic diversity analysis, the haplotype diversity within the four subspecies is lower than the total haplotype diversity (Hd = 0.792) of *M. thibetana,* and the nucleotide diversity (Pi = 0.042) of the Tibetan macaque is low. Both the haplotype diversity and the nucleotide diversity of *M. t. thibetana* (Hd = 0.576, Pi = 0.024) is higher the other three subspecies (*M. t. pullus:* Hd = 0.222, Pi = 0.002; *M. t. guizhouensis:* Hd = 0.478, Pi = 0.006; *M. t. huangshanensis:* Hd = 0.341, Pi = 0.004).

The calculation of genetic distances shows: (1) Mean genetic distances between four populations (0.0115 –0.1367) of *M. thibetana* are greater than the mean genetic distances within each population (0.0053–0.0019). (2) It must be noted that the mean genetic distances within *M. t. thibetana* population are greater than that between any two of other three subspecies. The genetic differentiation degree (Fst) among the four subspecies is significant ($P < 0.05$) (Table 3). Among the 44 sequences of the *M. thibetana* examined, 11 haplotypes were identified. Haplotype distribution is indicated in Table 4, and the four subspecies do not share haplotypes. In these haplotypes, four were present in *M. t. thibetana* population (Mt. Emei: Hap1, Hap2; Tangjiahe: Hap1; Mabian: Hap3, Hap4) and three in *M. t. huangshanensis* population (Mt. Hangshan: Hap5, Hap6, Hap7), two haplotypes were identified in *M. t. pullus* population (Mt. Wuyi: Hap8, Hap9) and two in *M. t. guizhouensis* (Jiangkou: Hap10, Hap11). The phylogenetic trees which were constructed with the haplotypes revealed two deep branching lineages (Fig. 2). *M. t. thibetana* population, is a sister group to all other subspecies, which compose a separate monophyletic group.

Discussion

Mitochondrial DNA variation in Tibetan macaques

Compared with other primates, the observed Hd is similar to golden monkeys (*Rhinopithecus roxellana*, Hd = 0.845; Li et al. 2007), and barbary macaques

Table 3. *Mean genetic distances between four subspecies (above the diagonal) and mean genetic distances within each subspecies (below the diagonal). Mean genetic differentiation degree between the four subspecies (Fst).*

	M. t. t.	*M. t. h.*	*M. t. p.*	*M. t. g.*
M. t. t.		0.1350	0.1352	0.1367
M. t. h.	0.683*		0.0151	0.0115
M. t. p.	0.493*	0.417*		0.0125
M. t. g.	0.575*	0.317*	0.469*	
Intraspecific	0.0053	0.0042	0.0019	0.0064

*$p < 0.05$.

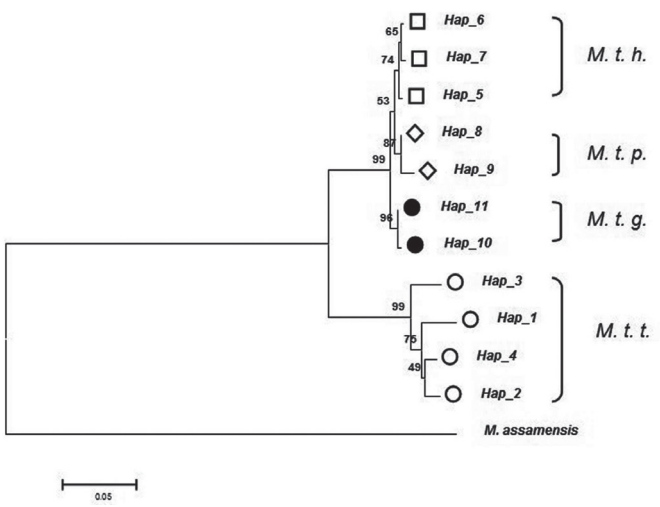

Fig. 2. *A phylogenetic tree of mtDNA haplotypes. This figure is a Neighbour Joining tree for 11 haplotypes which were calculated from 44 mitochondrial DNA control region sequences.*

(*M. sylvanus*, Hd = 0.872; Modolo et al. 2005), whereas Pi is higher than the reported values for those species (Pi = 0.0331, 0.026, respectively). These data suggest that the overall genetic diversity in *M. thibetana* is not low, which is congruent with Li's (2008) result. Haplotype diversity of *M. t. thibetana* population is higher than each of other three subspecies. These data imply that the populations belonging to the nominate subspecies have been separated from the other three subspecies for a relatively long time. The genetic structure of the other subspecies is most likely shaped by inbreeding, genetic drift, or rapid range expansion resulting in lower genetic diversity. However, a lack of variability in the mitochondrial control region does not necessarily reflect low levels of genetic diversity in the nuclear genome (Rosel & Rojas-Bracho 1999).

Phylogenetic relationships among Tibetan macaques

The Old World genus *Macaca* is one of the most successful groups of monkeys. Its representatives are distributed in both Asia and Africa, and the genus has the widest geographical range among primates after *Homo* (Fooden 1980). As a species of genus *Macaca*, Tibetan macaques (*M. thibetana*) are a threatened primate species endemic to China (Jiang et al. 1996). Our results presented here enable us to resolve the phylogenetic relationships within the *M. thibetana*. There are two clear branches of the NJ tree, one includes *M. t. huangshanensis*, *M. t. pullus*, *M. t. guizhouensis*, and the other is *M. t. thibetana*. This branching pattern may indicate geographical separation of ancestral populations along the River Yangtze. This riverine geographical barrier might contribute to the divergence between the nominate subspecies and other populations.

Implication for conservation

Tibetan macaques live in small isolated populations in east-central China (Li 1999). The IUCN Red list classifies them as near threatened/conservation dependent (IUCN 2009). Their habitat area has retreated with farmland development and their populations have decreased drastically (Wang 2000). In the light of the genetic variation among those four subspecies, we suggest defining the individual subspecies as four management units (MUs). According to the model proposed by Moritz (1994), evolutionary significant units (ESU) are designated on the basis of reciprocal monophyly at mitochondrial markers, whereas MUs are identified by significant differences in allele frequency distributions and significant divergence in mitochondrial or nuclear loci. Considering these criteria, populations with genotypes that are closely related to but not shared with other populations would be described as MUs. Additionally, molecular variance analysis consistently indicate that the four subspecies populations are considerably divergent. Furthermore, studies based on morphologic characters of the four subspecies have described significant differences

Table 4. *The haplotype distribution and abbreviation.*

Haplotype	*M. t. t.*			*M. t. p.*	*M. t. h.*	*M. t. g.*	Individual
	Mt. Emei, Sichuan	Tangjiahe, Sichuan	Mabian, Sichuan	Mt.Wuyi, Fujian	Jiangkou,Guizhou	Mt. Hangshan, Anhui	number
Hap1	+	+					4
Hap2	+						3
Hap3			+				2
Hap4			+				2
Hap5						+	7
Hap6						+	6
Hap7						+	2
Hap8				+			5
Hap9				+			4
Hap10					+		6
Hap11					+		3

(Jiang et al. 1996). Thus, we believe that these four subspecies should be conserved as different MUs.

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Literature

Deinard A. & Smith D.G. 2001: Phylogenetic relationships among the macaques: Evidence from the nuclear locus NRAMP1. *J. Human Evol. 41: 45–59.*

Eudey A.A. 1980: Pleistocene glacial phenomena and the evolution of Asian macaques. In: Lindburg D.G. (ed.), The macaques: Studies in ecology, behavior and evolution. *van Nostrand Reinhold, New York: 52–83.*

Fooden J. 1976: Provisional classification and key to living species of macaques (Primates: *Macaca*). *Folia Primatol. 25: 225–236.*

Fooden J. 1980: Classification and distribution of living macaques (*Macaca* Lacepede, 1799). In: Lindburg D.G. (ed.), The macaques: Studies in ecology, behavior, and evolution. *van Nostrand Reinhod, New York: 1–9.*

Fooden J. 1983: Taxonomy and evolution of the *sinica* group of macaques: 4. Species account of *Macaca thibetana*. *Fieldiana Zool. 17: 1–20.*

Groves C.P. 2001: Primate taxonomy. Washington. *Smithsonian Institution Press, Washington.*

Groves C.P. 2005: Order Primates. In: Mammal species of the world: A taxonomic and geographic reference, 3rd edition, Vol. 1, Wilson D.E. & Reeder D.M. (eds.). *Johns Hopkins University Press. Maryland: 111–184.*

IUCN 2009: IUCN Redlist of threatened species. *http://www.redlist.org*

Jiang X.L., Wang Y.X. & Wang Q.S. 1996: Taxonomy and distribution of Tibetan macaque (*Macaca tibetana*). *Zool. Res. 17: 361–369.*

Kimura M. 1980: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol. 16: 111–120.*

Kumar S., Tamura K. & Nei M. 2004: MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform 5: 150–163.*

Li J.H. 1999: The Tibetan macaque society: A field study. *Anhui University Press, Anhui.*

Li D., Fan L., Yin H., Wang H., Wu S. & Yue B. 2008: Genetic diversity analysis of *Macaca thibetana* based on mitochondrial DNA control region sequences. *Mitochondrial DNA 19: 446–452.*

Li M., Liu Z.J., Gou J.X., Ren B.P., Pan R.L., Su Y.J., Funk S.M. & Wei F.W. 2007: Phylogeography and population structure of the golden monkeys (*Rhinopithecus roxellana*): Inferred from mitochondrial DNA sequences. *Am. J. Primatol. 69: 1–15.*

Li Q.Q. & Zhang Y.P. 2004: A molecular phylogeny of *Macaca* based on mitochondrial control region sequences. *Zool. Res. 25: 385–390.*

Liu Y., Li J.H. & Zhao J.Y. 2006: Sequence variation of mitochondrial DNA control region and population genetic diversity of Tibetan macaques *Macaca thibetana* in the Huangshan Mountain. *Acta Zool. Sin. 52: 724–730.*

Modolo L., Salzburger W. & Martin R.D. 2005: Phylogeography of barbary macaques (*M. sylvanus*) and the origin of the Gibraltar colony. *Proc. Natl. Acad. Sci. USA. 102: 7392–7397.*

Moritz C. 1994: Applications of mitochondrial DNA analysis in conservation: A critical review. *Mol. Ecol. 3: 401–411.*

Rosel P.E. & Rojas-Bracho L. 1999: Mitochondrial DNA variation in the critically endangered vaquita *Phocoena sinus* (Norris and Mac-Farland, 1958). *Mar. Mamm. Sci. 15: 990–1003.*

Rozas J., Sánchez-DelBarrio J.C., Messeguer X. & Rozas R. 2003: DnaSP, DNA polymorphism analyses by the

coalescent and other methods. *Bioinformatics 19: 2496–2497.*
Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. & Higgins D.G. 1997: The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res. 24: 4876–4882.*
Wang S. 1998: China red data book of endangered animals (Mammalia). *Science Press, Beijing.*
Wang Q.S. 2000: *Macaca thibetana* of the Huangshan Mountain region. *Big Nat. 5: 4–5.*
Zhao J.Y. & Li J.H. 2005: Research on DNA extraction from old faeces of *Macaca thibetana*. *Acta Theriol. Sinica 25: 410–413.*