Phylogeography and population structure of the Yunnan snub-nosed monkey (*Rhinopithecus bieti*) inferred from mitochondrial control region DNA sequence analysis

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Abstract

Rhinopithecus bieti, **the Yunnan snub-nosed monkey, is the nonhuman primate with the highest altitudinal distribution and is also one of the 25 most globally endangered primate species. Currently,** *R. bieti* **is found in forests between 3000 and 4500 m above sea level, within a narrow area on the Tibetan Plateau between the Yangtze and Mekong rivers, where it is suffering from loss of habitat and shrinking population size (~1500). To assess the genetic diversity within this species, its population structure and to infer its evolutionary history, we sequenced 401 bp of the hypervariable I (HVI) segment from the mitochondrial DNA control region (CR) for 157 individuals from 11 remnant patches throughout the fragmented distribution area. Fifty-two variable sites were observed and 30 haplotypes were defined. Compared with other primate species,** *R. bieti* **cannot be regarded as a taxon with low genetic diversity. Phylogenetic analysis partitioned haplotypes into two divergent haplogroups (A and B). Haplotypes from the two mitochondrial clades were found to be mixed in some patches although the distribution of haplotypes displayed local homogeneity, implying a strong population structure within** *R. bieti*. **Analysis of molecular variance detected significant differences among the different geographical regions, suggesting that** *R. bieti* **should be separated into three management units (MUs) for conservation. Based on our results, it can be hypothesized that the genetic history of** *R. bieti* **includes an initial, presumably allopatric divergence between clades A and B 1.0–0.7 million years ago (Ma), which might have been caused by the Late Cenozoic uplift of the Tibetan Plateau, secondary contact after this divergence as a result of a population expansion 0.16–0.05 Ma, and population reduction and habitat fragmentation in the very recent past.**

Keywords: conservation, management unit, mitochondrial DNA, phylogeography, population structure, *Rhinopithecus bieti*

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Introduction

The genus of snub-nosed monkeys (*Rhinopithecus,* Colobinae, Cercopithecidae) comprises four species, three of which are endemic to China (the golden or Sichuan snub-nosed monkey, *R. roxellana*; the black or Yunnan

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snub-nosed monkey, *R. bieti*; and the gray or Guizhou snub-nosed monkey, *R. brelichi*). The fourth is distributed in northern Vietnam (Tonkin's snub-nosed monkey, *R. avunculus*). Fossil records indicate that snub-nosed monkeys were widely distributed in China and Vietnam during the Pleistocene (Jablonski 1998a, b). However, they are currently confined to very limited areas in isolated mountainous regions in Sichuan, Yunnan, Guizhou, Hubei, Shanxi, Gansu, Tibet and northern Vietnam (Kirkpatrick 1998; Quan & Xie 2002). Current census data suggests a

very worrying picture for three of these species, with 20 000 extant individuals of *R. roxellana* but only 1500 *R. bieti*, 800 *R. brelichi*, and 130–350 *R. avunculus* in the wild (Long *et al.* 1994 and 1996; Jablonski 1998a, b; Ren *et al.* 1998; Li *et al.* 2002; Quan & Xie 2002).

The divergence time among these four species has been estimated at 2.08–2.84 million years ago (Ma) based on analysis of the mitochondrial cytochrome *b* gene, and now the ranges of the extant species are disjunct (Jablonski 1998a, b; Quan & Xie 2002; Li *et al.* 2004). Previous studies based on morphological descriptions suggested that *R. bieti* diverged from other snub-nosed monkeys as a result of the uplift of Tibetan Plateau and became confined to a small region (Li & Lin 1983; Whyte 1984; Pan & Jablonski 1993; Pan & Oxnard 2001). Now *R. bieti* is confined to high-altitude forests between 3000 and 4500 m above sea level, within a narrow area on the Tibetan Plateau between the Yangtze and Mekong rivers (98°37′ to 99°41′E, 26°14′ to 29°20′N), making *R. bieti* the nonhuman primate inhabiting the highest known altitudes (Long *et al.* 1994 and 1996; Li *et al.* 2002). Recent field surveys found that wild *R. bieti* only remained in 15 remnant forest patches throughout the whole fragmented habitat range (Long *et al.* 1994 and 1996; Xiao *et al.* 2003). According to the landscape character, Xiao *et al.* (2003) have partitioned the whole habitat of *R. bieti* into four ranges: northern range, central range, southwest range and southeast range (Fig. 1). Now, wild *R. bieti* were suffering from a loss of habitat and were completely isolated in forest patches separated by busy highways, human-active regions and bare mountain slopes, making it impossible for individuals to move between each patch (Fig. 1) (Long *et al.* 1994 and 1996; Xiao *et al.* 2003; Ren personal communication). Because of its small numbers, major habitat reduction, serious fragmentation and threat of hunting, *R. bieti* has been identified as one of the most globally endangered 25 primate species during the 19th Congress of the International Primatological Society in Beijing in 2002.

Research on genetic variation in *R. bieti* has been limited and has not provided relevant information about this species. One study based on 52 allozyme loci detected very low genetic diversity (average heterozygosity, $H = 0.005$) within *R. bieti*, when compared with other primate species (*Macaca mulatta*, *Macaca fascicularis*, *Theropithecus gelada*, *Saimiri sciureus* etc. $H = 0.015–0.079$), implying a population bottleneck (Hu & Zhang 1996). However, Pan *et al.* (2006) sequenced the whole mitochondrial control region for 10 individuals and found five haplotypes. Until now, large-scale studies of the population genetics of *R. bieti* have not been carried out. For *R. bieti*, a threatened species with a limited population size, sound knowledge of its evolutionary history, contemporary genetics population structure and social structure is likely to contribute to appropriate conservation management strategies (Moritz 1994).

Critically, habitat destruction has not only reduced the overall population size to a very low level but has promoted marked isolation of the remaining populations. Therefore, a study of the genetic effects of population fragmentation is of central importance for long-term conservation guidelines (Modolo *et al.* 2005). In the present study, we analysed sequence variation of the mitochondrial control region, hypervariable region I (HV I) of 157 monkeys from 11 remnant patches, representing most of the remaining distribution and approximately 10% of the extant individuals. Our primary aims were: (i) to assess the level and partitioning of genetic variation within *R. bieti*, (ii) to analyse population demographic history and the events and factors which influenced it, (iii) to elucidate phylogeographical relationships of *R. bieti* among the remaining patches, (iv) to examine the extent to which habitat fragmentation has impacted on mitochondrial DNA (mtDNA) variation, and (v) to discuss the management strategies for this species.

Materials and methods

Sample collection and DNA extraction

Two blood, two muscle and 203 faecal samples were collected from 11 remnant patches throughout the current distribution range. Blood samples were collected when trapping individuals for radio-collaring and the samples were stored dry. Muscle samples were from dead individuals found in the wild and were stored in 95% ethanol. Faecal samples of different individuals were collected during direct behavioural observations and were also stored in 95% ethanol. To avoid resampling the same individual, each dropping was distinguished by freshness, size, shape, and colour, and faeces found less than 1.5 m apart were not sampled (Hayaishi & Kawamoto 2006). Blood and muscle samples were extracted using PCI (25:24:1 mix of phenol, chloroform, and isoamyl-alcohol) and chloroform (Sambrook *et al.* 1989). Faecal DNA samples were extracted using the DNA Stool Mini Kit (QIAGEN). In order to prevent contamination during DNA extraction, benches and plastic ware was cleaned with 10% bleach and sterile water and then exposed to ultraviolet (UV) light for 30 min. The surface of muscle samples was also exposed to UV light for 30 min. We used 20 extraction controls and none produced positive amplification during subsequent polymerase chain reaction (PCR). Nine human microsatellite loci, which are polymorphic in *R. bieti* (D1S207, D1S533, D2S1326, D5S1457, D6S493, D8S505, D11S2002, D17S1290 and D20S206; data not shown), were used to distinguish the origin of some faecal samples according to the method of Zhan *et al.* (2006), in the cases where behavioural observation could not identify individuals. We confirmed 153 different individuals from
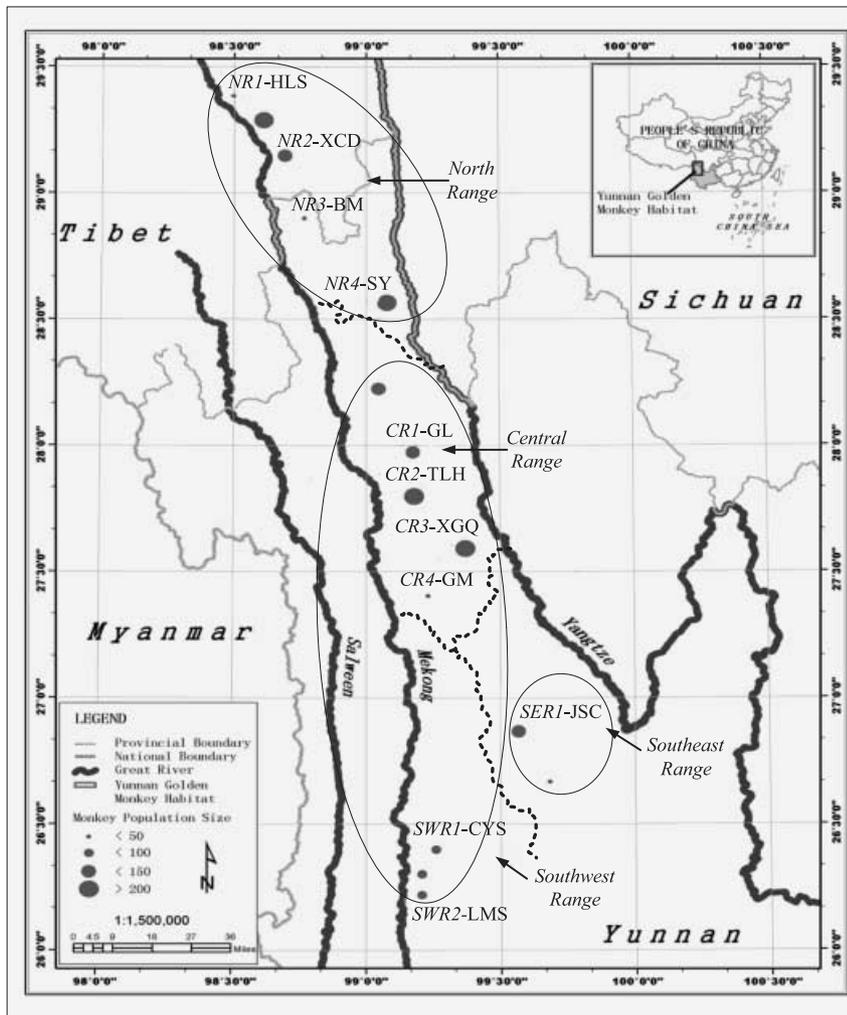
Fig. 1 Map of the distribution of *Rhinopithecus bieti* and sampling locations in the present study.

203 faecal samples through behavioural observation and molecular analysis. Together with blood and muscle samples, our study includes 157 individuals from 11 patches, which are labelled by serial number in the four ranges and acronyms of sampling locations: *NR1*-HLS (Northern Range 1-HLS; *n* = 5), *NR2*-XCD (8), *NR3*-BM (9), *NR4*-SY(18); *CR1*-GL (Central Range 1-GL; *n* = 26), *CR2*-TLH(7), *CR3*-XGQ (20), *CR4*-GM (20); *SWR1*-CYS (Southwest Range 1-CYS; *n* = 14), *SWR2*-LMS(10) and *SER1*-JSC (Southeast Range 1-JSC; *n* = 20) (Fig. 1 and Table 1).

HVI region amplification and Sequencing

Four-hundred and one base pairs (bp) of the CR HVI were amplified and sequenced. The primers were GH (5′-AACTGG CATTCTATTTAAACTAC-3′) and GL (5′-ATTGATTTCACGGAGGATGGT-3′) provided by Dr Christian Roos (German Primate Centre, Göttengen). Amplification was performed in a total volume of 50 μL containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM Mg^{2+}, 200 μmol of each dNTP, 0.2 μmol of each primer, 1.5 U Hotstart *Taq* DNA polymerase (QIAGEN), and 1 μg/μL BSA and ≤ 10 ng of genomic DNA. Forty cycles were used on a Perkin-Elmer Cetus 9700 DNA thermocycler with predenaturing at 95 °C for 15 min; denaturing at 95 °C for 60 s, annealing at 56 °C for 60 s, primer extension at 72 °C for 60 s; and a final 10 min of extension at 72 °C. Positive (blood-extracted DNA) and negative (water) controls were used to check PCR performance and contamination. The PCR products were purified using QIAquick PCR purification Kit (QIAGEN) and sequenced with the ABI PRISM BigDye Terminator Ready Reaction kit (Applied Biosystems) and run on an ABI 377 or an ABI-PRISM 3100 Genetic Analyser, according to the manufacturer's protocol. To avoid the errors in amplification and sequencing, PCR amplifications of entire samples were performed twice or more and the products were sequenced for both strands.

Table 1 Summary of mtDNA control region haplotype distributions, Haplotype diversity and nucleotide diversities indices for all sampling locations

Haplotypes	*NR1-*HLS	*NR2-*XCD	*NR3-*BM	*NR4-*SY	*CR1-*GL	*CR2-*TLH	*CR3-*XGQ	*CR4-*GM	*SWR1-*CYS	*SWR2-*LMS	*SER1-*JSC	Total
M01	1	3	5									9
M02	3											3
M03		2										2
M04		1										1
M05	1	2	4			1						8
M06				1								1
M07				3								3
M08				1								1
M09				3								3
M10				3								4
M11				4								4
M12					2							2
M13					1		1					2
M14							2					2
M15					7		1					8
M16					4		4	4				12
M17				3	3	4	2	3				15
M18					5		8	5				18
M19					1							1
M20					4	1	1	8				14
M21									2			2
M22							1		2			3
M23									1			1
M24									1			1
M25									8			8
M26										1		1
M27										4		4
M28										5		5
M29											6	6
M30											14	14
Sample number	5	8	9	18	26	7	20	20	14	10	20	157

Data analysis

Sequences were aligned by using the program CLUSTAL_X (Thompson *et al*. 1997) and rechecked by eye. Haplotypes were identified using CLUSTAL_X. Pairwise sequence differences among the haplotypes were calculated using MEGA 2.1 (Kumar *et al*. 2001). For phylogenetic analyses, we performed maximum-likelihood (ML) and maximum-parsimony (MP) analyses in parallel by using the program PAUP* 4.0 (Swofford 2002). MODELTEST 3.06 (Posada & Crandall 1998) was run to determine the appropriate model of molecular evolution in a likelihood ratio test framework, and *R. roxellana* and *R. brelichi* were used as outgroups. Gaps were treated as a fifth state in parsimony analyses. Bootstrap analyses were performed with 5000 replicates for maximum parsimony and 100 full heuristic replicates for maximum likelihood. For Bayesian phylogenetic inference, we used the program MRBAYES version 3.0

(Huelsenbeck & Ronquist 2001). Four Markov chain Monte Carlo (MCMC) were run for 100 000 generations, sampling every 10 generations; the initial 5% of trees were discarded as burn-in.

Genetic diversity within populations was estimated by haplotype (*h*) and nucleotide diversities (π) (Nei 1986) using DNASP 3.0 (Rozas & Rozas 1999). Hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) was performed to compare levels of genetic diversity within and among several possible population groupings of Yunnan snub-nosed monkey using ARLEQUIN 2.000 (Schneider *et al*. 2000) with 1000 permutations. The groupings that maximize values of Φ_{CT} and are statistically significant indicate the most parsimonious geographical subdivisions. Genetic differentiation between geographical subdivision was assessed by comparing average numbers of pairwise differences between distribution ranges (PiXY); average number of pairwise differences within populations (PiX

and PiY); and the corrected average pairwise difference (PiXY – (PiX + PiY)/2) using ARLEQUIN 2.000.

To test the hypothesis of demographic expansion, the population parameter $\theta = 2N_{ef}\mu$, where N_{ef} is the female effective population size and μ is the mutation rate per site per generation, was estimated using π according to the relationship $E(\theta) = \pi$ (Tajima 1983) and using Watterson's (1975) point estimator, denoted here as θ_W. DNASP 3.0 (Rozas & Rozas 1999) was used to compute π, θ_W and their standard deviations. The estimator π uses the recent population as the population of inference, whereas θ_W uses the historic population as the population of inference. We also calculated the maximum-likelihood estimates of θ for variable population sizes, denoted here as θ_{var}, jointly with the growth parameter g using the program FLUCTUATE 1.4 (Kuhner *et al.* 1998), which uses genealogical information in the data, and applying θ_W as a starting parameter for MCMC simulations. Stability of the parameter estimation was ensured by conducting 10 short MCMC of 4000 steps each and five long chains of length 400 000 with a sampling increment of 20, and one independent rerun. Population history was inferred by comparing π and θ_W (Crandall *et al.* 1999), by Tajima's (1989) *D*-test and Fu's (1997) F_S test of neutrality with ARLEQUIN 2.000 (Schneider *et al.* 2000) and DNASP 3.0 (Rozas & Rozas 1999). Fu's F_S test was selected because it is the most powerful coalescent-based neutrality test against population growth for larger sample sizes (Ramos-Onsins & Rozas 2002). If a population expansion was detected, we estimated its age according to the following equation modified from Harpending *et al.* (1993) recently used in a study of Japanese macaques (*Macaca fuscata*) (Marmi *et al.* 2004): $\tau = mlt$ [equation (1)], where τ is the time after expansion in mutational units, m is the mean divergence rate per nucleotide per year, l is the sequence length, and t is the number of years after the expansion episode. Finally, a minimum spanning network (Crandall & Templeton 1993) was constructed using TCS 1.13 (Clement *et al.* 2000) considering gaps as a fifth state. Haplotypes were hierarchically nested to visualize higher–order patterns of association (Templeton *et al.* 1987; Templeton & Sing 1993), and current and historic patterns of phylogenetic and geographical associations were statistically tested using nested clade analysis (NCA) implemented in GEODIS 2.0 (Posada *et al.* 2000). For those clades in which the null hypothesis of random geographical distribution was rejected, potential historical and biological associations were inferred from the inference key (Templeton 2004).

Results

Phylogenetic analysis and divergence time estimation

For the 401-bp CR HVI sequence generated from 157 individuals, 52 variable nucleotide sites, including three
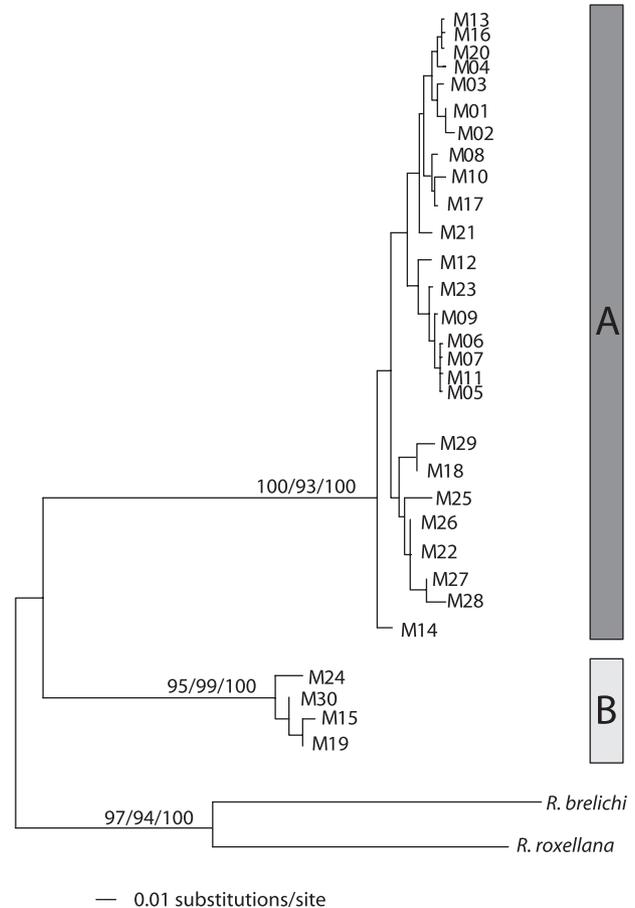
Fig. 2 Maximum-likelihood tree for all 30 haplotypes of *Rhinopithecus bieti* and for two outgroup taxa. Labels are haplotype identification numbers (see Table 1 and Table 5). Values above branches indicate support for each node based on maximum likelihood/maximum parsimony/Bayesian inference. Bootstrap values below 50% are not shown.

transversions (tv), 50 transitions (ts) (both ts and tv at the 109th and 202nd site) and 1 insertion/deletion, defined 30 haplotypes (deposited in GenBank under Accession no. DQ661651–DQ661680), of which three were identical with haplotypes identified by Pan *et al.* (2006): M15 (haplotype E in Pan's paper), M17 (A, B, C) and M30 (D). The HKY-G model was identified using MODELTEST as the best-fitting distance estimator, with a gamma-shape correction of 0.16, a transition/transversion (Ti/Tv) ratio of 7.92, and base frequencies of A = 0.295, C = 0.138, and G = 0.223. Parsimony analyses were performed under equal-weight (Ti/Tv = 1) and unequal-weight (Ti/Tv = 7.92) sets. All phylogenetic analyses resulted in almost identical tree topologies (Fig. 2), identifying two main haplogroups (Clades A and B). The mean pairwise distance between haplotypes in Clade A and B, at 0.11, was much higher than those either within Clade A (0.013) or Clade B (0.006). M15,
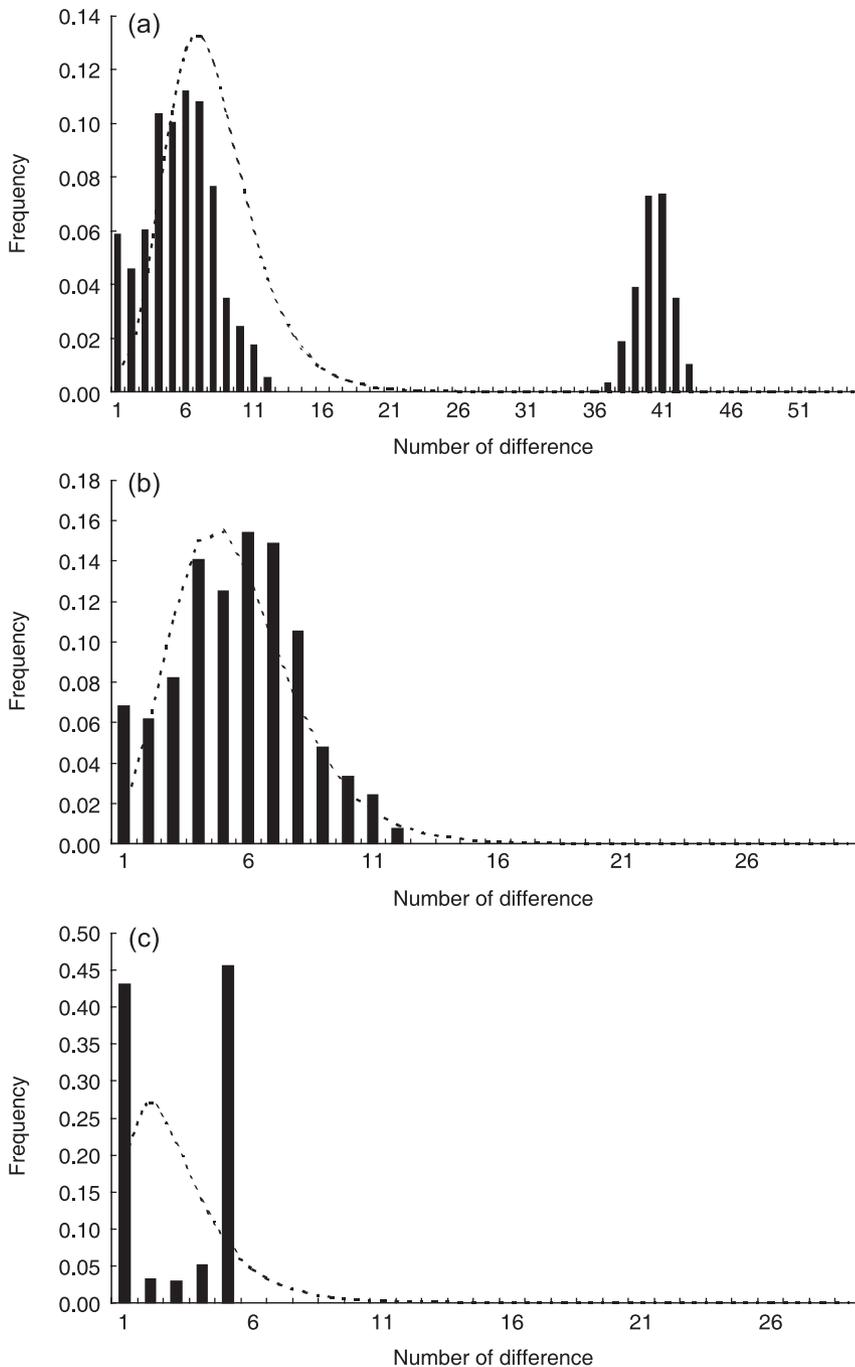
Fig. 3 Observed (solid pillar) and expected (discontinuous line) mismatch distributions showing the frequencies of pairwise differences: (Fig. 3a) considering all the samples; (Fig. 3b) within Clade A; (Fig. 3c) within Clade B.

found in *CR1*-GL and *CR3*-XGQ, M19 (*CR2*-TLH), M24 (*SWR2*-LMS), M30 (*SER1*-JSC) clustered into Clade B; with the other 26 haplotypes classified as Clade A. It should be noted that haplotypes in Clade B only occurred in the central and southern ranges of *R. beiti* habitat. Using a mutation rate range of 11.5 to 17.3% per million years for HVI, as estimated from the human-chimpanzee split (Vigilant *et al.* 1991) and used in the study of Japanese macaque (*Macaca fuscata*) (Marmi *et al.* 2004), the divergence time of Clades A and B was estimated to be between 1 and 0.70 Ma, which corresponds to the time of the most recent common ancestor (TMRCA) for extant haplotypes. The results of NCA and the inferences drawn are given in the Appendix. Nested design resulted in three hierarchical levels (Fig. 4); 13 of the 23 one-step, two-step, and three-step nested clades provided evidence for geographical
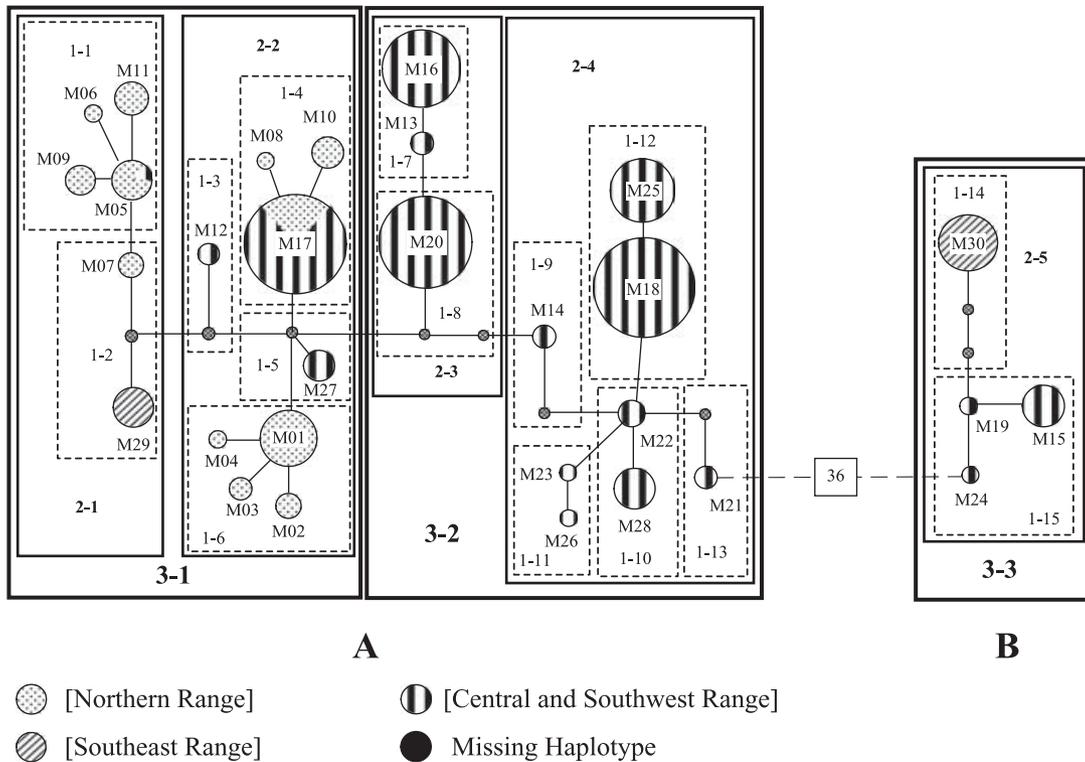
Fig. 4 Minimum spanning network based on Templeton *et al.* (1987; Templeton & Sing 1993) statistical parsimony. Nodes contain the haplotype number and are proportional to the haplotype frequency. Black nodes indicate undetected intermediate haplotype states separated by one mutational step. Boxes indicate one-step to three-step nesting levels for the nested clade analysis. The number in the box between A and B represent substitution steps.

Table 2 Summary of haplotype diversities (*h*), nucleotide diversity (π), the population parameter Φ, and g, and Tajima's *D* and Fu's (*Fs*) test for control region sequences

	n	N	$h \pm SD$	$\pi \pm SD$	$\theta_W \pm SD$	$\theta_{var} \pm SD$	$g \pm SD$	D (P value)	Fs (P value)
Clade A	133	26	0.936 ± 0.008	0.012 ± 0.0004	0.009 ± 0.003	1.250 ± 0.002	203.090 ± 55.929	-1.260 (0.087)	-3.48 (0.007)
Clade B	24	4	0.569 ± 0.074	0.003 ± 0.001	0.003 ± 0.002	0.003 ± 0.0007	-100.294 ± 155.261	-2.063 (0.009)	6.441(0.982)
Total	157	30	0.944 ± 0.006	0.034 ± 0.003	0.023 ± 0.006	0.032 ± 0.002	0.000002 ± 0.000	1.552 (0.891)	2.152 (0.854)

Note: *n*, number of individuals; N, number of haplotypes. The population parameter Φ was estimated using Watterson's (1975) θ_W, and Kuhner *et al.*'s (1998) $\theta_{g=0}$, which allows for population change, *g*. For θ_{var} and *g*, the standard deviation is approximated from the two-dimensional likelihood curve of the joint estimates of θ_{var} and *g*.

structure. At the lower nesting levels, results mainly pointed to restricted gene flow, isolation by distance and fragmentation. For clade 2–4, clade 3–2 and Clade A, continuous range expansions were inferred (see Appendix).

Population structure

Diversity indices, *h* and π, were summarized in Table 2. For the whole samples, *h* was 0.944 (± 0.006) and π was 0.034 (± 0.003), indicating a high haplotype and relatively

low nucleotide diversity. Haplotypes defined by mtDNA display very strong geographical specificity, consistent with patches clustering based on geographical partitioning: of the 30 haplotypes, only eight were shared among different patches (Table 1). The haplotypes found in *SER1*-JSC and *SWR2*-LMS were confined to their respective patches. The most abundant haplotype M18, shared by 18 individuals, was found in three patches (*CR1*-GL, *CR3*-XGQ and *CR4*-GM); and the most widespread haplotype, M17 (15 individuals), was found in five patches (*NR4*-SY,

CR1-GL, *CR2*-TLH, *CR3*-XGQ and *CR4*-GM). Other abundant and widespread haplotypes were M20 (14 individuals from *CR1*-GL, *CR3*-XGQ and *CR4*-GM), M30 (14 individuals from *SER1*-JSC), and M16 (12 individuals from *CR1*-GL, *CR2*-TLH, *CR3*-XGQ and *CR4*-GM). Interestingly, these haplotypes were only found in the central range of the *R. bieti* habitat, with the exception of M30 (from *SER1*-JSC) (Table 1 and Fig. 1). Mitochondrial DNA haplotypes of *R. bieti* displayed strong local homogeneity and population structure. M01–M11 were mainly found in the patches in the northern range; M12–M20 were mainly found in the patches in the central range; M21–M28 were mainly found in the southwest range; M29–M30 were only found in the southeast range (Table 1 and Fig. 1). But the grouping pattern '[*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS, *SWR2*-LMS] [*SER1*-JSC]' gave the highest Φ_{CT} value (0.400, $P < 0.001$). These results suggested that '[Northern Range] [Central and Southwest Range] [Southeast Range]' should be the most parsimonious geographical subdivision (Table 3 and Fig. 1), and the test of population differentiation showed that these three geographical populations are significantly differentiated from each other in pairwise comparisons ($P < 0.001$) (Table 4).

Population demographic history

For the whole samples and two haplogroups (Clade A & B), the point estimators π and θ_W were all similar (Table 2). It was noticeable that a significant negative Fu's F_S value (–3.48, $P = 0.007$), large population growth parameter g (203.090 ± 55.929) and θ_{var} (1.250 ± 0.002) strongly indicated population expansion within Clade A in the past (Table 2). Additionally, the mismatch distribution of Clade A was bell-shaped (Fig. 3b) and the following values were obtained: $\tau = 4.22$ (95% confidence interval = 3.15–7.41). The time after expansion of Clade A were estimated to be 0.16–0.05 Ma using equation (1). For Clade B, population growth parameter g (–100.294 ± 155.261) appears to indicate population contraction, similar to the red panda (*Ailurus fulgens*) (Kuhner *et al*. 1998; Li *et al*. 2005). Finally, a small θ_{var} value (0.032 ± 0.002) and small population growth parameter g (0.000002 ± 0.000) for the whole population indicate a stable demographic history (Table 2).

Discussion

Phylogenetic and geological history

Nuclear transferred mitochondrial fragments (numts) have been reported in the phylogenetic analysis of old world monkeys and Hominoids (Collura & Stewart 1995; Thalmann *et al*. 2004, 2005), and the possibility of amplification of numts will increase when using universal primers (Collura & Stewart 1995; van der Kuyl *et al*. 1995; Zhang & Hewitt 1996). The primers used in this study were redesigned using the published mitochondrial control region sequences of snub-nosed monkeys (Zhang & Ryder 1998; Pan *et al*. 2006). PCR amplification constantly produced one band, and the obtained sequences were unambiguous and did not show co-amplification of more than one locus, indicating they are not mixed with numts (Zhang & Hewitt 1996). Additionally, independent PCR amplifications for the same sample and differing DNA sources, tissue and faecal samples, of the same sampling locations showed identical haplotypes (e.g. M05 from *NR3*-BM, M30 from *SER1*-JSC). Furthermore, numts sequences typically show a different pattern in phylogenetic analysis comparing with accurate mitochondrial sequences (Saitou & Ueda 1994; Collura & Stewart 1995; Collura *et al*. 1996; Zhang & Hewitt 1996; Zhang & Gerstein 2003). All the sequences of the present study were consistent with the evolution pattern of published sequences of *R. bieti* in the phylogenetic analysis (see Results section and Fig. 2), making the co-amplification of numts in our analysis very unlikely.

Mitochondrial DNA data for *R. beiti* revealed significant genetic polarization among populations, with deep genetic divergence between the two lineages (Fig. 2). The phylogenetic relationship and divergence data of most primate species, including Asian colobines (*Rhinopithecus*, *Nasalis*, *Pygathrix*), were estimated referring to the time of human–chimpanzee divergence (Marmi *et al*. 2004; Raaum *et al*. 2005; Sterner *et al*. 2006). Consequently, we used a mutation rate range of 11.5 to 17.3% per million years for HVI estimated from the human–chimpanzee split (Vigilant *et al*. 1991), which was also used in Old World monkey (*Macaca fuscata*) (Marmi *et al*. 2004). Although the absolute timing of divergences may be debatable (because no appropriate fossils are available to date the ancestor of *Rhinopithecus* and we were only able to use a mutation rate of a cercopithcine, not a colobine monkey), the sequence of events and the relative timing depicted here are expected to well approximate the evolutionary history of *R. bieti*. The particular interest is the initial split occurring at 1.00–0.70 Ma, dividing the Clade B from the Clade A. This divergence event is strikingly consistent with the Late Cenozoic uplift of the Tibetan Plateau occurred 1.10–0.60 Ma (Li *et al*. 1981; Shi *et al*. 1998). The latest and most significant uplift of Tibetan plateau occurred in 1.10–0.60 Ma, caused the altitude of the Tibetan plateau to increase by up to 3000 m, and had great influence on propagation on the Tibetan plateau itself and its neighbouring areas (Harrison *et al*. 1992; Molnar *et al*. 1993; Shi *et al*. 1998). As a result of the uplift, the plateau plane began to diversify topographically by the intricate and continual development of mountains and watercourses during 1.20–0.50 Ma (Li *et al*. 1981; Wu 1989), which were expected to have formed boundaries between *R. bieti* populations formerly

Table 3 The results of AMOVA for groupings of sampled remnant patches of *Rhinopithecus bieti* estimated using Φ-statistics based on control region sequences; pops, populations

Groups	Among groups Φ_{CT}	Among pops within groups Φ_{SC}	Within pops Φ_{ST}	Percentage of variation (%) Among groups	Among pops within groups	Within pops
1 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS, *SWR2*-LMS] [*SER1*-JSC]	0.400***	0.120***	0.472***	40.0	7.2	52.8
2 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC] [*SWR1*-CYS] [*SWR2*-LMS]	0.368***	0.090**	0.425***	36.8	5.7	57.5
3 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SER1*-JSC] [*SWR1*-CYS] [*SWR2*-LMS]	0.368**	0.090**	0.425***	36.8	5.6	57.6
4 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS] [*SWR2*-LMS] [*SER1*-JSC]	0.364***	0.128***	0.445***	36.4	8.1	55.5
5 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SWR1*-CYS, *SWR2*-LMS] [*SER1*-JSC]	0.108	0.313***	0.388***	10.9	27.9	61.2
6 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC, *SWR1*-CYS, *SWR2*-LMS]	0.101	0.320***	0.389***	10.1	28.7	61.2
7 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SER1*-JSC, *SWR1*-CYS, *SWR2*-LMS]	0.092	0.343***	0.404***	9.2	31.2	59.6
8 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS, *SWR2*-LMS] [*SER1*-JSC]	0.383**	0.459***	0.123***	38.3	7.6	54.1
9 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SER1*-JSC] [*SWR2*-LMS] [*SWR1*-CYS]	−0.014	0.376***	0.367***	−1.4	38.1	63.3
10 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC] [*SWR1*-CYS, *SWR2*-LMS]	0.354***	0.097***	0.417***	35.4	6.3	58.3
11 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS] [*SWR2*-LMS] [*SER1*-JSC]	0.347**	0.133***	0.434***	34.7	8.7	56.6
12 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC, *SWR2*-LMS, *SWR1*-CYS]	0.055	0.343***	0.379***	5.5	32.4	62.1
13 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC] [*SWR2*-LMS] [*SWR1*-CYS]	0.358**	0.415*	0.089***	35.8	5.7	58.5
14 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS, *SWR2*-LMS, *SER1*-JSC]	0.021	0.364***	0.378***	2.1	35.6	62.3
15 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS, *SER1*-JSC] [*SWR2*-LMS]	−0.035	0.382***	0.360***	−3.5	39.5	64.0

Table 3 *Continued*

Groups	Among groups Φ_{CT}	Among pops within groups Φ_{SC}	Within pops Φ_{ST}	Percentage of variation (%)		
				Among groups	Among pops within groups	Within pops
16 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC] [*SWR1*-CYS, *SWR2*-LMS]	0.362**	0.133***	0.447***	36.2	8.5	55.3
17 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC] [*SWR1*-CYS] [*SWR2*-LMS]	0.359***	0.132***	0.443***	35.9	8.4	55.7
18 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC, *SWR1*-CYS, *SWR2*-LMS]	0.093	0.330***	0.392***	9.2	30.0	60.8
19 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM][*NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SER1*-JSC] [*SWR1*-CYS, *SWR2*-LMS]	0.240*	0.439***	0.262***	24.0	19.9	56.1
20 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SWR1*-CYS, *SWR2*-LMS] [*SER1*-JSC]	0.035	0.359***	0.381***	3.5	34.6	61.9
21 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SER1*-JSC, *SWR1*-CYS, *SWR2*-LMS]	0.138	0.326***	0.419***	13.8	28.1	58.1
22 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM] [*SWR1*-CYS] [*SWR2*-LMS] [*SER1*-JSC]	0.416	0.502***	0.148***	41.5	8.7	49.8
23 [*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY, *CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR2*-LMS, *SWR1*-CYS] [*SER1*-JSC]	0.566	0.171***	0.640***	56.6	7.4	36.0

(*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 4 Pairwise genetic differentiation for control region sequences of *Rhinopithecus bieti* based on Tajima & Nei's distance method. Above diagonal: average number of pairwise differences between populations (PiXY). Diagonal elements: average number of pairwise differences within population (PiX). Below diagonal: corrected average pairwise difference [PiXY – (PiX + PiY)/2]. Corrected average pairwise differences, which are statistically different, are indicated

	[Northern Range]	[Central and Southwest Range]	[Southeast Range]
[Northern Range]	3.63	8.87	29.24***
[Central and Southwest Range]	1.72***	10.68	28.37***
[Southeast Range]	18.36***	13.96***	18.13

(*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

connected. Interestingly, the haplotypes of Clade A and B coexist in central and southern ranges in the present, implying secondary contact after the initial divergence. Local co-occurrence of divergent lineages has also been found in *Macaca sylvanus* and other macaque species (e.g. Melnick & Hoelzer 1992; Tosi *et al.* 2000; Modolo *et al.* 2005), and can be explained by several factors such as the reconnection of formerly isolated habitats caused by geological change after initial divergence and subsequent population expansion over such geographical boundaries. Interestingly, a sudden population expansion of Clade A was detected through pairwise mismatch analysis and neutrality tests and the time was estimated to be at 0.16–0.05 Ma, which might explained the second connect of the two haplogroups (Clades A and B).

Mitochondrial DNA haplotypes in *R. bieti* display local homogeneity and strong population structure. Such distributions for mitochondrial DNA haplotypes have also been found in Japanese macaques (*M. fuscata*), which have been interpreted as being the result of female philopatry (Oi 1988; Hayaishi & Kawamoto 2006). In some species of colobine monkeys, some resident-male takeover events have been also reported (Hanuman langurs, *Presbytis entellus*, Rajpurohit *et al.* 2003; Sichuan snub-nosed monkey, *Rhinopithecus roxellana*, Wang *et al.* 2004), and there is a hint that female philopatry might contribute some part to genetic structure of *R. bieti*. Besides female philopatry, habitat fragmentation would also have contributed to enlarge the genetic difference between *R. bieti* in different patches. In addition, NCA analysis showed that *R. bieti* has suffered from fragmentation while at the same time demonstrating isolation-by-distance pattern with restricted gene flow. Therefore, based on our results and previous field surveys, it could be hypothesized that the genetic history of *R. bieti* might include (i) initial divergence between Clades A and B at 1.0–0.7 Ma, which might have been caused by the Late Cenozoic uplift of the Tibetan Plateau; (ii) a secondary contact occurred after the divergence, which might have been caused by the expansion of Clade A; and (iii) population reduction and habitat fragmentation until now.

Population demographic history

The deep phylogenetic tree, Tajima's *D* and Fu's *Fs* values (Table 5), and growth parameter *g* and θ_{var}, all indicate there was no single expansion or contraction in all populations of Yunnan snub-nosed monkey. Furthermore, the mismatch distribution analysis showed atypical distribution shapes, revealing a complicated demographic history for the populations. As the topographic features changed, climate oscillated and vegetation varied during the uplifting of the Tibetan Plateau and during glaciations in the Quaternary Period, the species may have suffered comprehensive population fluctuations, whereas the main population remained genetically healthy. It is notable that a sudden population expansion at 0.16–0.05 Ma was detected in Clade A. Quaternary climatic changes have shaped patterns of geographical distribution as well as demographic history (Avise 2000). For most species, repeated climatic oscillations have resulted in range contractions into separate refuge during climatic cooling and drying (Broccoli & Manabe 1992; Hewitt 1996). Crandall & Templeton (1993) predicted that older haplotypes should be more broadly distributed geographically, and the most widespread and frequent haplotypes would indicate the locations of refuge populations. Our study revealed that the most widespread and frequent haplotypes in Clade A (M16, M17, M18 and M20) are distributed in the central region of habitat, implying the refuge and the location of initial population expansion. The topographical diversity of the Tibetan Plateau might have created networks for refugia during glaciations. Additionally, geological research has found evidence for a warmer climatic phase (0.13–0.08 Ma) during the last interglacial and that might explain the population expansion similar to *M. sylvanus* (Kutzbach *et al.* 1993; Shi *et al.* 1998; Modolo *et al.* 1997, 2005).

Implications for conservation

According to the model proposed by Moritz (1994), evolutionary significant units (ESUs) are designated on the

Table 5 Variable sites of the *D*-loop region in *Rhinopithecus bieti*. Dots (.) indicate nucleotide identity and hyphens (-) deletions

```
                    Variable sites
                    [          1111111111 1111111111 1111111112 2222222233 33]
                    [1234466799 0011222222 3355556677 8889999990 0112334834 58]
Haplo-type          [1395634312 1989034568 7826895612 1250235892 5069670156 27]

M01                 TAGATGTACT TACTTGCCTG CTAAGCGAGG ATCGAGACTT ACAGCTTCGG G-
M02                 .......... .......... .......... ...C...... .......... .-
M03                 .G........ .......... .......... .......... .......... .-
M04                 .......T.. .......... .......... .......... .......... .-
M05                 .......... .....T..A. .......A.. .......... .T.A...... .-
M06                 .......T.. .....T..A. .......A.. .......... .T.A...... .-
M07                 .......... .....T..A. .......... .......... .T.A...... .-
M08                 .G........ .......... .......... ......G... ...A...... .-
M09                 ....C..... .....T..A. .......A.. .......... .T.A...... .-
M10                 ....C..... .......... .......... ......G... ...A...... .-
M11                 .......... .....T.CA. .......A.. .......... .T.A...... .-
M12                 C......... .........A .......... .......... ...A...... .-
M13                 .......T.. .......... .......... .......... ...A...... .-
M14                 CG........ .T........ .......... .......... .T.A...... .-
M15                 CG.GCACG.. .CTCCA.T.A TCGGAT.GAA G.TATA.TCA GTGATCCTAA A-
M16                 ..A.....T. .......... .......... .......... ...A...... .-
M17                 .......... .......... .......... ......G... ...A...... .-
M18                 CG........ CT........ .......... .......... .......... .-
M19                 CG.G.ACG.. .CTCCA.T.A TCGGAT.GAA G.TATA.TCA GTGATCCTAA A-
M20                 .G.....T.. .......... .......... .......... ...A...... .-
M21                 .......... .......... .......A.. .......... ...A...... .-
M22                 CG........ CT........ .......... .......... ...A...... .C
M23                 .......... .....T..A. ......A... .......... ...A...... .-
M24                 CGAG.ACG.. .CTCCA.T.A .CGGAT.GAA G.TATA.TCG GTGATCCTAA A-
M25                 CG........ CT.....A T .......... .......... ...A...... .-
M26                 CG........ CT........ .......... .......... ...A...... .-
M27                 CG........ CT........ .......... ...A...... ...A...... .-
M28                 CG.....C.. CT........ .......... ...A...... ...A...... .-
M29                 CG........ CT........ .......... .C........ .......... .-
M30                 CG.G.A.G.. .CTCCA.T.A TCGGAT.GAA G.TATA.TCA GTGATCCTAA A-
```

basis of reciprocal monophyly at mitochondrial markers, while management units (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. AMOVA suggested that '[*NR1*-HLS, *NR2*-XCD, *NR3*-BM, *NR4*-SY] [*CR1*-GL, *CR2*-TLH, *CR3*-XGQ, *CR4*-GM, *SWR1*-CYS, *SWR2*-LMS] [*SER1*-JSC]' should be the most parsimonious geographical subdivision and there is no shared haplotype between these three units (Table 3 and Fig. 1), except M05 (one individual) and M17 (three individuals). Thus we suggest that [Northern Range], [Central and Southwest Range] and [Southeast Range] should be regarded as three MUs (Fig. 1).

For the different MUs, we suggest that protection is required synchronously because of their genetic uniqueness. If the main goal of conservation strategies is only to retain the population size and just focus on one or two MUs, which is the present situation, unique haplotypes in other MUs will be lost and will cause a significant decrease in genetic diversity. Periodic translocations between these three MUs might be used to resume gene flow. Additionally, [Northern Range] merits special attention because it occupies the northernmost part of the species' distribution, in which only haplotypes of Clade A were found. Finally, more intense protection is also necessary for [Southeast Range] which possesses two unique haplotypes because this population is at the easternmost edge of the range and is significantly differentiated from other populations (Table 4).

The moderate level of the genetic diversity found in the present study indicates that *R. bieti* should not to be regarded as a taxon with low genetic diversity. Previous studies based on morphological characters and allozyme loci implied a very low genetic diversity for *R. bieti* (Pan *et al*. 1993; Hu & Zhang 1996), which might have resulted

from small sample sizes and less powerful markers. As far as we know, our study is the first to assess genetic diversity in *R. bieti* on a large scale, including samples from 11 out of the total 15 remnant patches. The 30 haplotypes indicated high haplotype diversity (0.944 ± 0.006), even though its nucleotide diversity was relatively low (0.034 ± 0.003). According to previous studies on other primate species, genetic diversity in *R. bieti* is higher than *Papio hamadryas* and *M. sylvanus* but lower than *M. fuscata* (Marmi *et al.* 2004; Winney *et al.* 2004; Modolo *et al.* 2005). Comparing with *R. roxellana* (the Sichuan golden monkey, a much more common species, Li *et al.* in press), haplotype diversity of *R. bieti* was higher but nucleotide diversity was slightly lower. Human activities (hunting and forest destruction), which are causing its habitat reduction and serious fragmentation, are thought to be the greatest threat to *R. bieti* (Long *et al.* 1996; Xiao *et al.* 2003; Ren, personal communication). Species characterized by limited mobility and strong population genetic structure, such as *R. bieti*, are more prone to suffering substantial losses in genetic diversity as a result of range reductions (Taylor *et al.* 1994; Bouzat *et al.* 1998; Wisely *et al.* 2002). Based on field surveys and previous studies (Xiao *et al.* 2003), the suitable habitat has decreased by 31% (1887 km^2) during the past 40 years and the size of forest patches has decreased from 15.6 to 5.4 km^2. Thus sustainable development should be planned to reduce the pressure of human activities on the habitat of *R. bieti*.

Acknowledgements

References

Avise JC (2000) *Phylogeography: the History and Formation of Species.* Harvard University Press, Cambridge, Massachusetts.

Bouzat JL, Cheng HH, Lewin HA, Westemeier RL, Brawn JD, Paige KN (1998) Genetic evaluation of a demographic bottleneck in the greater prairie chicken. *Conservation Biology*, **12**, 836–843.

Broccoli AJ, Manabe S (1992) The effects of orography on midlatitude Northern Hemisphere dry climates. *Journal of Climate*, **5**, 1181–1201.

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **10**, 1657–1660.

Collura RV, Auerbach MR, Stewart CB (1996) A quick, direct method that can differentiate expressed mitochondrial genes from their nuclear pseudogenes. *Current Biology*, **6**, 1337–1339.

Collura RV, Stewart CB (1995) Insertions and duplications of mtDNA in the nuclear genomes of old world monkeys and hominoids. *Nature*, **378**, 485–489.

Crandall KA, Posada D, Vasco D (1999) Effective population size: missing measures and missing concepts. *Animal Conservation*, **2**, 317–319.

Crandall KA, Templeton AR (1993) Empirical texts of some predictions from coalescent theory with applications to intra-specific phylogeny reconstruction. *Genetics*, **134**, 959–969.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplo-types: applications to human mitochondrial DNA restriction data. *Genetics*, **131**, 479–491.

Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, **147**, 915–925.

Harpending HC, Sherry ST, Rogers AR, Stoneking M (1993) The genetic structure of ancient human populations. *Current Anthropology*, **34**, 483–496.

Harrison TM, Copeland P, Kidd WSF, Yin A (1992) Raising Tibet. *Science*, **255**, 1663–1670.

Hayaishi S, Kawamoto Y (2006) Low genetic diversity and biased distribution of mitochondrial DNA haplotypes in the Japanese macaque (*Macaca fuscata yakui*) on Yakushima Island. *Primates*, **47**, 158–164.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnaean Society*, **58**, 247–276.

Hu ZA, Zhang YP (1996) *Genetic Diversity of Animals and Plants in China*, pp. 46–59. Science and Technology Publishing House, Zejiang, China.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics*, **17**, 754–755.

Jablonski NG (1998a) The evolution of the doucs and snub-nosed monkeys and the question of the phyletic unity of the odd-nosed colobines. In: *The Natural History of the Doucs and Snub-Nosed Monkeys* (ed. Jablonski NG), pp. 13–52. World Scientific Publishing, Singapore.

Jablonski NG (1998b) The response of catarrhine primates to Pleistocene environmental fluctuations in East Asia. *Primates*, **39**, 29–37.

Kirkpatrick RC (1998) Ecology and behavior in snub-nosed and douc langurs. In: *The Natural History of the Doucs and Snub-Nosed Monkeys* (ed. Jablonski NG), pp. 155–190. World Scientific Publishing, Singapore.

Kuhner MK, Yamato J, Felsenstein J (1998) Maximum likelihood estimation of population growth rates based on the coalescent. *Genetics*, **149**, 429–434.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA 2: molecular evolutionary genetics analysis software. Arizona State University, Temple, Arizona.

Kutzbach JE, Prell WL, Ruddiman WF (1993) Sensitivity of Eurasian climate to surface uplift of the Tibetan Plateau. *Journal of Geology*, **101**, 177–190.

Kutzbach JE, Ruddiman WF, Prell WL (1997) *Tectonic Uplift and Climate Change* (ed. Ruddiman WF), pp. 149–170. New York.

van der Kuyl AC, Kuiken CL, Dekker JT, Goudsmit J (1995) Phylogeny of African monkeys based upon mitochondrial 12S rRNA sequence. *Journal of Molecular Evolution*, **40**, 173–180.

Li JJ, Li BY, Wang FB (1981) The process of uplift of the Qinghai-Xizang Plateau. *Geological and Ecological Studies of Qinghai-Xizang Plateau*, pp. 111–118. Science Press, Beijing.

Li ZX, Lin ZY (1983) Classification and distribution of living primates in Yunnan, China. *Zoological Research*, **4**, 111–120.

Li M, Liu ZJ, Guo JX *et al.* (2007) Phylogeography and population structure of the golden monkey (*Rhinopithecus roxellana*): inferred from mitochondrial DNA sequences. *American Journal of Primatology*, in press.

Li BG, Pan RL, Oxnard CE (2002) Extinction of snub-nosed monkeys in China during the past 400 years. *International Journal of Primatology*, **23**, 1227–1244.

Li M, Wei F, Goossens B *et al.* (2005) Mitochondrial phylogeography and subspecific variation in the red panda (*Ailurus fulgens*): implications for conservation. *Molecular Phylogenetics and Evolution*, **36**, 78–89.

Li M, Wei FW, Huang CM, Pan RL, Ruiter J (2004) Molecular phylogeny of snub-nosed monkeys genus *Rhinopithecus* inferred from mitochondrial DNA cytochrome *b* and 12SrRNA sequences. *International Journal of Primatology*, **25**, 861–873.

Long YC, Kirkpatrick C, Zhong T, Xiao L (1994) Report on the distribution, population, and ecology of the Yunnan snub-nosed monkey. *Primates*, **35**, 241–250.

Long YC, Kirkpatrick C, Zhong T, Xiao L (1996) Status and conservation strategy of the Yunnan snub-nosed monkey. *Chinese Biodiversity*, **4**, 145–152.

Marmi J, Bertranpetit J, Terradas J, Takenaka O, Domingo RX (2004) Radiation and phylogeography in the Japanese macaque, *Macaca fuscata*. *Molecular Phylogenetics and Evolution*, **30**, 676–685.

Melnick DJ, Hoelzer GA (1992) Differences in male and female macaque dispersal lead to contrasting distributions of nuclear and mitochondrial DNA variation. *International Journal of Primatology*, **13**, 379–393.

Modolo L, Salzburger W, Martin RD (2005) Phylogeography of Barbary macaques (*Macaca sylvanus*) and the origin of the Gibraltar colony. *Proceedings of the National Academy of Sciences, USA*, **102**, 7392–7397.

Molnar P, England P, Martiod J (1993) Mantle dynamics, uplift of the Tibetan Plateau and the Indian monsoon development. *Reviews of Geophysics*, **34**, 357–396.

Moritz C (1994) Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, **3**, 401–411.

Nei M (1986) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Oi T (1988) Sociological study on the troop fission of wild Japanese monkeys (*Macaca fuscata yakui*) on Yakushima Island. *Primates*, **29**, 1–19.

Pan D, Fu YX, Zhang YP (2006) Population genetics of *Rhinopithecus bieti*: study of the mitochondrial control region. *Hereditas (Beijing)*, **28**, 432–436.

Pan YR, Jablonski NG (1993) Scaling of limb proportions and limb bone diameters in three species of Chinese snub-nosed langurs (genus *Rhinopithecus*). *Folia Primatology*, **60**, 56–62.

Pan RL, Oxnard CE (2001) Cranial morphology of the golden monkey (*Rhinopithecus*) and douc langur (*Pygathrix nemaeus*). *Human Evolution*, **16**, 199–223.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Posada D, Crandall KA, Templeton AR (2000) GEODIS: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487–488.

Quan GQ, Xie JY (2002) Research on the golden monkey. Science and Education Publishing House, Beijing.

Raaum RL, Sterner KN, Noviello CM, Stewart CB, Disotell TR (2005) Catarrhine primate divergence dates estimated from complete mitochondrial genomes: concordance with fossil and nuclear DNA evidence. *Journal of Human Evolution*, **48**, 237–257.

Rajpurohit LS, Chhangani AK, Rajpurohit RS, Mohnot SM (2003) Observation of a sudden resident-male replacement in a unimale bisexual troop of Hanuman langurs *Presbytis entellus* around Jodhpur (India). *Folia Primatology*, **74**, 85–87.

Ramos-Onsins SE, Rozas J (2002) Statistical properties of new neutrality tests against population growth. *Molecular Biology and Evolution*, **19**, 2092–2100.

Ren RM, Su YJ, Yan KH *et al.* (1998) Preliminary survey of the social organization of *Rhinopithecus* [*Rhinopithecus*] *roxellana* in Shennongjia National Natural Reserve, Hubei, China. In: *The Natural History of the Doucs and Snub-Nosed Monkeys* (ed. Jablonski NG), pp. 269–277. World Scientific Publishing, Singapore.

Rozas J, Rozas R (1999) DNASP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics*, **15**, 174–175.

Saitou N, Ueda S (1994) Evolutionary rates of insertion and deletion in noncoding nucleotide sequences of primates. *Molecular Biology and Evolution*, **11**, 504–512.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schneider S, Roessli D, Excoffier L (2000) ARLEQUIN 2.000: a software for genetic data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Shi YF, Li JJ, Li BY (1998) *Uplift and Environmental Changes of Qinghai-Xizang (Tibetan) Plateau in the Late Cenozoic*. Science and Technology Press, Guangzhou.

Sterner KN, Raaum RL, Zhang YP, Stewart CB, Disotell TR (2006) Mitochondrial data support an odd-nosed colobine clade. *Molecular Phylogenetics and Evolution*, **40**, 1–7.

Swofford DL (2002) PAUP*: *Phylogenetic Analysis Using Parsimony*, Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tajima F (1983) Evolutionary relationship of DNA sequences in finite populations. *Genetics*, **105**, 437–460.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.

Taylor AC, Sherwin WB, Wayne RK (1994) Genetic variation of microsatellite loci in a bottlenecked species: the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Molecular Ecology*, **3**, 277–290.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, **13**, 789–809.

Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343–351.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659–669.

Thalmann O, Hebler J, Poinar HN, Paabo S, Vigilant L (2004) Unreliable mtDNA data due to nuclear insertions: a cautionary tale from analysis of humans and other great apes. *Molecular Ecology*, **13**, 321–335.

Thalmann O, Serre D, Hofreiter M, Lukas D, Eriksson J, Vigilant L (2005) Nuclear insertions help and hinder inference of the evolutionary history of gorilla mtDNA. *Molecular Ecology*, **14**, 179–188.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **25**, 4876–4882.

Tosi AJ, Morales JC, Melnick DJ (2000) Comparison of Y chromosome and mtDNA phylogenies leads to unique inferences of macaque evolutionary history. *Molecular Phylogenetics and Evolution*, **17**, 133–144.

Vigilant L, Stoneking M, Harpending H, Hawkes K, Wilson AC (1991) African population and the evolution of human mitochondrial DNA. *Science*, **253**, 1503–1507.

Wang HP, Tan CL, Gao YF, Li BG (2004) A takeover of resident male in the Sichuan snub-nosed monkey (*Rhinopithecus roxellane*) in Qinling mountains. *Acta Zoologica Sinica*, **50**, 859–862.

Watterson GA (1975) On the number of segregating sites. *Theoretical Population Biology*, **7**, 256–276.

Whyte P (1984) *The Evolution of the East Environment*. Centre of Asian Studies, Hong Kong.

Winney BJ, Hammond RL, Macasero W *et al.* (2004) Crossing the Red Sea: phylogeography of the hamadryas baboon, *Papio hamadryas hamadryas*. *Molecular Ecology*, **13**, 2819–2827.

Wisely SM, Buskirk SW, Fleming MA, McDonald DB, Ostrander EA (2002) Genetic diversity and fitness in black-footed ferrets before and during a bottleneck. *Journal of Heredity*, **93**, 231–237.

Wu XH (1989) On morphologic boundary of the southeastern Qinghai-Xizahng Plateau and development of Jinsha river system. *Mountain Research*, **7**, 75–84.

Xiao W, Ding W, Cui LW, Zhou RL, Zhao QK (2003) Habitat degradation of *Rhinopithecus bieti* in Yunnan, China. *International Journal of Primatology*, **24**, 389–398.

Zhan XJ, Li M, Zhang ZJ *et al.* (2006) Molecular censusing doubles giant panda population estimate in a key nature reserve. *Current Biology*, **16**, 451–452.

Zhang Z, Gerstein M (2003) Patterns of nucleotide substitution, insertion and deletion in the human genome inferred from pseudogenes. *Nucleic Acids Research*, **31**, 5338–5348.

Zhang DX, Hewitt GM (1996) Nuclear integrations challenges for mitochondrial DNA markers. *Trends in Ecology & Evolution*, **11**, 247–251.

Zhang YP, Ryder OA (1998) Mitochondrial cytochrome *b* gene sequences of Old World monkeys: with a special reference on evolution of Asian colobines. *Primates*, **39**, 39–49.

Appendix

Nested clade analysis of geographical distances

Nested clade	P	Interior clades	D_C	D_N	Inference
Clade 1-2	0.006	Clade 07 (interior)	0.00	147.75 L	1-19-20-2-3-4-9 AF
		Clade 21 (tip)	0.00 S	55.42 S	
		I-T	0.00	92.34 L	
Clade 1-6	0.001	Clade 01 (interior)	36.02 L	37.00 L	1-2-3-4 RGF and IBD
		Clade 02 (tip)	0.00	25.67	
		Clade 03 (tip)	0.00	17.39	
		Clade 04 (tip)	0.00	17.39	
		I-T	36.02 L	15.47 L	
Clade 1-10	0.008	Clade 24 (interior)	55.09 L	44.57 L	1-19-20-2-3-4-9 AF
		Clade 30 (tip)	0.00 S	11.01	
		I-T	55.09 L	33.56 L	
Clade 1-11	0.000	Clade 25 (interior)	0.00	0.00	INC
		Clade 26 (tip)	0.00	0.00	
		I-T	0.00	0.00	
Clade 1-12	0.044	Clade 18 (interior)	22.03	18.97 S	1-2-11-17-4 RGF and IBD
		Clade 27 (tip)	0.000	128.44 L	
		I-T	22.03	109.48 S	
Clade 2-1	0.000	Clade 1-1 (tip)	55.47 S	69.31 S	1-2-3-4 RGF and IBD
		Clade 1-2 (interior)	80.60	147.60 L	
		I-T	25.13	78.30 L	
Clade 2-2	0.000	Clade 1-3 (interior)	0.000	30.96 S	1-2-3-5-6-7 RGF/RGD and LDD
		Clade 1-4 (tip)	57.34 S	88.99 S	
		Clade 1-5 (interior)	0.000 S	224.77 L	
		Clade 1-6 (tip)	30.53 S	105.38	
		I-T	−48.90	66.02 L	
Clade 2-3	0.008	Clade 1-7 (tip)	28.32	26.49	INC
		Clade 1-8 (interior)	21.75	23.55	
		I-T	−6.57	−2.93	
Clade 2-4	0.000	Clade 1-9 (interior)	0.00	80.32	1-2-11-12 CRE
		Clade 1-10 (interior)	20.40 S	57.12	
		Clade 1-11 (tip)	0.00	43.88	
		Clade 1-12 (tip)	62.56	59.51	
		Clade 1-13 (interior)	0.00	43.88	
		I-T	−45.01 S	−0.22	
Clade 3-1	0.000	Clade 2-1 (tip)	90.26	92.30	INC
		Clade 2-2 (interior)	105.89	105.52	
		I-T	15.63	13.23	
Clade 3-2	0.000	Clade 2-3 (interior)	26.05 S	52.46 S	1-2-11-12 CRE
		Clade 2-4 (interior)	61.85	73.29 L	
Clade 3-3	0.000	Clade 2-5 (interior)	62.76	99.75 L	1-19-20-2-3-4-9 AF
		Clade 2-6 (tip)	0.00 S	45.94 S	
		I-T	62.76 L	53.81 L	
Clade 4-1	0.000	Clade 3-1(tip)	92.61	110.19	1-2-11-12 CRE
		Clade 3-2(interior)	65.49 S	91.59 S	
		I-T	−27.12 S	−18.61 S	
Total cladogram	0.000	Clade 4-1 (tip)	94.08	93.32	INC
		Clade 4-2 (tip)	67.83	114.33	

Note: Clade (D_C) and nested clade (D_N) distances are given for all nested clades containing geographical and genetic variation. An 'S' indicates that the distance is significantly small ($P < 0.05$), and an 'L' indicates that it is significantly large ($P < 0.05$). Interior vs. tip distances, D_C and D_N are given for nested clades where the interior/tip status is known and for which both tips and interiors exist. Within the same nesting group, the clade name is shaded for interior clades. Inferences are given following Templeton's (2004) inference key. AF, allopatric fragmentation; INC, inconclusive outcome; RGF, restricted gene flow; IBD, isolation by distance; RGD, restricted gene dispersal; LDD, long-distance dispersal; CRE, continuous range expansion.