Evolution and dispersal of three closely related macaque species, *Macaca mulatta*, *M. cyclopis*, and *M. fuscata*, in the eastern Asia

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Abstract

Macaca mulatta, *M. cyclopis* and *M. fuscata* are three closely related species in the *fascicularis* species group. *M. mulatta* is wide-spread in Asia, while *M. cyclopis* and *M. fuscata* are restricted to Taiwan and Japan, respectively. Both *M. cyclopis* and *M. fuscata* are thought to be derived from ancient '*mulatta*' populations in the eastern Asia. In this study, we analyzed sequences of mitochondrial DNA control region to provide genetic evidence for the evolution and dispersal scenario of the three species proposed by Fooden and Albrecht [Fooden, J., Albrecht, G.H. 1999. Tail-length evolution in *fascicularis*-group macaques (Cercopithecidae: *Macaca*). Int. J. Primatol. 20, 431–440]. Our results indicated that several localities in the southern China and Vietnam harbored multiple divergent mtDNA lineages that may not have evolved sympatrically. These divergent mtDNA lineages may have originated from different ancient northern populations that retreated into southern localities during glacial periods. However, the age of the southward retreat and the northward recolonization may be dated back to a more ancient past during late middle Pleistocene (0.12–0.18 mya) instead of during the LGM (0.018 mya). Times of gene divergence between *M. mulatta* and the two island species, estimated by mean nucleotide difference, suggest the ancestral populations colonized Taiwan and Japan around 0.38–0.44 mya. In addition, a more recent age of *mulatta–cyclopis–fuscata* population divergence (when ancient populations were isolated), estimated to be 0.17 mya by net nucleotide divergence, is suggested.
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1. Introduction

The 19 extant species of the genus *Macaca* (Brandon-Jones et al., 2004) have been organized into three to six species groups according to morphological or genetic differences (Delson, 1980; Fooden, 1976; Fooden and Lanyon, 1989; Groves, 2001; Hayasaka et al., 1996; Li and Zhang, 2005; Zhang and Shi, 1993). Among these species groups, the evolutionary relationship of the *fascicularis* species group, composed of *Macaca fascicularis*, *M. mulatta*, *M. cyclopis* and *M. fuscata*, is of particular interest because paraphyly has been observed from genetic data (Hayasaka

et al., 1996; Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2000, 2002, 2003). Based on maternally, paternally and bi-parentally inherited gene sequences, evolutionary relationship of the *fascicularis* species group has been described by Melnick and colleagues (Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2000, 2002, 2003). According to their studies, *M. fascicularis* is the ancestral form among the four species. *M. mulatta* may have originated from a *fascicularis*-like ancestor 2.5 million years ago (mya) and became widely distributed within a relatively short period. The rapid expansion was followed by mitochondrial differentiation between the eastern and western parts of the species range (about 0.75 mya). After the intraspecific divergence, populations of eastern *M. mulatta* (China–Burma) colonized Taiwan and Japan during glacial periods when land bridges were available. Mitochondrial

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differentiation between *M. mulatta* and *M. cyclopis* or *M. fuscata* was estimated to be 0.25 and 0.50 mya, respectively.

Female philopatry of *Macaca* species maintains a geographic structuring of the maternally inherited mitochondrial DNA (mtDNA) that reflects patterns of cladogenic events (Melnick et al., 1993). It explains the observation that mtDNA haplotypes of eastern *M. mulatta* (those from China) are more similar to haplotypes of the two insular species (*M. cyclopis* and *M. fuscata*) than to haplotypes of western *M. mulatta* (those from India–Pakistan), or mtDNA paraphyly of *M. mulatta* (Melnick et al., 1993; Morales and Melnick, 1998). However, such pattern was not detected in Y-chromosome (Tosi et al., 2000). This can be attributed to male-mediated gene flow between eastern and western *M. mulatta* that homogenized nuclear genetic variation across geographic range (Tosi et al., 2002, 2003). Moreover, a southward introgression by male *M. mulatta* to populations of *M. fascicularis* generated a paraphyletic Y-chromosome pattern in *M. fascicularis* (Tosi et al., 2002, 2003).

According to Allen's rule, size of extremities such as ears and tails of mammals increases from colder to warmer climates in the same or closely related species, especially for those inhabiting a large latitudinal range. The composite distribution range of members in the *fascicularis* species group encompasses a wide latitudinal zone from 10°S to 40°N. The relative tail length (RTL, tail length/head and body length) in the species group generally decreases with increasing latitudes just as predicted by Allen's rule (Fooden, 1997). However, the correlation does not apply to *M. mulatta* inhabiting the 15°N–25°N latitudinal zone, i.e. the southern populations (Fooden and Albrecht, 1999). RTL of extant southern *mulatta* populations is less than that of *M. cyclopis* living in similar latitudes, but is similar to that of the conspecific northern populations. Fooden and Albrecht (1999) also in Fooden (2000) suggested that modern southern *mulatta* populations did not originate within their present range; instead, they may be descendents of the populations retreating from farther north. They proposed a hypothetical scenario for the evolution and dispersal of *M. mulatta* based on variation of RTL in the *fascicularis* species group as well as the evolutionary history of the *mulatta* group (including *M. mulatta*, *M. cyclopis* and *M. fuscata*), which will be briefly described in the following.

By the observed trend between latitude and RTL in the *fascicularis* species group, the ancient *mulatta* populations originally inhabited between 15°N and 25°N were probably more similar to *M. cyclopis* in RTL (mean RTL = 0.80, the long-tailed populations), while those inhabiting farther north were more similar to *M. fuscata* in RTL (mean RTL = 0.15 at 40°N, the short-tailed populations). The latitudinal gradient in RTL was formed gradually during northward population expansion at least 0.04 mya. At this stage, the ancient *mulatta* populations colonized Taiwan and Japan when dispersing routes were available. Extant *M. fuscata* and *M. cyclopis* are two relictual populations descended from the ancient short-tailed populations and long-tailed populations, respectively. During the last glacial

maximum (LGM, about 0.018 mya), when climatic deterioration compressed suitable habitats southward, the short-tailed populations (mean RTL < 0.50) retreated from north in the eastern Asia and replaced the long-tailed populations. During the southward range shift, a west–east gradient of decreasing RTL had been established (mean RTL = 0.45 at 100°E, mean RTL = 0.30 at 120°E). However, the factors responsible for the gradient are not clear. Subsequent to the LGM, i.e. during the Holocene expansion, both the eastern and western populations of *M. mulatta* dispersed northward.

If the southward dispersal of *M. mulatta* in the eastern Asia occurred during the LGM as described by Fooden and Albrecht (1999), the phylogenetic relationship of the extant local populations may not correspond to geographic affinity and the dispersal pattern may be hard to trace. Furthermore, dispersal pattern of *M. mulatta* during Holocene expansion (about 0.01 mya) should be apparent from the geographic distribution of closely related mtDNA haplotypes. However, the low divergent level of mitochondrial ribosomal RNA genes that Morales and Melnick (1998) used may not be sensitive enough to reveal recent differentiation. A more polymorphic mtDNA marker is needed.

Mitochondrial DNA polymorphism is commonly used to reveal phylogenetic and phylogeographic relationships between populations of a species or closely related species (Avise et al., 1987). The control region (CR), a unique non-coding nucleotide sequence, is the most variable portion of mtDNA in mammals (Saccone et al., 1993). The sedentary nature of female macaques and the maternal inheritance of mtDNA make the molecule an ideal tool to elucidate population history (Melnick et al., 1993). Given the scenario hypothesized by Fooden and Albrecht (1999), objectives in this study are: (1) to find genetic evidence that supports the southward retreat in *M. mulatta*; (2) to trace the possible dispersal patterns of northward range expansion of *M. mulatta*; and (3) to estimate the time of divergent events of ancestral *M. cyclopis* and *M. fuscata*.

2. Materials and methods

2.1. Sample collection

In this study, mtDNA CR sequences of the four species in the *fascicularis* species group were analyzed. Samples of 148 *M. cyclopis* (107 fecal, 32 hair and 9 tissue samples) collected from five regional populations in Taiwan (GenBank accession nos. AY878873–AY878925 and DQ143984–DQ143987) and 5 *M. fascicularis* of unknown origin (GenBank accession nos. AY884307–AY884311) were sequenced. Sequences of 98 *M. mulatta* sampled from 11 provinces of China and Vietnam (GenBank accession nos. AF135271–AF135368, unpublished data by Ding et al.) and 50 *M. fuscata* sampled from six local populations in Japan (GenBank accession nos. AJ419855–AJ419864, Marmi et al., 2004) were included. Localities of samples with known geographic origins are indicated in Fig. 1.
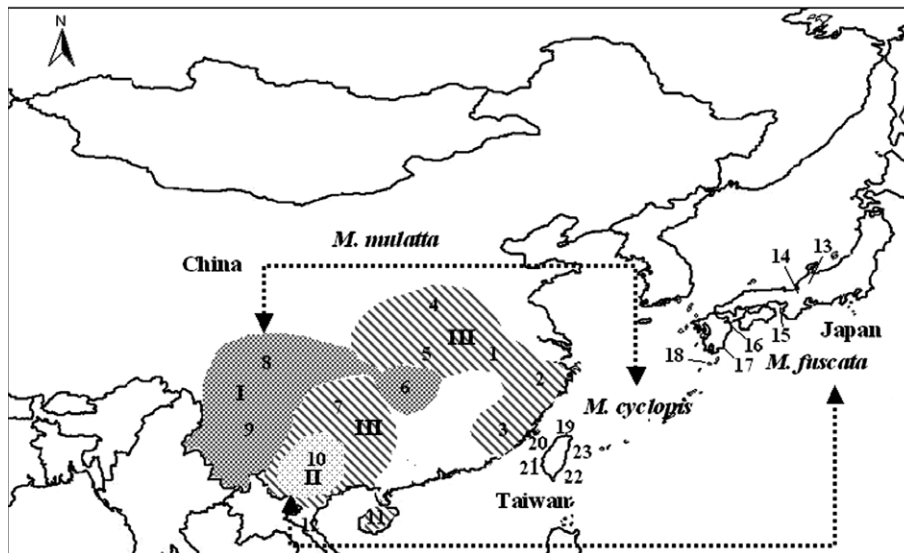
Fig. 1. Sampling localities of *Macaca mulatta*, *M. fuscata* and *M. cyclopis*. (1) Anhui, (2) Zhejiang, (3) Fujian, (4) Henan, (5) Hubei, (6) Hunan, (7) Guizhou, (8) Sichun, (9) Yunnan, (10) Guangxi, (11) Hainan, (12) Vietnam, (13) Hakusan, (14) Takahama, (15) Awajishima, (16) Takasakiyama, (17) Koshima, (18) Yakushima, (19) North Taiwan, (20) Central Taiwan, (21) South Taiwan, (22) East Taiwan and (23) Northeast Taiwan. Geographic distribution of the three phylogenetic clades of *M. mulatta*, haplogroups I, II and III, is also shown on the map. Arrows indicate that *M. cyclopis* and *M. fuscata* may be genetically more related to *M. mulatta* of haplogroups I and II, respectively.

2.2. DNA extraction, PCR amplification and nucleotide sequencing

Total genomic DNA was extracted by traditional phenol–chloroform procedure (Kocher et al., 1989). Some of the DNA samples extracted from feces were further eluted by a silica pellet method (Geneclean III, Bio101) when the first PCR attempt failed. The mtDNA CR was amplified by PCR using a primer set of DL1 (forward): 5′-CCAGAAATGAA CACCCTTCCTAGGGC-3′ (Chu et al., 2005) and Saru5 (reverse): 5′-GCCA GGACCAAGCCTATTT-3′ (Hayasaka et al., 1991) that produced a 1.4 Kb fragment composed of the whole CR region and the conservative peripheral regions. The long PCR product may reduce the possibility to amplify a nuclear CR pseudogene. The PCR mixture contained 10 pmol of each primer, 200 μM dNTP, 1.5 mM MgCl$_2$, 1× PCR reaction buffer (20 mM Tris–HCl, pH 8.4, 20 mM KCl), 1 unit *Taq* polymerase (GIBCO BRL) and about 1–3 μL DNA extract in a total volume of 50 μL. A DNA thermal cycler (Biometra) was programmed to perform initial denaturation at 95 °C for 10 min and 32 cycles of 1 min at 95 °C, 1 min at 58 °C, and 2 min at 72 °C. A 10 min final extension at 72 °C was also included. PCR elutes were partially sequenced by ABI 377 or ABI 3100 automated DNA sequencer using DL1 (forward) and another internal primer DH1 (reverse): 5′-CGGAGATGGGGGGTGGGGGGGGTTTG-3′ (Chu et al., 2005).

2.3. Analysis of mitochondrial DNA sequences

Sequences were aligned by Pileup program in GCG (Wisconsin Package Version 10.1, Genetics Computer Group, Madison, WI). A fragment of 386 nucleotide sites was available for analyses after alignment. For a comparison of genetic polymorphism of the four species, nucleotide diversity (π) and mean nucleotide difference within species (d_x) were calculated using Arlequin 2.0 (Schneider et al., 2000).

Phylogenetic relationship of unique haplotypes was constructed using neighbor-joining (NJ) method (Saitou and Nei, 1987) and Bayesian approach and was rooted by sequences of *M. fascicularis*. NJ tree was constructed by MEGA 3.1 (Kumar et al., 2004). When constructing phylogenetic trees, distance estimated by a sophisticated model often generates a large variance and thus reduces the resolution of the tree topology when conducting bootstrap (Nei and Kumar, 2000b). The situation could be worse when the dataset is composed of a large number of taxa with short nucleotide sequence (Nei and Kumar, 2000a). To avoid such problem, a simple *p*-distance model was used to calculate genetic distance between distinct sequences. Statistical support of the tree topology was estimated by interior branch analysis with 1000 bootstrap replicates.

Bayesian analysis of the sequence data was performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003), with starting trees generated randomly. A total of 5,000,000 generations were run and the likelihood scores stabilized after 500,000 generations. A tree was saved every 100 generations to give 50,000 trees, of which the first 10,000 were rejected (corresponding to those obtained before the likelihood scores stabilized). Three Markov Chain Monte Carlo runs each having four simultaneous chains were performed independently. The joint posterior probability distribution of each run was congruent, suggesting that the chains were run for a sufficient number of generations to sample the posterior probability landscape adequately. Bootstrap values or likelihood probabilities of 90% and 70% were considered as thresholds of high and moderate supports for a clade, respectively.

2.4. Demographic inferences

Mismatch distribution analysis (MDA) was conducted to infer possible population expansion of a species. MDA assumes the pairwise nucleotide differences of haplotypes drawn from a population to have a unimodal distribution if it has experienced a demographic expansion event; otherwise, the distribution is multimodal reflecting a stochastic shape of gene trees (Rogers and Harpending, 1992). Goodness of fit, i.e. the sum of square deviations (SSD) between the observed and the expected mismatch (Schneider and Excoffier, 1999), and Harpending's raggedness index (Harpending, 1994) were calculated to examine the statistical support of the expansion event. Fu's F_s test (Fu, 1997) was conducted to further confirm the demographic expansion. Because insufficient sampling tends to bias the analysis, each unique haplotype was used once regardless of its frequency of occurrence in samples by assuming the increase of haplotypes refers to a population expansion event (Harpending et al., 1993). The approach may ignore recent expansions but can detect relatively ancient ones.

Time of a confirmed expansion was estimated by $\tau = 2ut_e$, where τ is estimated by MDA, u is the sum of per nucleotide mutation rate in the DNA region under study and t_e is time since expansion. Mutation rates of the mtDNA CR estimated from various phylogenetic studies (reviewed by Parsons et al., 1997) were in a range of 0.025–0.26 site^{-1} myr^{-1}. In this study, we adopted the medium of the reported values, 0.14 site^{-1} myr^{-1}, which corresponds to a divergence rate of 28% per million years in the mtDNA CR. Hence, time since expansion was estimated by t_e (myr) $= \tau/(2 \times 0.14$ site^{-1} myr$^{-1} \times 386$ sites). A 95% confidence interval for τ was obtained by a parametric bootstrap approach (Schneider and Excoffier, 1999).

Initial times of the mtDNA CR divergence between the four species in the *fascicularis* species group were estimated by $d_{xy} = 2ut_d$, where d_{xy} is the mean nucleotide difference between haplotypes of two species, t_d is time since divergence and u is the same as described above. Because ancestral populations of *M. cyclopis* and *M. fuscata* may have originated from different local populations of ancient *M. mulatta*, genetic diversity in each of the two species is supposed to be a subset of genetic diversity of *M. mulatta* in the eastern Asia. Thus, samples of *M. mulatta* were divided into three groups as revealed from the phylogenetic analysis for further comparison. The initial periods of gene divergence between *M. cyclopis* or *M. fuscata* and each of the three groups of *M. mulatta* were also estimated by $d_{xy} = 2ut_d$. To correct the discrepancy between "gene divergence" and "population divergence" due to the presence of ancestral polymorphism in populations (Edwards and Beerli, 2000), times of population divergence (i.e., when the two ancestral populations were isolated to each other) between species or populations in the *mulatta* group were estimated by net nucleotide divergence, $d_A = 2ut$ (Nei and Li, 1979). The most conservative time of population divergence was estimated from the minimum value of d_A. Here

$d_A = d_{xy} - 1/2(d_x + d_y)$, where d_{xy} is the mean nucleotide difference between haplotypes of species (or populations) x and y, d_x and d_y are the mean nucleotide differences between haplotypes within species (or populations) x and y, respectively.

Since the distribution of the haplotypes from eastern China in the phylogenetic tree was not in accordance to their geographic affinity, pairwise nucleotide differences among haplotypes sympatric in Guangxi (14 haplotypes), Zhejiang (eight haplotypes) and Fujian (six haplotypes) provinces were examined for the possible ages of divergence. Distribution of pairwise nucleotide differences was calibrated on a time scale of Pleistocene events (Jenkins, 2001) by $d_{xy} = 2ut_d$.

3. Results

3.1. Mitochondrial DNA CR variation

Among the total sequences examined ($N = 301$), 124 distinct haplotypes were identified. Basic genetic polymorphism parameters of the four species are summarized in Table 1. In total, 188 variable sites were found, including 169 transitions, 44 transversions, and 20 indels. Nucleotide diversity was the highest in *M. fascicularis* (0.105 ± 0.065, $\pi \pm$ SD), followed by *M. mulatta* (0.081 ± 0.039), and was smaller in *M. fuscata* (0.062 ± 0.031) and in *M. cyclopis* (0.061 ± 0.030). The trend of genetic diversity agrees with the phylogenetic relationship of the four species that ancestral taxon tends to retain higher genetic diversity than derived taxa.

3.2. Phylogeny reconstruction

Branching patterns and the statistic supports in the NJ and the Bayesian trees were similar except for some tip clusters. Hence, only the NJ tree is illustrated in Fig. 2 to show the relationship between haplotypes. The NJ tree comprised five phylogenetic clades with high bootstrap support (>90%) and a single lineage (Zhejiang4 of *M. mulatta*) outward the major clades (Fig. 2A). *M. cyclopis* and *M. fuscata* each formed a monophyletic clade, while *M. mulatta* comprised three haplogroups (Haplogroup I to III) and the single lineage. The clustering patterns of the haplotypes within the two island species clades corresponded to their geographic affinity (Fig. 2B), while the patterns within the Haplogroups I and

Table 1

Sample size (N), number of haplotypes (H), number of polymorphic sites (P), and nucleotide diversity (π) in the 5′ end partial segment of the mtDNA CR of the four *Macaca* species in the *fascicularis* species group

Species	N	H	P	π (SD)
M. fascicularis	5	5	86	0.105 (0.065)
M. mulatta	98	57	138	0.081 (0.039)
M. cyclopis	148	53	107	0.061 (0.030)
M. fuscata	50	9	61	0.062 (0.031)
Total	301	124	188	

II of *M. mulatta* corresponded to geographic affinity as well. The Haplogroup I consisted of haplotypes from Hunan, Yunnan and Sichuan in China, while the Haplogroup II comprised of haplotypes from Guangxi in China and Vietnam. As for the Haplogroup III, it contained haplotypes from a wide range from Henan, Hubei, Anhui, Zhejiang, Fujian, Guangxi to Hainan in China and Vietnam (Fig. 1). In this haplogroup, closely related haplotypes were distributed in several provinces of China or Vietnam, and some sympatric haplotypes in Henan, Zhejiang, Fujian, Guangxi, and Vietnam were too divergent to be clustered together (Fig. 2A). In addition, since bootstrap supports among the five phylogenetic clades were less than 90%, the relationship of these clades could not be concluded. However, under the
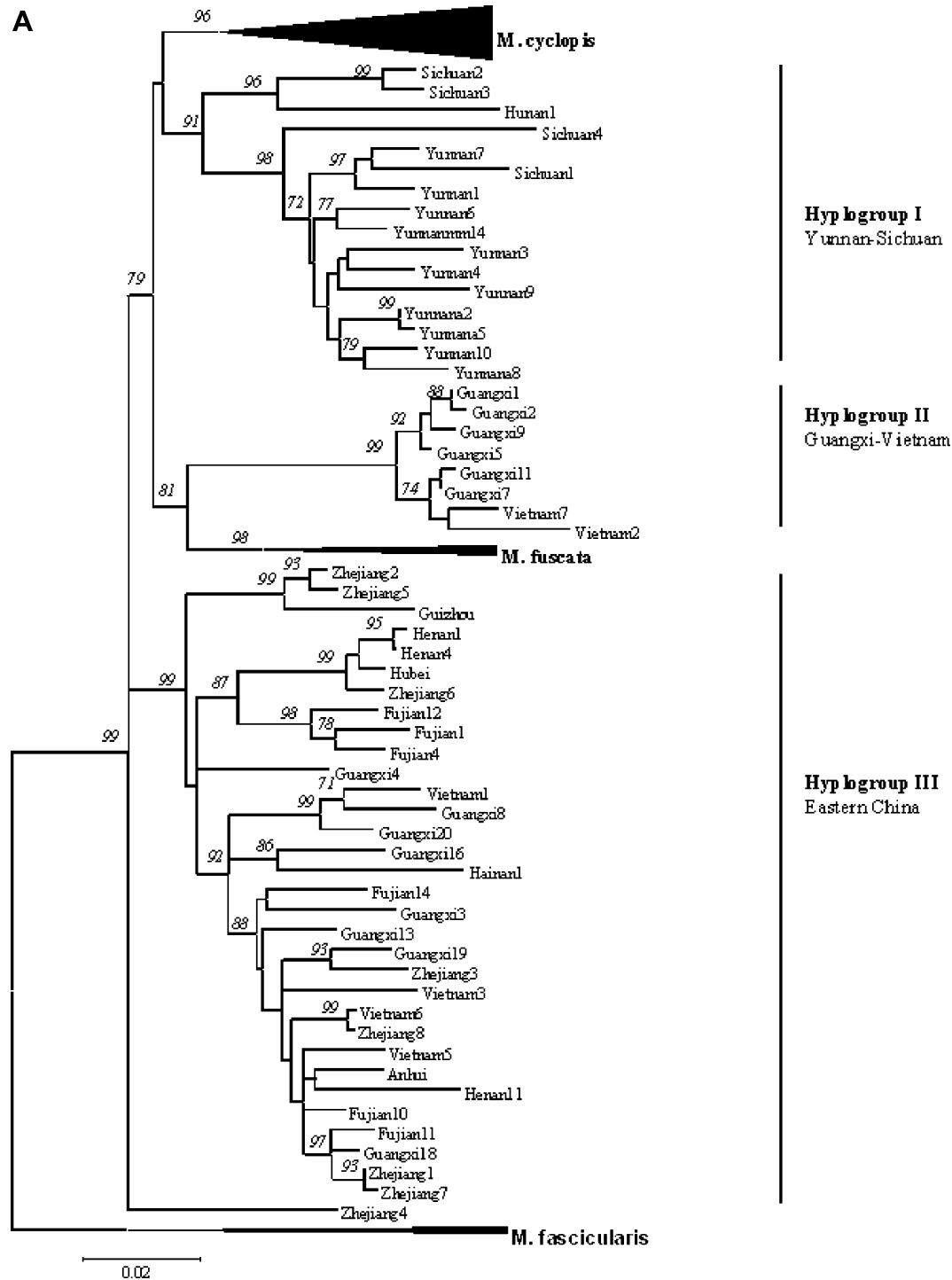

Fig. 2. Neighbor-joining tree illustrating the phylogenetic relationship among the 124 mtDNA CR haplotypes of the four *Macaca* species in the *fascicularis* species group. The percentage bootstrap support (1000 replicates) greater than 70 is listed for interior branches. The haplogroups of *M. cyclopis* (53 haplotypes) and *M. fuscata* (nine haplotypes) are compressed in (A) and the haplogroups of *M. mulatta* are compressed in (B) in order to simplify the tree topology. *M. fascicularis* (five haplotypes) is the outgroup to root the tree. Haplotype codes of *M. mulatta* follow Ding et al. published in the Genbank.
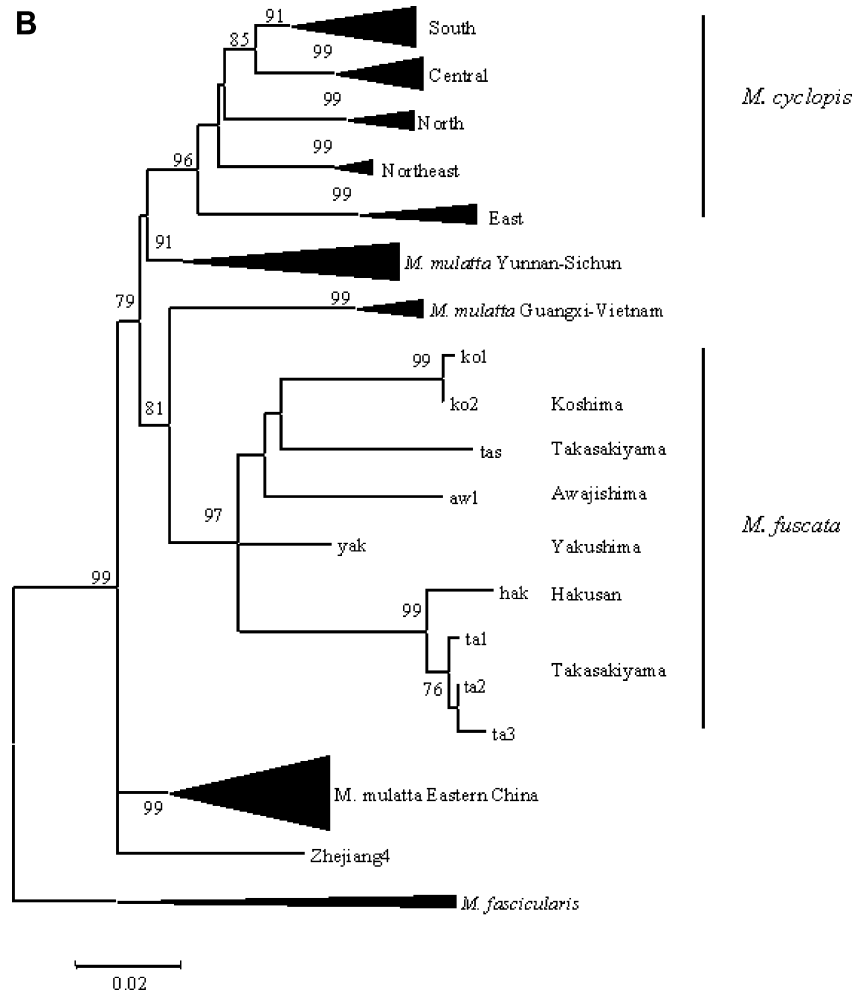
Fig. 2 (*continued*)

criterion of moderate support (bootstrap value > 70%), *M. fuscata* was grouped with Haplogroup II into a cluster (bootstrap value = 81%), which belonged to a larger assemblage with *M. cyclopis* and the Haplogroup I included (bootstrap value = 79%).

3.3. Demographic inferences

Statistical parameters of MDA did not reject a smooth, unimodal distribution of pairwise nucleotide differences between unique haplotypes in each of the four species in the *fascicularis* species group (Table 2). It indicated that population expansion occurred in all of the four species. Only the

ages of expansion in *M. mulatta* and *M. cyclopis* were described because samples of *M. fascicularis* (five haplotypes) and *M. fuscata* (nine haplotypes) were under-represented. Mismatch distributions of *M. mulatta* and *M. cyclopis* are illustrated in Fig. 3. Expansion events of the two species were further confirmed by the large negative values of Fu's F_s (Table 2). The values of τ were 33.5 in *M. mulatta* and 23.9 in *M. cyclopis*, which gave the estimated ages of expansion to be 0.31 mya (95% CI of $t_e = 0.24$–0.41 mya) and 0.22 mya (95% CI of $t_e = 0.15$–0.37 mya) for the two species, respectively.

The mean number of nucleotide differences (d_x) within species showed the same decreasing trend from the basal taxon

Table 2
Population expansion parameters and Fu's F_s test of the four *Macaca* species in the *fascicularis* species group

Species	τ (95% CI)	t [a] (95%CI)	SSD[b] (p)	RI[c] (p)	Fu's Fs (p)
M. fascicularis	49.5 (35.4–62.1)	0.46 (0.33–0.57)	0.075 ($p = 0.75$)	0.200 ($p = 0.53$)	1.27 ($p = 0.47$)
M. mulatta	33.5 (26.2–44.8)	0.31 (0.24–0.41)	0.002 ($p = 0.83$)	0.001 ($p = 1.00$)	−24.13 ($p < 0.01$)
M. cyclopis	23.9 (16.6–40.2)	0.22 (0.15–0.37)	0.004 ($p = 0.34$)	0.003 ($p = 0.70$)	−24.12 ($p < 0.01$)
M. fuscata	30.6 (23.1–36.0)	0.28 (0.21–0.33)	0.035 ($p = 0.25$)	0.099 ($p = 0.14$)	−1.08 ($p = 0.18$)

[a] The time unit of t is in myr.

[b] SSD: The sum of square deviations between the observed and the expected mismatch (Schneider and Excoffier, 1999).

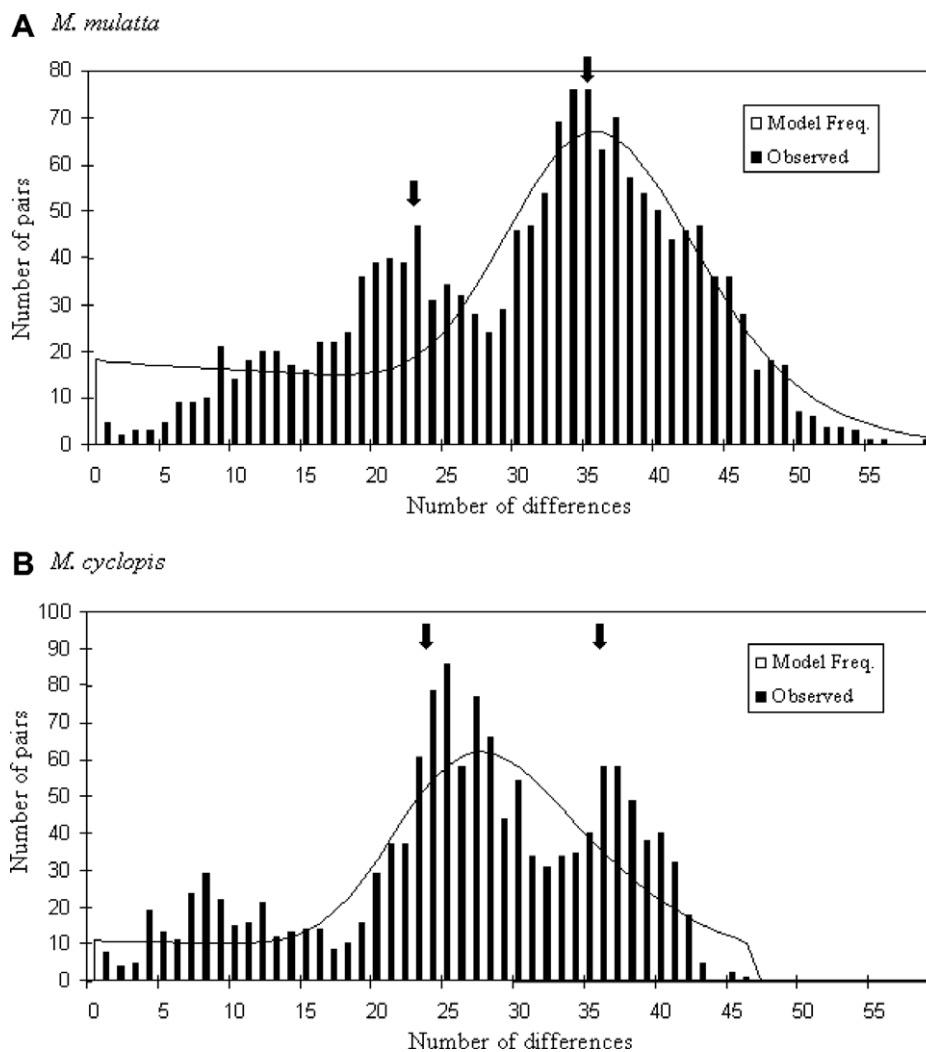[c] RI: Harpending's raggedness index (Harpending, 1994).

Fig. 3. Observed and expected mismatch (model frequency) distributions given the null distribution (unimodal, bell-shaped) corresponding to population expansion for (A) *M. mulatta*, and (B) *M. cyclopis*. Arrows indicate similar modes of population expansion.

(*M. fascicularis*, $d_x = 40.5$) to the tip taxa (*M. cyclopis* and *M. fuscata*, $d_x = 26.2$ and 24.7, respectively) in this species group (Table 3). The values of d_{xy} between *M. cyclopis* and each of the three haplogroups of *M. mulatta* ranged from 42.7 to 47.1 (Table 3), which corresponded to an initial gene divergent time of 0.40–0.44 mya. Similarly, the values of d_{xy} between *M. fuscata* and each of the three haplogroups of *M. mulatta* ranged from 40.8 to 45.4, which corresponded to an initial gene divergent time of 0.38–0.42 mya. The minimum values of d_A between *M. cyclopis* or *M. fuscata* and *M. mulatta* were 18 and 19.5 (Table 4), which indicated the most conservative time of population divergence to be 0.17 and 0.18 mya, respectively. In addition, various divergent levels were found between hap-

lotypes sympatric in Guangxi, Zhejiang and Fujian. The pair-wise nucleotide differences of these sympatric haplotypes showed coincident modes around 0.01, 0.06–0.12 and 0.18–0.32 mya (Fig. 4), the timing of which approximated to various interglacial periods in the Pleistocene.

4. Discussion

Previous authors have reported mtDNA paraphyly among *mulatta* lineages from India and China (Hayasaka et al., 1996; Melnick et al., 1993; Morales and Melnick, 1998; Tosi et al., 2003). We also found that *M. mulatta* is not a mitochondrially monophyletic taxon. However,

Table 3

Mean number of nucleotide differences among (d_{xy}, above diagonal), within (d_x, diagonal) and net nucleotide differences among (d_A, below diagonal) the four *Macaca* species in the *fascicularis* group in a segment of 386 nucleotide sites of the mtDNA CR

	M. fascicularis	M. mulatta	M. cyclopis	M. fuscata
M. fascicularis	40.5	59.8	62.4	66.2
M. mulatta	24.2	30.8	43.2	44.1
M. cyclopis	29.0	14.7	26.2	45.6
M. fuscata	33.6	16.3	20.4	24.7

Table 4
Mean number of nucleotide differences among (d_{xy}, above diagonal), within (d_x, diagonal) and net nucleotide differences among (d_A, below diagonal) the five *Macaca* populations in the *mulatta* group in a segment of 386 nucleotide sites of the mtDNA CR

	M. cyclopis	M. mulatta I	M. mulatta II	M. mulatta III	M. fuscata
M. cyclopis	26.2	42.7	47.1	42.8	45.8
M. mulatta I[a]	18.0	23.2	44.7	38.5	43.4
M. mulatta II[b]	30.2	29.2	7.7	34.7	40.8
M. mulatta III[c]	19.3	16.5	20.4	20.8	45.4
M. fuscata	20.4	19.5	24.6	22.7	24.7

Minimum values of d_A between *M. cyclopis* or *M. fuscata* to each of the three haplogroups of *M. mulatta* are underlined.
[a] Haplogroup I (Yunnan-Sichun).
[b] Haplogroup II (Guangxi-Vietnam).
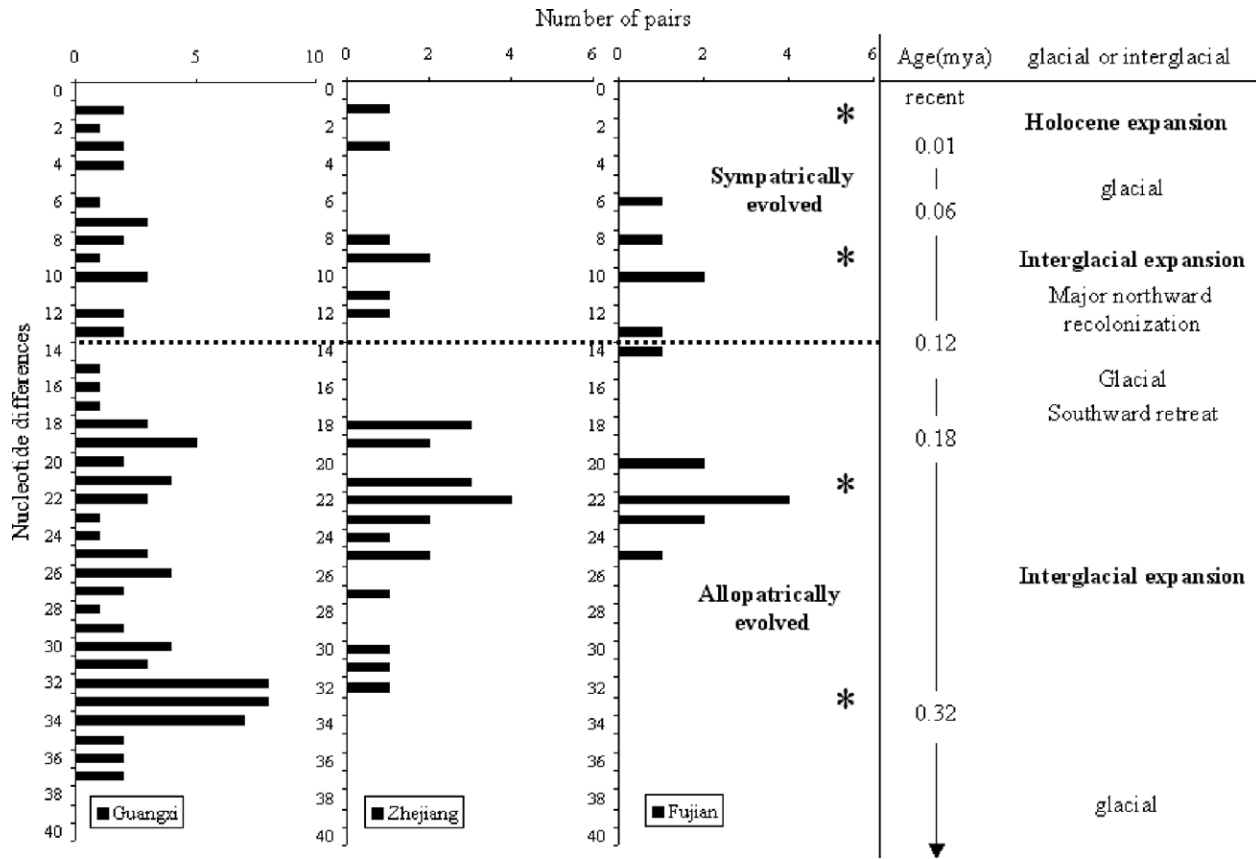[c] Haplogroup III (Eastern China).

Fig. 4. Distribution of pairwise nucleotide differences of *M. mulatta* haplotypes in Guangxi, Zhejiang, and Fujian provinces of China aligned with the time frame of glacial and interglacial periods in the Pleistocene. Dotted line indicates the pairwise nucleotide difference of 13, corresponding to divergence around 0.12 mya. Sympatric haplotypes differing by more than 13 nucleotides may have evolved allopatrically. Asterisks indicate possible expansion modes during interglacial periods in the Pleistocene.

although we did not have the sequences from India–Pakistan to re-examine the reported pattern, our result revealed a different paraphyletic pattern in mtDNA among *mulatta* lineages unreported elsewhere. By applying a highly polymorphic mtDNA marker and a larger panel of mtDNA sequences than previous studies (Deinard and Smith, 2001; Hayasaka et al., 1996; Morales and Melnick, 1998; Tosi et al., 2000; 2003; Zhang and Shi, 1993), we found that western (Yunnan–Sichuan) and eastern Chinese lineages were separated from each other in the phylogenetic tree with respect to *M. cyclopis* and *M. fuscata* (Fig. 2A). The result supports the recent divergence of *M. cyclopis* and *M.*

fuscata from Chinese *M. mulatta* and the retention of genetic polymorphism in the Chinese *M. mulatta* as described elsewhere (Hoelzer, 1997; Melnick et al., 1993; Morales and Melnick, 1998). Moreover, the monophyly of *M. cyclopis* and *M. fuscata* suggests postglacial vicariance after the ancestral *mulatta* populations dispersed into Taiwan and Japan, respectively.

4.1. Retreat-recolonization history of M. mulatta

In this study, no distinct geographic pattern was found in haplotypes of *M. mulatta* distributed in the eastern

China (Fig. 2A). Multiple divergent mtDNA lineages (especially those differing by more than 20 nucleotides) were found sympatric in Henan, Zhejiang, Fujian and Guangxi provinces of China and Vietnam, but no haplotype closely related to these lineages in nearby localities. It shows a discrepant phylogeographic pattern against isolation by distance (IBD) model (Wright, 1943). By assuming mtDNA haplotypes accumulate during population expansion and a mutation rate of $0.14 \, \text{site}^{-1} \, \text{myr}^{-1}$ in the mtDNA CR, possible nucleotide differences between sympatric haplotypes evolved from a single mtDNA matriline during different temporal ranges can be estimated. Applying the time frame of glacial and interglacial events described by Jenkins (2001), the divergent time of haplotypes differing by about two nucleotides is less than 0.01 my (i.e. during Holocene). Accordingly, differences of 6–13 and more than 20 nucleotides between haplotypes correspond to divergence since the last and the second last interglacials (0.06–0.12 mya and >0.2 mya), respectively (Fig. 4).

However, it is less likely for multiple mtDNA lineages to coexist for such a long time under the process of lineage sorting (Avise et al., 1984; Hoelzer et al., 1998; Wallman et al., 1996) when effective population size decreased dramatically during glacial periods (e.g. 0.01–0.06 mya and 0.12–0.18 mya, etc.). Moreover, even if these divergent lineages evolved sympatrically from the same mtDNA matriline, phylogenetic relationship between them should still follow the IBD model not observed in this study. Hence, it is less likely that these divergent mtDNA haplotypes evolved sympatrically.

Two other scenarios may lead to sympatry of multiple divergent mtDNA lineages observed in this study. It may have resulted either from southward retreat that caused a stochastic collection of divergent lineages in a locality (i.e. a refugium) during glacial period, or from population expansion that brought divergent mtDNA lineages into contact during interglacial period. In the former case, it infers "forced dispersal" for populations characterized by female philopatry. A great scale of mtDNA lineage extinction and range shift may have occurred during the retreat process due to competition or simply deteriorated environments. A corollary to it is a great gap of nucleotide difference between sympatric haplotypes and the lack of closely related haplotypes nearby. In the latter case, it infers secondary admixture due to population expansion. Since population expansion tends to maintain mtDNA lineages, haplotypes closely related to these divergent mtDNA lineages can be found in nearby populations.

Results in this study indicate that phylogenetic relationship of haplotypes distributed in the eastern China is against the IBD model. Multiple divergent mtDNA lineages were found coexisting in several localities without closely related lineages nearby. This suggests a complex amalgamation of distant mtDNA lineages due to the southward retreat in the eastern China. Thus, the genetic data supports that there have been a southward retreat as proposed by Fooden and Albrecht (1999). In addition, it also

indicates that possible glacial refugia may include regions in Zhejiang, Fujian and Guangxi provinces that harbor divergent mtDNA lineages in China (probably in the mountain regions such as Wuyi-Shan and Nan Ling).

Fooden and Albrecht (1999) proposed that the southward retreat and the subsequent recolonization of *M. mulatta* in the eastern Asia occurred during and after the LGM (0.018 mya), respectively. If ages of the two events were as proposed, the dispersal pattern of retreat may be hard to trace, while that of recolonization should be quite apparent. The resulting pattern should be some closely related mtDNA haplotypes (differed by one or two nucleotides) overlaying in a wide range (e.g. Chen et al., 2005; Conroy and Cook, 2000; Fu et al., 2005; Hatase et al., 2002; Ritchie et al., 2004; Ruedi and Castella, 2003). However, we did not detect any such pattern. Instead, geographic affinity observed in most sub-clades consisting of haplotypes differing by less than 13 nucleotides suggests to date the recolonization event back to a more ancient age. In addition, nucleotide difference between those sympatric but divergent mtDNA lineages (>20 nucleotides) also suggests a more ancient time of the retreat event than during LGM. Hence, the scenario presented by Fooden and Albrecht (1999) was supported by our genetic data, however, the chronology of the scenario is arguable. In the following, we propose plausible ages for the southward retreat and the northward recolonization of *M. mulatta*, based on the divergent level of mtDNA CR haplotypes.

First, the major southward retreat, which generated a complex pattern of CR haplotypes in the eastern China, occurred during the second last glacial (0.12–0.18 mya or earlier, corresponding to nucleotide difference >13 between sympatric haplotypes) instead of the LGM (0.018 mya). The retreat apparently displaced the native populations (the long-tailed *mulatta* populations) inhabiting the 15°N–25°N latitudinal zone and resulted in a discrepant phylogenetic relationship between *M. cyclopis* and *M. mulatta*. That is, *M. cyclopis* is not mitochondrially more related to *M. mulatta* in the eastern China (such as populations in Fujian and in Zhejiang). Instead, it is more related to *M. mulatta* in the southwest China (Yunnan, Haplogroup I) (Figs. 1 and 2). However, the connection between *M. cyclopis* and the Haplogroup I is not well supported (bootstrap = 28%) probably due to the extinction of the "transitional populations". The extant populations of *M. mulatta* in Yunnan and *M. cyclopis* may be two relictual groups descended from ancestors more similar to the long-tailed *mulatta* populations. The mountain area in the southwestern China proposed as a refugium for various animals and plants in China (Harrison et al., 2002) has protected Yunnan populations from displacement. Nonetheless, a decreasing west–east gradient of RTL from 90°E to 115°E (Fooden, 2000) and a more or less homogeneous pattern of allozymes (Fooden and Lanyon, 1989) and microsatellites (Morin et al., 1997) in *M. mulatta* indicate that male-mediated gene flow from the eastern China population to the Yunnan population was common. Besides, postglacial

vicariance may prevent gene flow between China and Taiwan and thus retained the morphological character of a longer tail in *M. cyclopis*.

Subsequently, during the last interglacial (0.06–0.12 mya, corresponding to nucleotide differences about 6–13 between haplotypes), the major northward recolonization occurred that covered the current range of *M. mulatta* in China. Population expansion during this period can be inferred from a similar level of lineage divergence within provinces of Yunnan and Fujian, as well as between Guangxi, Fujian, Zhejiang and Vietnam. During the last glacial, populations may have been isolated in multiple refugia located in Henan, Zhejiang, Fujiang and Guangxi, instead of performing another large-scaled retreat. Similar locations of glacial refugia in the southern China have also been suggested for a conifer species, *Cunninghamia konishii* (Lu et al., 2001).

Afterward, the Holocene expansion was mostly confined locally. It was inferred from the observation that the same or related haplotypes were found within provinces of China, e.g. haplotypes of Haplogroup II in Guangxi, haplotypes in Zhejiang, Henan, and Yunnan (Fig. 2A). However, the occurrence of the same or related haplotypes in distant localities provides some exceptions (detailed data not showed). For example, haplotype Sichuan1 occurs in Sichun and Hainen, while haplotype Yunnana5 occurs in Yunnan and Hunan, as well as two closely related haplotypes, Zhejiang9 and Vietnam6, occur in two distant localities Zhejiang and Vietam. The exceptions need to be re-examined for the possibility of anthropogenic introduction.

4.2. Divergence and demographic history of ancient mulatta populations

Mean nucleotide divergences between *M. mulatta* and each of the two island species, *M. cyclopis* and *M. fuscata* (d_{xy}, Table 3), suggest that ancestral *mulatta* populations colonized Taiwan and Japan in approximately the same time period around 0.38–0.44 mya during the early stage of the third last glacial (0.32–0.44 mya). The result is also supported by fossil records that suggest the existence of *M. cyclopis* in Taiwan and *M. fuscata* in Japan at least 0.3 mya (Fooden and Wu, 2001) and 0.4 mya (Kamei, 1969), respectively. Considering ancestral polymorphism, the most conservative estimates of the population divergent time between *M. mulatta* and each of the two species are around 0.17 and 0.18 mya, respectively. The estimates indicate the times of population (or species) isolation were during the end of the second last interglacial (0.18–0.32 mya) and the beginning of the second last glacial (0.12–0.18 mya). The observed monophyly in *M. cyclopis* and *M. fuscata* suggests that ancestral population of each species originated from different regional population in mainland at around 0.4 mya, and, during 0.17–0.4 mya, dispersed from mainland to Taiwan or Japan when dispersal routes were available. Furthermore, the divergent level between the three species also indicates that successful dispersal was unlikely

since the second last glacial. It is concordant with the palynological evidence that most of the exposed continental shelf connecting Taiwan and China was covered by savanna-like vegetation (Liew et al., 1998) that is not suitable for macaques. The cool and arid climate even extended to a lower latitudinal zone of the Sunda region (Gathorne-Hardy et al., 2002; Heaney, 1991).

The MDA did not reject a smooth, unimodal distribution of nucleotide differences between haplotypes in *M. mulatta* and *M. cyclopis*. It suggests that the population expansion occurred around 0.22 and 0.31 mya for *M. cyclopis* and *M. mulatta*, respectively (Table 2). Besides, there are multiple modes (arrows placed in Fig. 3) embedded in the empirical curves of both species. The similarity of the modal patterns in the two species indicates that they have experienced similar demographic history during Pleistocene. The expansion that dated back to a more ancient past (0.31 mya) also indicates earlier population colonization.

Molecular dating of any event (e.g. divergence of populations or population expansion) is subject to the referred mutation rate and needs to be interpreted with caution (Hillis et al., 1996). Nevertheless, the estimated ages of population expansion in the study are consistent with the glacial history, and the divergent times between *M. cyclopis*, *M. fuscata* and *M. mulatta* are within the ranges reported elsewhere (Hayasaka et al., 1996; Marmi et al., 2004; Morales and Melnick, 1998; Tosi et al., 2003). The hypothetic scenario suggested by Fooden and Albrecht (1999) is supported by our mtDNA CR data. However, the ages of the retreat and the recolonization of ancestral *M. mulatta* may be dated back to a more ancient time during the late middle Pleistocene (0.13–0.18 mya) and late Pleistocene (0.06–0.12 mya) instead of the LGM (0.018 mya). In addition, by applying the net nucleotide divergence (d_A, Nei and Li, 1979) and a more complete panel of mtDNA sequences than previous studies, a more recent population divergent time, 0.17 mya, for the three species of the *mulatta* group is suggested.

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