Available online at www.sciencedirect.com
ScienceDirect
Molecular Phylogenetics and Evolution 44 (2007) 838–849
MOLECULAR PHYLOGENETICS AND EVOLUTION
www.elsevier.com/locate/ympev

Phylogenetic relationships and morphometric affinities of the Arunachal macaque *Macaca munzala*, a newly described primate from Arunachal Pradesh, northeastern India

Debapriyo Chakraborty [a,b], Uma Ramakrishnan [c], Jikom Panor [d], Charudutt Mishra [b,e], Anindya Sinha [a,b,f,*]

[a] *National Institute of Advanced Studies, Indian Institute of Science Campus, Bangalore 560012, India*
[b] *Nature Conservation Foundation, 3076/5, 4th Cross, Gokulam Park, Mysore 570002, India*
[c] *National Centre for Biological Sciences, GKVK Campus, Bangalore 560065, India*
[d] *Zoological Park, Itanagar 791113, India*
[e] *International Snow Leopard Trust, 4649, N. Sunnyside Avenue, Suite 325, Seattle, Washington 98103, USA*
[f] *Indian Association for Cultivation of Science, 2A-2B Raja S C Mullick Road, Jadavpur, Kolkata 700032, India*

Received 16 September 2006; revised 10 March 2007; accepted 5 April 2007
Available online 20 April 2007

Abstract

A new species of primate, the Arunachal macaque *Macaca munzala*, belonging to the *sinica* species-group of the genus, was described from northeastern India in 2005, and, based on its appearance and distribution, hypothesised to be closely related to *M. assamensis* and *M. thibetana*. We subsequently obtained an entire adult male specimen and tissue remains from two other *M. munzala* individuals. Molecular analyses establish the distinct identity of the species and indicate a time of origin of *c*. 0.48 mya for it. The species also shows close phylogenetic affinities with the allopatric *M. radiata* and with the geographically closer *M. assamensis* and *M. thibetana*, possibly mediated by male introgression from an ancestral *M. assamensis–M. thibetana* stock into an ancestral *M. munzala* stock. Morphometric analyses, on the other hand, reiterate its close similarity only with *M. assamensis* and *M. thibetana*, presumably resulting from convergent evolution under similar ecological conditions and along a latitudinal gradient, as predicted by Bergmann's and Allen's rules.

Keywords: Arunachal macaque; *Macaca munzala*; *Macaca radiata*; *Macaca assamensis*; *Macaca thibetana*; *Macaca sinica*; Cytochrome *b*; D-loop; TSPY; Molecular phylogeny; Morphometry; Convergent evolution

1. Introduction

A previously undescribed species of primate belonging to the genus *Macaca* was recently discovered in the eastern Himalayan state of Arunachal Pradesh in north-east India, and described as a new species, the Arunachal macaque *Macaca munzala* (Sinha et al., 2005). The holotype and paratypes of the species were depicted by photographs, and the distinctive identity of *M. munzala* was diagnosed based on a combination of appearance and colouration, relative tail length, and geographical distribution (Sinha et al., 2005). The species, a member of the *sinica* species-group of the genus *Macaca* (classified according to penis morphology), was thought to be closely related to the Tibetan macaque *M. thibetana* and the Assamese macaque *M. assamensis* based on their morphological similarities (Sinha et al., 2005).

On March 7, 2005, we obtained an entire specimen of an adult male *M. munzala* in the Zemithang region of western Arunachal Pradesh, which had entered a house and had

* Corresponding author. Address: National Institute of Advanced Studies, Indian Institute of Science Campus, Bangalore 560012, India. Fax: +91 80 22185024.
E-mail addresses: asinha@nias.iisc.ernet.in, anindyaranasinha@yahoo.com (A. Sinha).

1055-7903/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2007.04.007

been killed by the villagers in self-defense. The specimen was treated at the Itanagar Zoo, tissues collected for molecular analyses, and the skull, baculum, and caudal vertebrae measured and described. The skin, skull, and all retrieved bones and vertebrae were deposited with the Arunachal Pradesh Forest Department, for preservation at the State Forestry Research Institute in Itanagar (Accession Nos.: 2005.01.01–2005.01.10). During our field surveys, two other tissue samples of *M. munzala* were also obtained from Gyamdong and Lhou, villages close to Zemithang, from where the species was originally reported.

In addition to *M. munzala*, *M. assamensis*, and *M. thibetana*, the *sinica* species-group also includes the bonnet macaque *M. radiata* and the toque macaque *M. sinica*, which are endemic to peninsular India and Sri Lanka, respectively. Although the evolutionary history of *Macaca* has been broadly studied using molecular phylogenetic approaches (Hayasaka et al., 1988; Morales and Melnick, 1998; Tosi et al., 2000, 2003), specific phylogenetic relationships and the evolutionary history of the *sinica* species-group of the macaques remain unknown. To test whether *M. munzala* is indeed closely related to *M. thibetana* and *M. assamensis*, and to determine its phylogenetic position within the *sinica* group, we conducted molecular analyses of mitochondrial (cytochrome *b* and D-loop) and nuclear (TSPY gene on the Y-chromosome) sequences of *M. munzala* (three individuals) and *M. radiata* (four individuals), and compared them with available published sequences of *M. thibetana*, *M. assamensis* and *M. sinica*. In order to assess the role of ecology in the evolution of this group of primates, we also compared morphological (body mass, relative tail length) and anatomical (skull, caudal vertebrae, and baculum morphometrics) characteristics of these five species. Evolutionary trends within these morphological and anatomical traits were examined across a latitudinal gradient in accordance with the predictions made by Bergmann's and Allen's rules (Allen, 1877, see Meiri and Dayan, 2003 for a review of Bergmann's rule).

2. Methods

2.1. DNA extraction and amplification

DNA was extracted from three *M. munzala* skin samples and four *M. radiata* blood samples using a DNeasy tissue kit (Qiagen, Hilden, Germany) following procedures recommended by the supplier, with the exception that DNA was incubated for 15 min in 100 μl elution buffer before elution. To study the evolutionary origin of *M. munzala* and its relationship with other members of the *sinica* species-group, we used both maternally (mitochondrial) and paternally (Y-chromosomal) inherited markers. All the polymerase chain reactions (PCRs) were carried out in a reaction volume of 10 μl. The final concentration of the reaction mixture was: 1× *Taq* Buffer B without $MgCl_2$, 2 mM $MgCl_2$, 1.5 U *Taq* DNA Polymerase (Bangalore Genei, Bangalore, India), 0.25 mM dNTP mix (Eppendorf, Hamburg, Germany), 0.1 μM of each primer (Sigma–Aldrich Chemicals, Bangalore, India), and about 1–1.5 μl of DNA extract. All the reactions were carried out in two Eppendorf mastercylers (Eppendorf, Hamburg, Germany), one gradient and the other non-gradient.

We chose two different segments of the mitochondrial DNA rather than a single large one in order to better represent its genetic variability. We amplified a 424-bp long $tRNA^{Glu}$-cytochrome *b* segment of the coding region of the mitochondrial DNA using the L14724 (5′-CGAAGC TTGATATGAAAAACCATCGTTG-3′) and H15149 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCT CA-3′) primer set from Li and Zhang (2005). In order to also sample a non-coding region of the mitochondrial genome, we amplified a 534-bp long D-loop region using the LqqF (5′-TCCTAGGGCAATCAGAAAGAAAG-3′) and TDKD (5′-CCTGAAGTAGGAACCAGATG-3′) primer set from Li and Zhang (2004). We conducted a standard 35-cycle PCR to amplify the target regions with a denaturation of 20 s at 94 °C, annealing of 30 s at 51 °C (cytochrome *b*) or at 59 °C (D-loop), and an extension of 20 s (cytochrome *b*) or 35 s (D-loop) at 72 °C. Finally, we amplified two segments of the TSPY (Testis-Specific Protein, Y-chromosome) gene, with a total length of ~1.4 kb, using the TSPY-A (5′-AGCCAGGAAGGCCT TTTCTCG-3′) and 740R (5′-GATCATGTAGCTCAGC ATGTCT-3′), and the E700F (5′-GTCCGTCTTATCCA TGYCGA-3′) and TSPY5R (5′-CTGTGCATAAGACCA TGCTGAG-3′) primer sets, respectively (Tosi et al., 2000). Each of the segments was separately amplified at an annealing temperature of 58 °C for 30 s and an extension temperature of 72 °C for 1 min keeping the other conditions as above.

2.2. DNA sequencing

All the PCR products were checked visually by running 1 μl of the product in 1% Agarose gels (Bangalore Genei, Bangalore, India) and were purified using Qiagen PCR purification kit (Qiagen, Hilden, Germany). The concentrations were measured by loading 2 μl of the PCR products in a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Delaware, USA). Sequencing was accomplished using an ABI 310 Genetic Analyzer, and the raw sequences analysed with the ABI 310 Genetic Analyzer Version 3.1 software (Applied Biosystems, Foster City, USA). Both forward and reverse sequencing reactions were analyzed.

2.3. Phylogenetic analyses

The *M. munzala* and *M. radiata* sequences were edited manually and assembled using the Mega 3.1 sequence editor (Kumar et al., 2004). They were multiple aligned together with *M. assamensis*, *M. thibetana*, and *M. sinica* sequences, downloaded from GenBank, using ClustalW in Mega 3.1 Alignment Explorer with default parameters.

Appropriate sequences of *Papio hamadryas*, *M. sylvanus* and *M. arctoides* were also downloaded and used in the phylogenetic analysis to root the reconstructed trees. The origins, sources and abbreviations of all the individuals subjected to molecular phylogenetic analyses are listed in Tables 1a and 1b.

Average genetic distances between and within the five species of the *sinica* group were calculated using the Kimura-2-parameter model in Mega 3.1 (Kumar et al., 2004). The nucleotide diversity of the mitochondrial and nuclear DNA sequences were calculated using DnaSP Version 4.10 (Rozas et al., 2003). Phylogenetic analyses were conducted using the maximum likelihood (ML) and Bayesian inference (BI) methods in PAUP* (Swofford, 2002) and MrBayes Version 3.1 (Ronquist et al., 2005), respectively. In all cases, trees were built independently for the cytochrome *b* and D-loop sequences, the combined mitochondrial DNA data, and for the combined segments of the TSPY marker. Prior to phylogenetic analyses, ModelTest Version 3.8 (Posada, 2006) was used to determine the best-fit model (based on Akike Information Criterion) for the separate and combined datasets. These analyses revealed GTR+G+I to be the most appropriate evolutionary model for both cytochrome *b* and D-loop, TVM+I+G for the combined mitochondrial DNA data, and GTR for the TSPY marker. The ML trees were reconstructed using the above evolutionary models and tested with 500 bootstrap replicates each. The BI trees were reconstructed assuming only a GTR evolutionary model and by allowing the program to generate all the other parameters independently. The phylogenetic analyses were run for 10^6 generations for the mitochondrial data and 2×10^6 generations for the nuclear data to ensure convergence. Samples were collected every 1000 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 'burnin' (Ronquist et al., 2005).

2.4. Estimation of divergence times

The relative evolutionary rates of the different lineages within all the phylogenetic trees were calculated using the software RRTree (Robinson-Rechavi and Huchon, 2000). The null hypothesis, that the rate of evolution is homogeneous among all branches within each phylogeny, was rejected for many of the lineages and a strict molecular clock was thus considered inappropriate. Divergence times were calculated using the software r8s Version 1.71 (Sanderson, 2003). The divergence times for the mitochondrial and Y-chromosomal trees were estimated separately. As many of the lineages exhibited heterogeneity in their

Table 1a
Origin, source and abbreviation of the individuals subjected to mitochondrial DNA analysis

Species	Origin	GenBank Accession No.		Abbreviation	Reference
		Cytochrome *b*	D-loop		
Macaca assamensis	Myanmar	AY685859	AY682619	Myanmar1	Cytochrome *b*: Li and Zhang (2005)
	Myanmar	AY685861	AY682621	Myanmar2	D-loop: Li and Zhang (2004)
	Myanmar	AY685860	AY682620	Myanmar3	
	Myanmar	AY685862	AY682622	Myanmar4	
	Vietnam	AY685852	AY682611	Vietnam1	
	Vietnam	AY685853	AY682613	Vietnam2	
	Vietnam	AY685857	AY682617	Vietnam3	
	Yunnan, China	AY685854	AY682614	Yunnan1	
	Yunnan, China	AY685855	AY682615	Yunnan2	
	Yunnan, China	AY685856	AY682616	Yunnan3	
	Yunnan, China	AY685863	AY682612	Yunnan4	
	South China	AY685858	AY682618	South China	
Macaca thibetana	Sichuan, China	AY685864	AY682610	Sichuan1	Cytochrome *b*: Li and Zhang (2005)
	Sichuan, China	AY685865	AY682607	Sichuan2	D-loop: Li and Zhang (2004)
	Sichuan, China	AY685866	AY682608	Sichuan3	
	Sichuan, China	AY685867	AY682609	Sichuan4	
	Sichuan, China	AY685868	AY682606	Sichuan5	
Macaca munzala	Tawang, India	DQ859977	DQ859982	Tawang1	Cytochrome *b*: This study
	Tawang, India	DQ859975	DQ859980	Tawang2	D-loop: This study
	Tawang, India	DQ859976	DQ859981	Tawang3	
Macaca radiata	Karnataka, India	DQ859973	DQ859978	Karnataka1	Cytochrome *b*: This study
	Karnataka, India	DQ859974	DQ859979	Karnataka2	D-loop: This study
Macaca sylvanus	Unknown	AJ309865	AJ309865	*M. sylvanus*1	Cytochrome *b*: Arnason et al. (2000)
	Unknown	NC_002764	NC_002764	*M. sylvanus*2	D-loop: Arnason et al. (2000)
Papio hamadryas	Unknown	NC_001992	NC_001992	*P. hamadryas*1	Cytochrome *b*: Arnason et al. (1998)
	Unknown	Y18001	Y18001	*P. hamadryas*2	D-loop: Arnason et al. (1998)

Table 1b
Origin, source and abbreviation of the individuals subjected to TSPY sequence analysis

Species	Origin	GenBank Accession No.	Abbreviation	Reference
Macaca assamensis	Southern China	AY224236	South China1	Tosi et al. (2003)
	Southern China	AF284244	South China2	Tosi et al. (2000)
Macaca thibetana	Southeastern China	AY224237	Southeast China1	Tosi et al. (2003)
	Southeastern China	AF284276	Southeast China2	Tosi et al. (2003)
Macaca munzala	Tawang, India	EF222279	Tawang1	This study
	Tawang, India	EF222280	Tawang2	This study
	Tawang, India	EF222281	Tawang3	This study
Macaca radiata	India	AF284271	India1	Tosi et al. (2000)
	India	AF284270	India2	Tosi et al. (2000)
	Karnataka, India	EF222277	Karnataka1	This study
	Karnataka, India	EF222278	Karnataka3	This study
Macaca sinica	Polonnaruwa, Sri Lanka	AF284234	Sri Lanka1	Tosi et al. (2000)
	Polonnaruwa, Sri Lanka	AF284273	Sri Lanka2	Tosi et al. (2000)
		AF284233	Sri Lanka3	Tosi et al. (2000)
Macaca arctoides	Malaysia	AF284240	Malaysia1	Tosi et al. (2003)
	Unknown	AF284241	Unknown	Tosi et al. (2003)
	Thailand	AF284242	Thailand1	Tosi et al. (2003)
	Vietnam	AF284243	Vietnam1	Tosi et al. (2003)
Macaca sylvanus	Northwestern Africa	AF284274	*M. sylvanus*3	Tosi et al. (2000)
	Northwestern Africa	AF284275	*M. sylvanus*4	Tosi et al. (2000)
	Northwestern Africa	AF425281	*M. sylvanus*5	Tosi et al. (2002)
Papio hamadryas	Eastern Africa	AF284277	*P. hamadryas*3	Tosi et al. (2000)

evolutionary rates, we used the NPRS method (Sanderson, 1997) and the POWELL algorithm to reconstruct their divergence times. For the mitochondrial DNA trees, we have constrained the root (the *Papio–Macaca* split) to lie between 8.6 and 10.9 million years ago (mya), as determined by an analysis of the entire mitochondrial genome (Raaum et al., 2005). It should be noted that the palaeontologically determined date of 10 mya for this split (Delson, 1980) falls within this range. Along with this constraint, we fixed the next split between *M. sylvanus* and the other taxa within the genus at 5.5 mya (Delson, 1996). We have, however, used only the palaeontologically determined dates of 10 and 5.5 mya, respectively, to calibrate the TSPY gene tree.

2.5. Morphological and anatomical comparisons

We evaluated the morphological and anatomical affinities of *M. munzala* by comparing 25 craniodental and four baculum measurements, and the centrum lengths of the caudal vertebrae of this species (Sinha et al., 2006) with those of *M. assamensis*, *M. thibetana*, *M. radiata* and *M. sinica* (craniodental measurements from Pan and Oxnard, 2004; baculum and caudal vertebrae measurements from Fooden, 1988). All craniodental measurements, the mean values for which are listed in Pan and Oxnard (2004), except OCCH and GLENOL, were used in this analysis. In the case of baculum, data were available for four *M. assamensis*, two *M. thibetana*, five *M. radiata*, and seven *M. sinica* adult males. One set of *M. assamensis* baculum data reported in Fooden (1988) was presumably from a subadult with significantly smaller dimensions, and was excluded from the final analysis. To assess the similarity in morphometric measurements across species, matrices of Euclidean distances were calculated, and cluster analyses performed separately for craniodental (mean values) and baculum (individual values) measurements. Joining-trees and single-linkage clustering algorithms were used, and the tree plots were standardised to a percent scale by expressing the linkage distance between any two cases as a percent of the maximum linkage distance.

Available data on body mass and relative tail length of *M. munzala* (body mass from Sinha et al., 2006; relative tail length from Sinha et al., 2005) and each of the other four species (Fooden, 1988) were plotted against the latitudinal midpoints of their known distributions and examined visually for monotonic relationships, as predicted by Bergmann's and Allen's rules, respectively. We also used the greatest skull length of adult males as a surrogate for body mass (data on *M. munzala* from Sinha et al., 2006; on the other four species from Fooden, 1988), and tested for significant monotonic relationships with latitude, again as predicted by the Bergmann's rule (James, 1970; Blackburn et al., 1999), using Pearson's product-moment correlations. Similar tests were performed to examine whether relative tail lengths showed a significant negative monotonic relationship with latitude, as predicted by Allen's rule (Allen, 1877, reported in Ray, 1960). Differences in the relationship between the linear sequence and size (centrum lengths) of the caudal vertebrae of the five species were examined

graphically. While investigating patterns in body size and relative tail length along latitudinal gradients, we used both community-level (individuals of all species pooled together) and species-level (each species analysed separately) approaches (Brehm and Fiedler, 2004). All statistical analyses were performed using Statistica 6 (StatSoft Inc., Tulsa, USA).

3. Results

3.1. Phylogenetic analyses

Interspecific genetic distances for the cytochrome *b*–D-loop combined sequences and the TSPY sequence for all the species of the *sinica* group of the genus *Macaca* are greater than the respective intraspecific distances (Table 2), indicating relatively long-term genetic separation of the species within this group. The only exception is the interspecific distance for the TSPY sequence between *M. munzala* and *M. assamensis*. It must be noted, however, that the genetic variability within the TSPY sequence is very low (nucleotide diversity per site, Pi = 0.00656), as compared to that for the mitochondrial cytochrome *b*–D-loop sequences (Pi = 0.10339). For the combined mitochondrial sequences, the interspecific distances of *M. munzala* from each of the other three species are greater than that between *M. assamensis* and *M. thibetana* (the species-pair with lowest average interspecific genetic distance). The intraspecific distance for *M. munzala*, in contrast, is rather small, comparable to those of *M. radiata* and *M. thibetana*, and almost an order of magnitude less than that of *M. assamensis*.

All the phylogenetic trees that were constructed with the cytochrome *b*–D-loop combined sequences reveal *M. munzala* to be a distinct clade genetically closer to *M. radiata* than to *M. assamensis* or *M. thibetana* (Fig. 1a and b). Qualitatively similar tree topologies were also obtained for cytochrome *b* sequence data independently (data not shown). The distinction of the *M. munzala* clade from the *M. assamensis–M. thibetana* clade is maintained for the D-loop sequence data as well, although, in this case, it appears to be paraphyletic with both the *M. radiata* and the *M. assamensis–M. thibetana* clades (data not shown). *M. munzala* and *M. radiata* display a deep genetic divergence from each other, which possibly occurred 3.20 million years ago (mya). Both these taxa nevertheless appear to be of fairly recent origin (at 0.48 and 0.17 mya, respectively). In contrast to this, the older *M. assamensis–M. thibetana* paraphyletic clade appears to have originated 3.39 mya, with the nested *M. thibetana* clade separating out at 2.20 mya.

The phylogenetic trees constructed with the TSPY sequence, on the other hand, show *M. munzala* to be monophyletic with the *M. assamensis–M. thibetana* clade (Fig. 1c and d). The principal evolutionary divide in the *sinica* species-group, according to the nuclear DNA analysis, occurred between the *M. munzala–M. assamensis–M. thibetana* and the *M. radiata–M. sinica* clades at 2.66 mya. As also observed with the mitochondrial DNA analysis, the *M. assamensis–M. thibetana* clade is older in its origin (1.57 mya) than the *M. radiata–M. sinica* clade (0.98 mya).

3.2. Morphological and anatomical comparisons

Cluster analysis of the baculum measures reveals a clear pattern separating out a group comprising the three northern species (*M. munzala*, *M. assamensis*, and *M. thibetana*) from the southern group, including *M. radiata* of peninsular India and *M. sinica* of Sri Lanka. Within the former group, there is considerable interspersion between *M. thibetana* and *M. assamensis*, while the *M. munzala* stands out as the most distant species within this cluster (Fig. 2a). The pattern seen in the cluster analysis of the craniodental measures is qualitatively similar to that obtained with the baculum (Fig. 2b).

Amongst the five species examined, the body mass of the relatively northern species appears to be greater than those occurring at lower latitudes, providing support for Bergman's rule (Fig. 3). Furthermore, the community-level analyses of all species including *M. munzala* show a significant increase in greatest skull length (used as a surrogate for body mass) with latitude (Table 3). In the species-level analyses (excluding *M. munzala*), three of the four species (except *M. sinica*) again exhibit significant positive relationships between greatest skull length and latitude as predicted by the Bergmann's rule (Table 3). There is also a significant negative correlation between relative tail length and latitude in the community-level analysis, thus

Table 2
Average genetic distances for cytochrome *b*–D-loop combined sequences (upper section of the matrix) and the TSPY sequence (lower section of the matrix) between and within the five species of the *sinica* species-group calculated using the Kimura-2-parameter model

	Macaca munzala	*Macaca assamensis*	*Macaca thibetana*	*Macaca radiata*	*Macaca sinica*	Intraspecific distances cytochrome *b*–D-loop
Macaca munzala		0.109	0.114	0.100	—	0.007
Macaca assamensis	0.000		0.064	0.135	—	0.046
Macaca thibetana	0.002	0.002		0.124	—	0.004
Macaca radiata	0.002	0.003	0.004		—	0.008
Macaca sinica	0.003	0.003	0.005	0.001		—
Intraspecific distances TSPY	0.000	0.001	0.000	0.000	0.000	

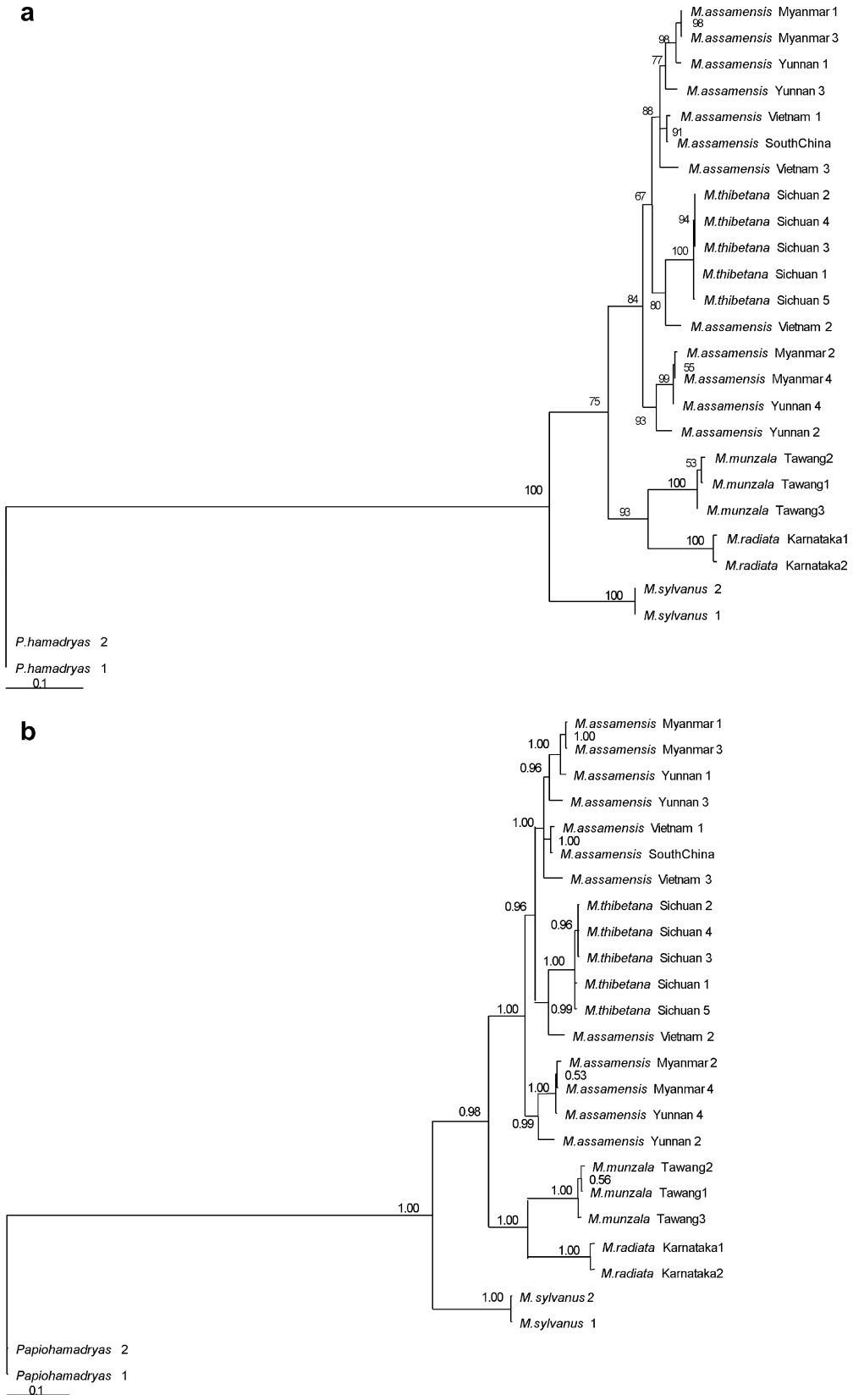
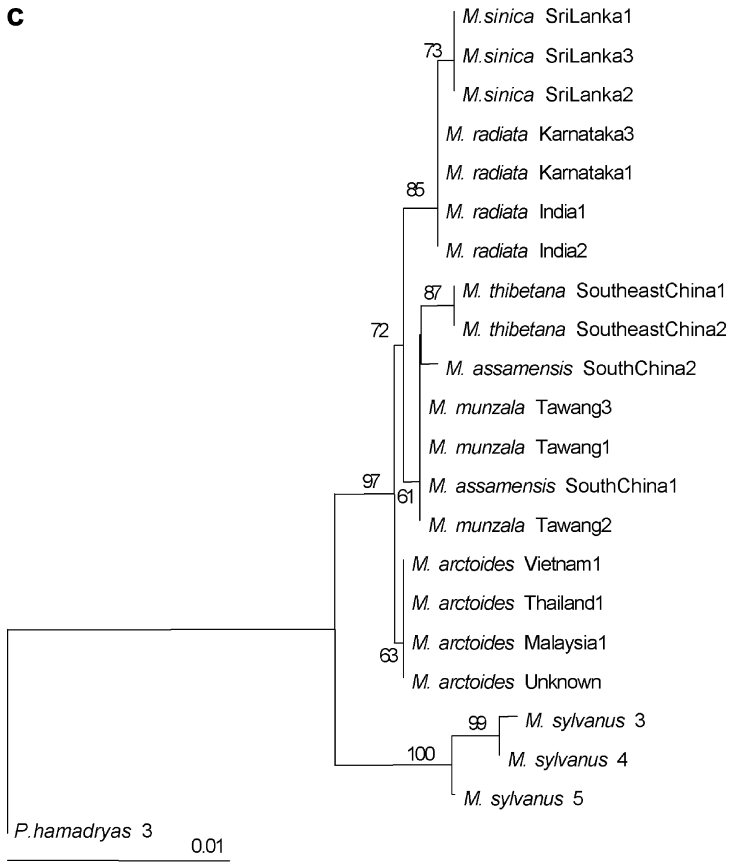


Fig. 1. Phylogenetic relationships among the five species of the *sinica* species-group revealed by (a) maximum likelihood and (b) Bayesian inference analyses of the combined cytochrome *b*–D-loop sequence data, and (c) maximum likelihood and (d) Bayesian inference analyses of the TSPY sequence data. In all cases, 50% majority rule consensus trees are shown. Numbers next to each node represent bootstrap values (a and c) and posterior probabilities (b and d). For details of the individuals analysed, see Tables 1a and 1b, respectively.



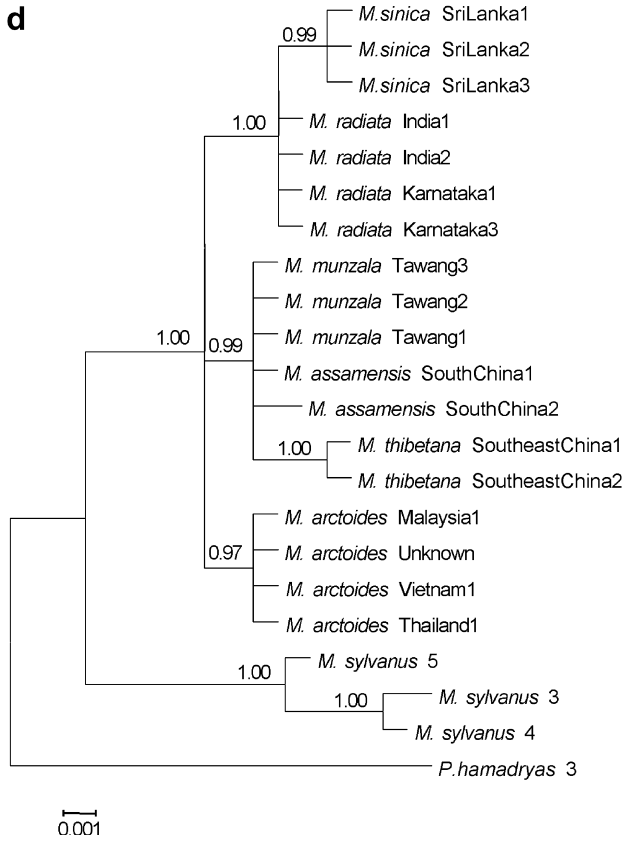


Fig. 1 (*continued*)

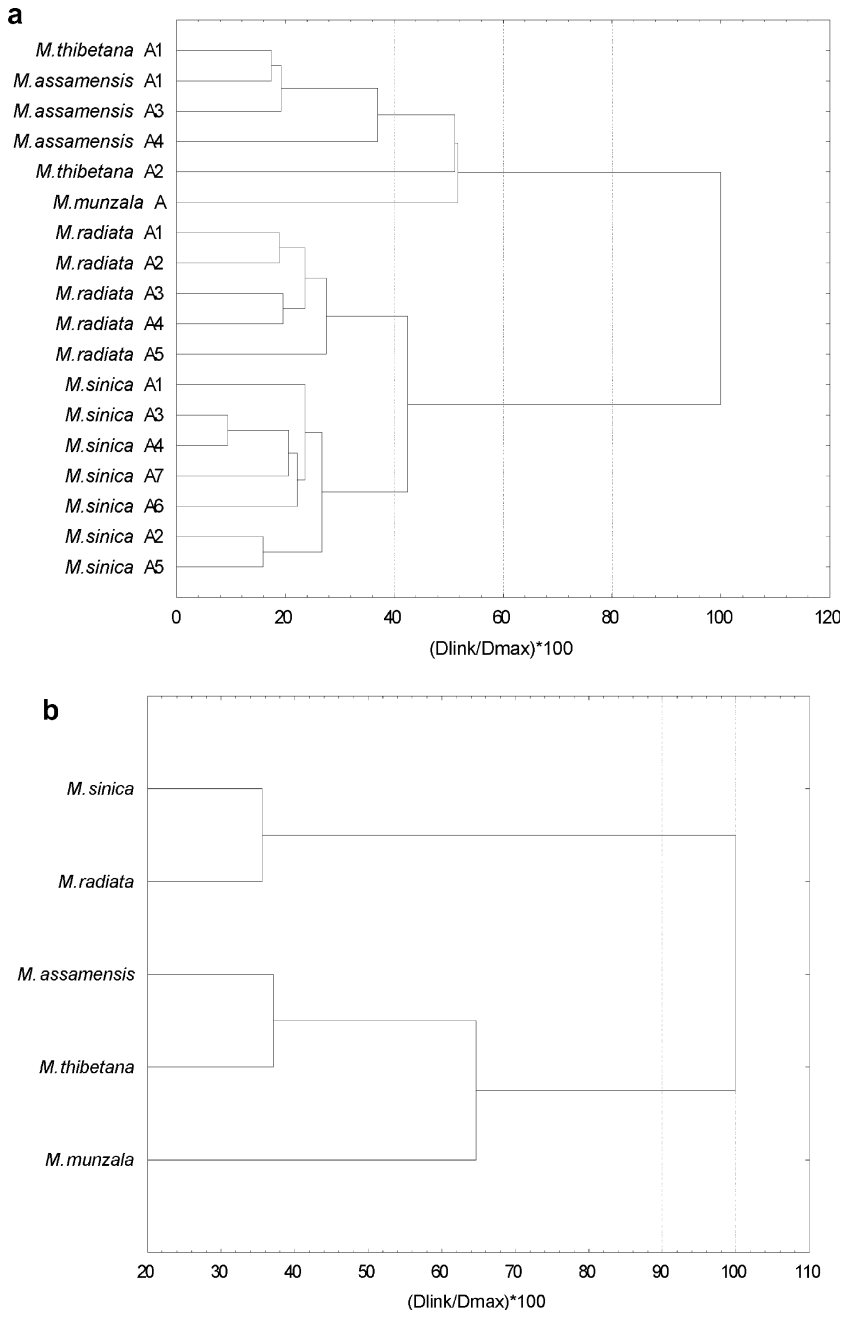


Fig. 2. (a) Cluster analysis (single-linkage Euclidean distances) of baculum measurements (four variables; Sinha et al., 2006) of the *M. munzala* adult male specimen, and seven adult *M. sinica*, five *M. radiata*, three *M. assamensis* and two *M. thibetana* males (obtained from Fooden, 1988). One *M. assamensis* individual (A2) was removed from the original analysis because it could have been a subadult given its significantly smaller dimensions (see Section 2). (b) Cluster analysis (single-linkage Euclidean distances) of skull morphometric measurements of the *M. munzala* adult male specimen (25 variables; Sinha et al., 2006) and mean values of measurements for adult males of *M. sinica*, *M. radiata*, *M. assamensis* and *M. thibetana* (obtained from Pan and Oxnard, 2004).

providing support for Allen's rule (Table 3 and Fig. 3). However, in the species-level analysis, the relationship is significantly negative for *M. assamensis* and *M. sinica* but not for *M. thibetana* or *M. radiata* (Table 3). Analysis of the structure and linear arrangement of the caudal vertebrae nevertheless showed that both their numbers as well as their lengths seem to reduce with increasing latitude (Fig. 4). *M. munzala*, however, is an exception, which, although fitting the pattern well in terms of the total number of vertebrae (intermediate between the immediately northern *M. thibetana* and southern *M. assamensis*), has relatively longer vertebrae (Fig. 4).

4. Discussion

Macaca munzala has recently been identified as a distinct species within the *sinica* species-group of the genus

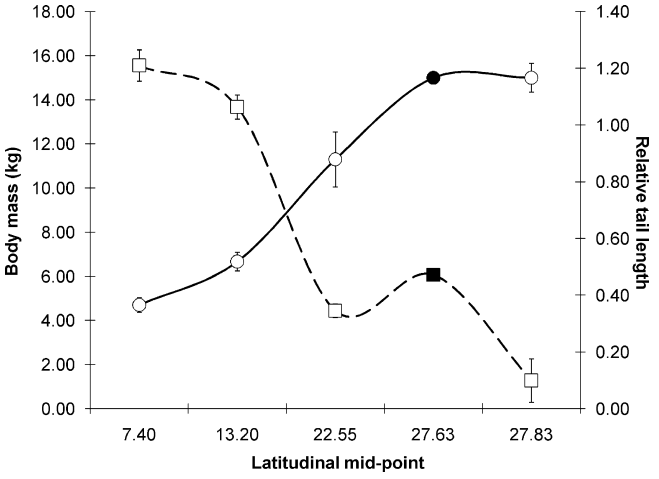


Fig. 3. Mean (±SD) body mass (solid line) and relative tail length (dashed line) of adult male macaques of the *sinica* species-group plotted against the latitudinal centre points of the known species distributions. The open circles and squares represent, from left to right, *M. sinica*, *M. radiata*, *M. assamensis* and *M. thibetana* (data derived from Fooden, 1988) while the closed circle and square represent the body mass and relative tail length for the single specimen of *M. munzala* available.

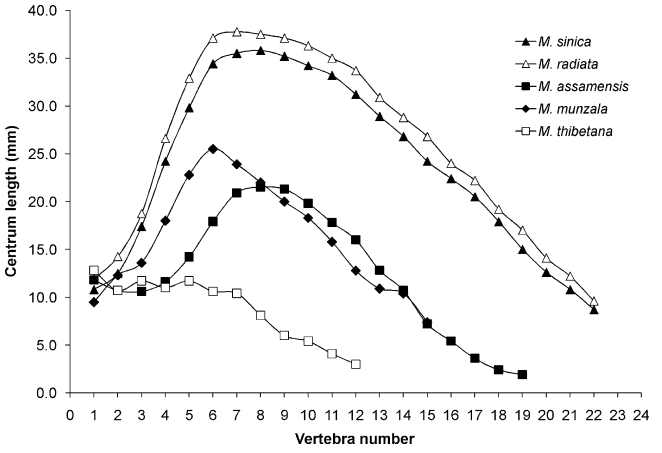


Fig. 4. Centrum lengths of the caudal vertebrae of the adult male *M. munzala* specimen (closed diamonds) compared with the mean centrum lengths of the four other species of the *sinica* species-group (obtained from Fooden, 1988), plotted against vertebra number. The species from top to bottom in the legend are ordered according to increasing latitudinal midpoints of their distribution (see Fig. 3).

Table 3
Relationship between morphological attributes and latitudinal distribution among macaque species of the *sinica* species-group, showing Pearson's correlation coefficients between (i) greatest skull length and latitude, and (ii) relative tail length (ratio of tail length to head and body length) and latitude for adult males

Species	Greatest skull length	Relative tail length
All species	0.941 (80)***	−0.931 (59)***
Macaca assamensis	0.485 (28)**	−0.646 (16)**
Macaca thibetana	0.534 (18)*	−0.177 (08)
Macaca radiata	0.632 (12)**	0.520 (12)
Macaca sinica	0.111 (21)	−0.684 (21)***

Significant monotonic and positive relationships in (i) would indicate agreement with Bergmann's rule, while significant monotonic and negative relationships in (ii) would indicate agreement with Allen's rule. The overall correlation in the top row represents a community-level analysis for all species combined including *M. munzala*, for which sample sizes were limited. The subsequent rows represent species-level analyses (see Section 2). Data for all species other than *M. munzala* have been derived from Fooden (1988). Sample sizes are indicated in parentheses.

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Macaca based on its unique morphology and geographical distribution (Sinha et al., 2005).

Our molecular genetic analyses of the mitochondrial DNA sequences confirm this finding by placing it as a distinct, coherent clade, separate from the *M. radiata* and *M. assamensis–M. thibetana* clades, within the *sinica* species-group. The *M. munzala* clade is also characterised by a low intraspecific genetic divergence of 0.7%, comparable to *M. radiata* and *M. thibetana*. This, together with the large interspecific genetic distances between *M. munzala* and each of the other species within the group (varying from 10.0% to 11.4%), thus confirms its identity as a distinct species by the genetic species concept as well (Bradley and Baker, 2001). Additionally, these interspecific distances of *M. munzala* are also much greater than that of 6.4% between *M. assamensis* and *M. thibetana*, which have long been recognised as distinct species within the *sinica* group on the basis of their morphological and anatomical features (reviewed in Fooden, 1988).

The nuclear TSPY gene tree, on the other hand, places *M. munzala* within the *M. assamensis–M. thibetana* clade. It, however, fails to resolve the 'soft' polytomy between these three species adequately. This is due to the low nucleotide diversity of this sequence, possibly due to low evolutionary rates. This has also been noted in earlier studies of other macaques (Tosi et al., 2003; Ziegler et al., 2007). Furthermore, TSPY, being a protein-coding gene, is likely to be under strong selection and thus inappropriate for the reconstruction of recent phylogenetic relationships. These results, nevertheless, provide critical insights into the evolutionary origin of *M. munzala*, which is discussed below.

Based on similarities in morphological characteristics and ecological distribution (in the Eastern Himalaya and adjoining areas of south China and southeast Asia) between *M. munzala*, *M. assamensis*, and *M. thibetana*, Sinha et al. (2005) had suggested the possibility of close phylogenetic relationships between these species. Although this is supported by our analysis of the TSPY gene tree, our mitochondrial DNA analyses, surprisingly, establish a much closer relationship of *M. munzala* with the geographically and ecologically more distant *M. radiata* of tropical peninsular India.

Our morphometric analyses of the *M. munzala* adult male specimen and its comparison with the other four species also suggest affinities between this species and the *M. assamensis–M. thibetana* clade. Although structurally distinct, both the baculum and the skull of *M. munzala*

bear greater similarity with those of *M. assamensis* and *M. thibetana* and cluster with them rather than with *M. radiata* and *M. sinica*. The relative tail length of *M. munzala* is also comparable to that of *M. assamensis*. The absolute tail length of *M. assamensis* (mean ± SD of 212 ± 18 mm, $n = 22$; Fooden, 1988) is, however, significantly smaller than that of *M. munzala* (252 ± 17 mm, $n = 2$; Mann–Whitney *U*-test, two-tailed, $p < 0.025$). Moreover, the detailed structural analysis of the caudal vertebrae of these taxa reveals that the *M. munzala* vertebrae stand out in their relatively large size. The caudal vertebral structures of the five species, in fact, shows that *M. munzala*, *M. radiata* and *M. sinica* are similar to each other in that their centrum lengths begin to increase from the second vertebra itself (although they subsequently decrease), while the first four vertebrae of *M. assamensis* and *M. thibetana* are comparable to each other in their centrum lengths. This similarity in caudal vertebral structure of *M. munzala* with that of *M. radiata* (and *M. sinica*), despite differences in their absolute tail lengths (where *M. munzala* is closer to *M. assamensis*), supports the results from our molecular phylogenetic analyses of the mitochondrial DNA sequences that establish the significant genetic divergence between the *M. assamensis–M. thibetana* and the *M. munzala–M. radiata–M. sinica* clades.

Although this morphological convergence of *M. munzala* with the *M. assamensis–M. thibetana* clade could be attributable to their common ancestry, as revealed by the TSPY gene tree, the differences in their caudal structure suggest that the morphological convergence is more likely due to similar selective forces in their comparable ecological environments. These three species have been reported from subtropical and temperate regions (with *M. assamensis* also occurring in some tropical forests), while *M. radiata* and *M. sinica* are largely confined to the warm tropical areas of peninsular India and Sri Lanka, respectively.

Morphological patterns within and between closely related species have been described by Bergmann's and Allen's rules along temperature/latitudinal gradients (see Meiri and Dayan, 2003 for a review of Bergmann's rule). Our analysis reveals a significant latitudinal increase in body mass and greatest skull length within the *sinica* species-group of macaques, both at the community- and the species-level, as predicted by the Bergmann's rule (see Fooden, 1988 for a similar species-level regression analysis). Furthermore, although a decline in relative tail lengths of these species along a latitudinal gradient, as predicted by Allen's rule, was less pronounced (but see Fooden, 1988), analysis of their caudal vertebral structure and organisation again suggests a simultaneous decrease in both the number and size of these vertebrae with increasing latitude. The only exception in this regard was *M. sinica*, with caudal vertebrae consistently shorter in length than those of *M. radiata*; the origin of this southernmost species thus possibly involved a further reduction in vertebral length

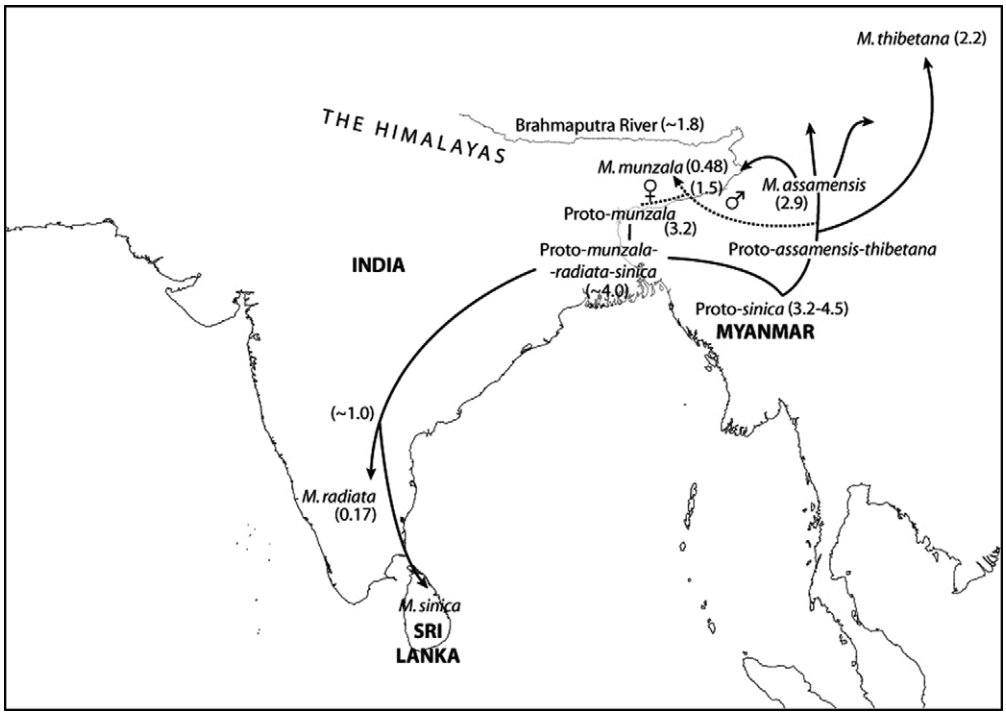


Fig. 5. Hypothetical reconstruction of principal stages in evolution and dispersal of the *sinica* species-group of macaques on the bases of both palaeontological evidence (Delson, 1980) and molecular phylogeny (this study). The dashed lines represent the tentative male introgression from the proto-*assamensis–thibetana* stock into the proto-*munzala* stock culminating in the modern-day *M. munzala*. The numbers in parentheses represent the time in mya. Please note that the different evolutionary stages, being hypothetical entities, cannot be precisely located geographically in the absence of firm palaeontological evidence.

from that of the ancestral *M. radiata–M. sinica* stock. These patterns thus point to the important role that ecology may have played in bringing about the adaptive morphological and anatomical convergence of *M. munzala* with the two northern species of this group.

Our genetic analyses permit us to explore the possible evolutionary origins of *M. munzala*. The mitochondrial DNA trees clearly indicate the common ancestry of *M. munzala* and *M. radiata*, while the Y-chromosomal analysis suggests a much closer evolutionary relationship between the *M. munzala* and the *M. assamensis–M. thibetana* lineages. Lineage sorting is expected to occur more rapidly for Y-chromosomes than for mitochondrial genes (Tosi et al., 2003). In our analyses, however, there appears to have been complete mitochondrial lineage sorting in this species-group, while the Y-chromosome lineages remain unresolved. This suggests that male introgression and hybridization may have played an important role in the ancestry of *M. munzala*.

The phylogenetic patterns obtained in our study also allow us to address models of speciation within the *sinica* species-group (Fig. 5). Our results broadly support the evolutionary scenario proposed by Delson (1980) for this group of macaques based on fossil evidence. In his model, a long-tailed proto-*sinica* species arose in or west of present-day Myanmar, and moved west and south into peninsular India approximately at 1.5–2 million years ago (mya). Our molecular analysis suggests a date of origin of *c*. 3.2 (TSPY) to 4.5 (mitochondrial DNA) mya for such an ancestor. During the westward movement, a branch possibly emigrated north towards China, differentiating into a relatively large-bodied, short-tailed form represented by some of the earlier fossils of *Macaca* cf. *anderssoni* (*c*. 3 mya; Delson, 1980) and its present-day descendants, *M. assamensis* and *M. thibetana*.

The ancestral *sinica* species that moved west might have colonised northeastern India approximately at 3–4 mya with a group (the proto-*radiata–sinica* stock) subsequently migrating southward into peninsular India. Our mitochondrial DNA analysis suggests that the immediate ancestor of *M. munzala* diverged from the proto-*radiata* stock at *c*. 3.2 mya. The modern-day *M. munzala* stock seems to have subsequently originated at *c*. 1.5 mya (TSPY analysis) following male introgression from a proto-*assamensis–thibetana* stock, followed by the appearance of the species, as we know it today, at *c*. 0.48 mya. The present-day *M. munzala* also appears to have evolved heavier bodies and shorter tails in parallel, given its distribution in the northern latitudes, thus displaying a further morpho–anatomical convergence with the *M. assamensis–M. thibetana* stock that had diverged earlier.

Additionally, the geological history of the river Brahmaputra indicates it to be a young river with its present configuration taking shape only during the late Pliocene to Early Pleistocene, at *c*. 1.8 mya (Barman, 1981). It is possible that the isolated forest refugia associated with Pleistocene glacials may have provided rich opportunity for the introgression and hybridization, which led to the origin of *M. munzala*, to have occurred in this region (Eudey, 1980; Jablonski, 1993). Today, the river is considered a geographical divide between the Indo-Burmese and Indian biogeographic regions serving as a physical barrier to secondary contact between the modern-day macaque species.

Finally, the southern branch of the proto-*sinica* group may have given rise to *M. radiata* of southern India and *M. sinica* of Sri Lanka, possibly during the Middle Pleistocene (at 2.0–0.1 mya), a time of alternating climatic amelioration and decay when the modern-day populations of *M. thibetana* and *M. munzala* may have also evolved and occupied their present ranges. Such a scenario is strongly supported by (i) fossil evidence of ancestral *Macaca* populations (Delson, 1980), (ii) the apparently less-derived penile morphology and cranial hair-flow patterns of *M. assamensis* and *M. thibetana* relative to *M. radiata* or *M. sinica* (Hill and Bernstein, 1969), and (iii) our molecular clock estimates of the divergence times of these species. Further phylogenetic analyses with fast-evolving, neutral autosomal markers would provide a better understanding of the spatio-temporal aspects of speciation within the *sinica* species-group of the genus.

Acknowledgments

We wish to thank the Forest Department of Arunachal Pradesh for their support as well as research permissions. Special thanks are due to the Chief Wildlife Warden and Pekyom Ringu for their continued support for our research. We acknowledge grants from the Rufford Maurice Laing Foundation, the Whitley Fund for Nature, the Wildlife Conservation Society—India Program, the Department of Science and Technology—Government of India, and the National Centre for Biological Sciences—Tata Institute of Fundamental Research, which made this work possible. We also thank the Primate Research Laboratory, Indian Institute of Science for blood samples of bonnet macaques. We are extremely grateful to two anonymous referees whose valuable suggestions have significantly improved the quality of the paper. Finally, we express our gratitude to R. Suresh Kumar, Uttara Mendiratta, M.D. Madhusudan, Mayukh Chatterjee, Kakoli Mukhopadhyay, Aparajita Datta, Sonia Joseph, R. Raghunath, Sujoy Chaudhuri, members of the Uma Ramakrishnan laboratory, and V.L. Roth of Duke University for their help, support and encouragement in the field and in the laboratory.

References

Allen, J.A., 1877. The influence of physical conditions in the genesis of species. Radic. Rev. 1, 108–140.

Arnason, U., Gullberg, A., Janke, A., 1998. Molecular timing of primate divergences as estimated by two nonprimate calibration points. J. Mol. Evol. 47, 718–727.

Arnason, U., Gullberg, A., Burguete, A.S., Janke, A., 2000. Molecular estimates of primate divergences and new hypotheses for primate dispersal and the origin of modern humans. Hereditas 133, 217–228.

Barman, G., 1981. Geomorphology of the Brahmaputra basin, its flood problems and their possible remedial measures. In: Proceedings of the Seminar on Fluvial Process and Geomorphology of the Brahmaputra Basin, Miscellaneous Publication No. 46, Geological Survey of India, Calcutta (now Kolkata), pp. 21–31.

Blackburn, T.M., Gaston, K.J., Loder, N., 1999. Geographic gradients in body size: a clarification of Bergmann's rule. Divers. Distrib. 5, 165–174.

Bradley, R.D., Baker, R.J., 2001. A test of the genetic species concept: cytochrome-*b* sequences and mammals. J. Mamm. 82, 960–973.

Brehm, G., Fiedler, K., 2004. Bergmann's rule does not apply to geometrid moths along an elevational gradient in an Andean montane rainforest. Global Ecol. Biogeogr. 13, 7–14.

Delson, E., 1980. Fossil macaques, phyletic relationships and a scenario of deployment. In: Lindburg, D.G. (Ed.), Macaques: Studies in Ecology, Behavior and Evolution. Van Nostrand Reinhold, New York, pp. 10–30.

Delson, E., 1996. The oldest monkeys in Asia. In: Takenaka, O. (Ed.), Abstracts, International Symposium on Evolution of Asian Primates. Freude and Kyoto University, Primate Research Institute, Inuyama, Aichi, Japan, p. 40.

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, Amsterdam, pp. 52–83.

Fooden, J., 1988. Taxonomy and evolution of the *sinica* group of macaques: 6. Interspecific comparisons and synthesis. Fieldiana Zool. (N.S.) 45, 1–44.

Hayasaka, K., Gojobori, T., Horai, S., 1988. Molecular phylogeny and evolution of primate mitochondrial DNA. Mol. Biol. Evol. 5, 626–644.

Hill, W.C.O., Bernstein, I., 1969. On the morphology, behaviour and systematic status of the Assam macaque (*Macaca assamensis* McClelland, 1839). Primates 10, 1–17.

Jablonski, N.G., 1993. Quaternary environments and the evolution of primates in East Asia, with notes on two new specimens of fossil Cercopithecidae from China. Folia Primatol. 60, 118–132.

James, F.C., 1970. Geographic size variation in birds and its relationship to climate. Ecology 51, 365–390.

Kumar, S., Tamura, K., Nei, M., 2004. MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. 5, 150–163.

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

Li, Q., Zhang, Y., 2005. Phylogenetic relationships of the macaques (Cercopithecidae: *Macaca*), inferred from mitochondrial DNA sequences. Biochem. Genet. 43, 375–386.

Meiri, S., Dayan, T., 2003. On the validity of Bergmann's rule. J. Biogeogr. 30, 331–351.

Morales, J.C., Melnick, D.J., 1998. Phylogenetic relationships of the macaques (Cercopithecidae: *Macaca*), as revealed by high resolution restriction site mapping of mitochondrial ribosomal genes. J. Hum. Evol. 34, 1–23.

Pan, R., Oxnard, C., 2004. Craniodental variation in the African macaque, with reference to various Asian species. Folia Primatol. 75, 355–375.

Posada, D., 2006. ModelTest Server: a web-based tool for the statistical selection of models of nucleotide substitution online. Nucleic Acids Res. 34, W700–W703.

Raaum, R.L., Sterner, K.N., Noviello, C.M., Stewart, C.B., Disotell, T.R., 2005. Catarrhine primate divergence dates estimated from complete mitochondrial genomes: concordance with fossil and nuclear DNA evidence. J. Hum. Evol. 48, 237–257.

Ray, C., 1960. The application of Bergmann's rule and Allen's rule to the poikilotherms. J. Morphol. 106, 85–108.

Robinson-Rechavi, M., Huchon, D., 2000. RRTree: relative-rate tests between groups of sequences on a phylogenetic tree. Bioinformatics 16, 296–297.

Ronquist, F., Huelsenbeck, J.P., van der Mark, P., 2005. MrBayes 3.1 Manual, Draft 5/26/2005. http://mrbayes.csit.fsu.edu/mb3.1_manual.pdf. Accessed on May 6, 2006.

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.

Sanderson, M.J., 1997. A nonparametric approach to estimating divergence times in the absence of rate constancy. Mol. Biol. Evol. 14, 1218–1231.

Sanderson, M.J., 2003. r8s: inferring absolute rates of molecular evolution and divergence times in the absence of a molecular clock. Bioinformatics 19, 301–302.

Sinha, A., Datta, A., Madhusudan, M.D., Mishra, C., 2005. *Macaca munzala*: a new species from western Arunachal Pradesh, northeastern India. Int. J. Primatol. 26, 977–989.

Sinha, A., Kumar, R.S., Mishra, C., 2006. Ecology and Conservation of the Arunachal Macaque *Macaca munzala*. NCF Technical Report No. 15. Nature Conservation Foundation, National Institute of Advanced Studies and International Snow Leopard Trust, Mysore, India.

Swofford, D.L., 2002. PAUP*: phylogenetic analysis using parsimony (* and other methods). Version 4.0b10. Sinauer, Sunderland, MA, USA.

Tosi, A.J., Morales, J.C., Melnick, D.J., 2000. Comparison of Y chromosome and mtDNA phylogenies lead to unique inferences of macaque evolutionary history. Mol. Phylogen. Evol. 17, 133–144.

Tosi, A.J., Morales, J.C., Melnick, D.J., 2002. Y-chromosome and mitochondrial markers in *Macaca fascicularis* indicate introgression with Indochinese *M. mulatta* and a biogeographic barrier in the Isthmus of Kra. Int. J. Primatol. 23, 161–178.

Tosi, A.J., Morales, J.C., Melnick, D.J., 2003. Paternal, maternal and biparental molecular markers provide unique windows into the evolutionary history of macaque monkeys. Evolution 57, 1419–1435.

Ziegler, T., Abegg, C., Meijaard, E., Perwitasari-Farajallah, D., Walter, L., Keith Hodges, J., Roos, C., 2007. Molecular phylogeny and evolutionary history of Southeast Asian macaques forming the *M. silenus* group. Mol. Phylogenet. Evol. 42, 807–816.