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Mitogenomic analysis of Chinese snub-nosed monkeys: Evidence of positive selection in NADH dehydrogenase genes in high-altitude adaptation

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ABSTRACT

Chinese snub-nosed monkeys belong to the genus *Rhinopithecus* and are limited in distribution to six isolated mountainous areas in the temperate regions of Central and Southwest China. Compared to the other members of the subfamily Colobinae (or leaf-eating monkeys), these endangered primates are unique in being adapted to a high altitude environment and display a remarkable ability to tolerate low temperatures and hypoxia. They thus offer an interesting organismal model of adaptation to extreme environmental stress. Mitochondria generate energy by oxidative phosphorylation (OXPHOS) and play important roles in oxygen usage and energy metabolism. We analyzed the mitochondrial genomes of two Chinese snub-nosed monkey species and eight other colobines in the first attempt to understand the genetic basis of high altitude adaptation in non-human primates. We found significant evidence of positive selection in one Chinese snub-nosed monkey, *Rhinopithecus roxellana*, which is suggestive of adaptive change related to high altitude and cold weather stress. In addition, our study identified two potentially important adaptive amino acid residues (533 and 3307) in the *NADH2* and *NADH6* genes, respectively. Surprisingly, no evidence for positive selection was found in *Rhinopithecus bieti* (the other Chinese snub-nosed monkey analyzed). This finding is intriguing, especially considering that *R. bieti* inhabits a higher altitudinal distribution than *R. roxellana*. We hypothesize that a different adaptive genetic basis to high altitude survival exists in *R. bieti* from those seen in other mammals, and that positive selection and functionally associated mutations in this species may be detected in nuclear genes related to energy and oxygen metabolism. More information on the structure, function, and evolution of mitochondrial and nuclear genomes in Chinese snub-nosed monkeys is required to reveal the molecular mechanisms that underlie adaptations to high altitude survival in non-human primates.

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1. Introduction

Snub-nosed monkeys are enigmatic and threatened primates that belong to the subfamily Colobinae (Kirkpatrick, 1998; Ren et al., 1998). The snub-nosed monkey genus *Rhinopithecus* is comprised of four distinct allopatric species (Groves, 2001): *Rhinopithecus brelichi* (the gray snub-nosed monkey), *Rhinopithecus bieti* (the black snub-nosed monkey), *Rhinopithecus roxellana* (the golden snub-nosed monkey) and *Rhinopithecus avunculus* (the Tonkin snub-nosed monkey). With the exception of *Rhinopithecus avunculus*, which is distributed in low-medium altitude subtropical forests in northwestern Vietnam (<1200 m above sea level; Boonratana and Le, 1998), *Rhinopithecus* species are endemic to temperate areas of China and inhabit six isolated mountainous regions at high altitude (Kirkpatrick, 1998). For example, *R. bieti* ranges in the Himalayas of southwestern China in altitudes as high as 3400–4600 m where temperatures drop below freezing, making it the non-human primate with the highest known altitudinal distribution (Kirkpatrick et al., 1998). Compared with other colobines and most primates, Chinese snub-nosed monkeys are therefore better adapted to high altitude and display a remarkable ability to tolerate low temperatures and hypoxia. They offer an interesting organismal model of adaptation to extreme environmental stress.

Despite substantial work on the physiological, morphological and behavioral characters of snub-nosed monkeys, little research has examined potential adaptations to high altitude survival in these species, or in any other non-human primate, at the molecular level. It is therefore interesting and necessary to investigate the key genes for aerobic metabolic pathways to understand the molecular mechanisms that underlie their adaptations to cold stress and hypoxia.

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Mitochondria, as the "energy factories" of animals, generate energy by oxidative phosphorylation (OXPHOS) (Luo et al., 2008) and play important roles in oxygen usage and energy metabolism (Xu et al., 2007). Earlier studies have revealed a substantially greater amount of mitochondria in the cells of species native to high altitude environments compared to those that occupy lowland habitats. This is believed to be related to the fact that cold temperature and low oxygen pressure are the two most remarkable characters of highland areas that affect animal physiology (Luo et al., 2008; Ning et al., 2010), and high concentrations of mitochondria are needed to increase oxygen utilization and produce more energy to improve aerobic metabolic fitness. Hence, the mitochondrial genome, which encodes for 13 essential OXPHOS system proteins (7 subunits of the NADH dehydrogenase complex, the cytochrome *b* subunit of the cytochrome bc_1 complex, 3 subunits of the cytochrome *c* oxidase, and 2 subunits of ATP synthase) (Saraste, 1999; Lopez-Barneo et al., 2001; da Fonseca et al., 2008) represents a particular useful genetic marker for investigating the molecular basis of organismal adaptation to high altitude environments. Indeed, several mtDNA analyses have detected signatures of adaptive evolution in the cytochrome *c* oxidase genes of plateau pikas, camelids and Tibetan antelope (Xu et al., 2005; Luo et al., 2008; Di Rocco et al., 2009), the NADH dehydrogenase genes of Tibetan horses (Xu et al., 2007; Ning et al., 2010), the cytochrome *b* gene of alpacas (da Fonseca et al., 2008), and the ATP synthase genes of Caprini antelope (Hassanin et al., 2009). In the present study, we sequenced and compared mitochondrial genome sequences of two Chinese snub-nosed monkey species (*R. bieti* and *R. roxellana*) with those of their closest low altitude living relative (*R. avunculus*) and other colobines in the first investigation of adaptation to high altitude survival in non-human primates. Given that positive selection has been found in the mitochondrial genes of other high altitude adapted mammals (see above), we hypothesize that signatures of adaptive evolution will be found in OXPHOS system related mitochondrial genes of the high altitude living Chinese snub-nosed monkeys when compared to their lower altitude living relatives.

2. Methods

2.1. DNA samples and sequence determination

Currently, complete mitochondrial genome sequences of snub-nosed monkey species that live at high altitude are only available for *R. roxellana* (NC_008218; Sterner et al., 2006). We thus sequenced the mitochondrial genome of the highest altitude living snub-nosed monkey species (*R. bieti*) from an individual collected from Lanping district (3500 m) in Yunnan Province, China and the mitochondrial genome of a lower altitude living snub-nosed monkey (*R. avunculus*) collected from Eastern Vietnam (750 m) for comparison. In addition to the mitochondrial genomes of the *Rhinopithecus* species, those of seven other lower altitude living colobine genera available in GenBank were also included in the analyses (*Pygathrix nemaeus*, NC_008220; *Presbytis melalophos*, NC_008217; *Nasalis larvatus*, NC_008216; *Semnopithecus entellus*, NC_008215; *Procolobus badius*, NC_008219; *Trachypithecus obscurus*, NC_006900; and *Colobus guereza*, NC_006901; Raaum et al., 2005; Sterner et al., 2006). The taxonomic and geographical information for these colobines as well as their mitochondrial genome GenBank accession numbers are provided in Table 1.

For *R. bieti* and *R. avunculus* samples, we extracted total DNA from frozen tissues using a standard proteinase K, phenol/chloroform extraction (Sambrook et al., 1989). Complete mitochondrial genomes were then amplified in 4 overlapping segments using the Long and Accurate PCR™ Kit (Takara Biotechnology Co., Ltd). The primer information is listed in Table 2. These long-PCR primers were designed using conserved mtDNA regions found within Colobinae mitochondrial genome sequences available in GenBank. PCR amplification was carried out using the following parameters: 94 °C hot

Table 1
Species used in this study. **1** — Oates et al. (1994); Nijman and Meijaard (2000); Bennett and Sebastian (1988); Yeager (1989); Napier and Napier (1985). **2** — Lippold (1977, 1998). **3** — Li et al. (2003); Ren et al. (2000); Tan et al. (2007). **4** — Li et al. (2008); Kirkpatrick et al. (1998). **5** — Boonratana and Le (1998). **6** — Brandon-Jones (1999); Kawamura (1979). **7** — Gupta and Chivers (1999); Bernstein (1967); Curtin and Chivers (1978). **8** — Koenig et al. (1997); Sugiyama (1976); Oppenheimer (1977); Molur et al. (2003). **9** — Fashing and Oates (in press); Oates (1977). **10** — Ting (2008).

	Scientific name	Common name	Distribution and habitat	Altitude range	Accession nos.
Asian colobines	*Nasalis larvatus* (Oates et al., 1994; Nijman and Meijaard, 2000; Bennett and Sebastian, 1988; Yeager, 1989; Napier and Napier, 1985)	Proboscis monkey	Borneo, SE Asia — lowland rainforest	0–350 m	NC_008216
	Pygathrix nemaeus (Lippold, 1977, 1998)	Red-shanked douc	Mainland SE Asia — primary evergreen forest	0–2000 m	NC_008220
	Rhinopithecus roxellana (Li et al., 2003; Ren et al., 2000; Tan et al., 2007)	The golden snub-nosed monkey	Qinling, Sichuan/Gansu and Shennongjia Mountains in China — montane, temperate forest	2000–3400 m	NC_008218
	Rhinopithecus bieti (Li et al., 2008; Kirkpatrick et al., 1998)	The black snub-nosed monkey	Himalayan Mountain of southwestern China — montane, temperate forest	3400–4600 m	HM125579 (this study)
	Rhinopithecus avunculus (Boonratana and Le, 1998)	The Tonkin snub-nosed monkey	Vietnam — subtropical rainforest	200–1200 m	HM125578 (this study)
	Presbytis melalophos (Brandon-Jones, 1999; Kawamura, 1979)	Mitered leaf monkey	Malay Peninsula, Sumatra, and western Borneo — tropical lowland and montane rainforest	0–2500 m	NC_008217
	Trachypithecus obscurus (Gupta and Chivers, 1999; Bernstein, 1967; Curtin and Chivers, 1978)	Dusky or spectacled leaf monkey	SE Asia — tropical lowland and montane rainforest	Unknown, low-medium altitudes	NC_006900
	Semnopithecus entellus (Koenig et al., 1997; Sugiyama, 1976; Oppenheimer, 1977; Molur et al., 2003)	Hanuman langur	Himalayan foothills and throughout India — extreme variety of habitats	0–4000 m	NC_008215
African colobines	*Colobus guereza* (Fashing and Oates, in press; Oates, 1977)	*Guereza colobus*	West Central — East Africa, tropical lowland to montane rainforest	200–3300 m	NC_006901
	Procolobus badius (Ting, 2008)	Western red colobus	West African Guinean primary rainforest	Unknown, low-medium altitudes	NC_008219

Table 2
Long PCR primers for amplifying the complete mitochondrial genomes of *Rhinopithecus bieti* and *Rhinopithecus roxellana*.

No.	Primer (5′–3′)
1	CAAGACACTGAAAATGCCTAGACGGGTT
	TCAGCCTATGTGTGTGATTGAAGAGTATGC
2	CAAACTCTCGCTCCACAGAAGCTGCTACC
	TAGATGGCCTGCTGTAATATTGGCGGTTAG
3	GGACTCTTACCCCACTCATTTACACCCAC
	GAGGGTAGTCAAGGGTGAAGGCCAAGTTG
4	GACGCCAACACAGCAGCCATTCAAGCA
	GCGGGGATGCTTGCATGTGTAATCTTGCT

start (1 min 30 s), 32 cycles of 97 °C denaturation (10 s), 58 °C (for Primer1), 65 °C (for Primer3), or 68 °C (for Primer2 and Primer4) annealing (5 min 30 s). At the end, a final 10-min extension at 72 °C was performed. Long PCR products were sequenced in both directions using a primer walking strategy and a total of 66 sequencing primers. The sequencing primer information is available upon request. Sequencing was performed in an ABI PRISM™ 3700 DNA sequencer following the manufacturer's protocol.

2.2. Phylogenetic reconstruction

The concatenated nucleotide sequences of 13 protein-coding genes, 22 tRNAs, 2 rRNAs and the D-loop of 10 colobine species (two Chinese snub-nosed monkeys and eight other colobines) were aligned with the CLUSTAL X program (Thompson et al., 1997) and refined by eye.

Phylogenetic trees were reconstructed with the concatenated nucleotide alignment using MEGA4 (Kumar et al., 2008) for neighbor-joining (NJ) analysis, PAUP*4.0b10 (Swofford, 2002) for maximum likelihood (ML) analysis, and MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) for Bayesian inference. In the NJ analysis, the Kimura 2-parameter nucleotide model with a pairwise deletion option for gaps was used. In the ML analysis, the best-fitting model of sequence evolution was determined using the program Modeltest 3.06 (Posada and Crandall, 1998). The chosen model and its parameters were used in PAUP* to infer the ML tree with the heuristic algorithm, 10 random-addition sequence replicates, and TBR branch swapping. The reliability of the tree topologies was evaluated using bootstrap support (Felsenstein, 1985; BS) with 1000 replicates for the NJ analysis and 100 replicates for the ML analysis. The parameters estimated by Modeltest were also used as priors for the Bayesian analysis. Four Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses were run for 2×10^6 generations, sampling trees every 1000 generations. The first 25% of the sample was discarded as the burnin. A 50% majority-rule consensus of post burn-in trees was constructed to summarize posterior probabilities (PP) for each branch. In all analyses, two African colobine species, *P. badius* and *C. guereza*, were used as outgroups based on the general consensus that they are the sister group to the other taxa in the sample, which are all found in Asia (Xing et al., 2005; Sterner et al., 2006).

2.3. Selection constraint analyses

The nonsynonymous to synonymous rate ratio ω (*dn*/*ds*) provides an indication of a change in selective pressure. A *dn*/*ds* ratio of 1, <1 and >1 will indicate neutral evolution, purifying selection and positive selection on the protein involved, respectively. Here, we used the codon-based likelihood approach implemented in the CODEML program of the PAML4 package (Yang, 2000) to assess potential adaptive evolution in mitochondrial protein-coding genes. The combined dataset of 13 protein-coding genes was used. All models correct the transition/transversion rate and codon usage biases (F3×4). Different starting ω values were also used to avoid local optima on the likelihood surface (Suzuki and Nei, 2001).

To detect if there are significant changes in selective pressure between the high altitude and lower altitude colobines, the "two-ratios" model was used, which assumes that the branches of interest have different ratios from the background ratio ω_0 (Yang, 1998, 2002; Yang and Nielsen, 1998) in the branch-specific models. The "two-ratios" model assumes a ω ratio for the two Chinese snub-nosed monkeys and the background ratio for all other lineages in the phylogeny. In addition, we also used the "one-ratio" (M0) and "free-ratios" (M1) models. The M0 model assumes the same ω ratio for all branches while the M1 model assumes an independent ω ratio for each branch (Yang, 1998). We constructed likelihood ratio tests (LRT) comparing the M0 and "two-ratios" models, and the M0 and M1 models. Significant differences between the models were evaluated by calculating twice the log-likelihood difference following a χ^2 distribution, with the number of degrees of freedom equal to the difference in the numbers of free parameters between the models.

Considering that positive selection may act in very short episodes during the evolution of a protein (Gillespie, 1991) and affect only a few sites along a few lineages in the phylogeny, recently developed likelihood models accommodating ω ratios to vary both among lineages of interest and amino acid sites, that is, an improved version of the "branch-site" model, were considered here (Zhang et al., 2005). We used branch-site Model A for stringent testing and identification of sites under positive selection along the lineages of interest (Zhang et al., 2005). The presence of sites with ω>1 is suggested when the positive-selection model (Model A) fits the data significantly better than the corresponding null model (M1a). The conservative Bayes Empirical Bayes (BEB) approach (Yang et al., 2005) was then used to calculate the posterior probabilities of a specific codon site and to identify those most likely to be under positive selection.

3. Results

3.1. Characteristics of Colobinae mt genomes

The general characteristics of the mitochondrial genomes are summarized in Table 3. The complete mitochondrial genomes of 10 species range from 16,532 bp to 16,648 bp in size, with the newly determined *R. bieti* and *R. avunculus* mt genomes being 16,551 bp and 16,548 bp long, respectively. Genome length differences are largely due to the variation in tandem repeats of the control region. All genomes share not only 13 protein-coding genes, 22 tRNA genes, 2 rRNAs, and a control region, but also the same gene order. These mitochondrial genomes are apparently AT-biased (ranging from 59.25% to 62%; average = 61.13%). The sequence divergence among ingroup taxa ranged from 12.9% (*COXI*) to 19.1% (*NADH3*) for the protein-coding dataset (average 16.1%), from 7.6% (12*S rRNA*) to 9.2% (16*S rRNA*) for the rRNA dataset (average 8.6%), 8.12% for the *tRNA* dataset, 18.3% for the control region, and 14.5% for the complete dataset. The mitochondrial genomes of *R. bieti* and *R. avunculus* shared the highest similarity to that of *R. roxellana*.

3.2. Phylogenetic analyses

The mitogenomic phylogenetic analyses produced inconsistent tree topologies with varying levels of support based on the three tree-building methods (Fig. 1). These trees differed primarily in the relationships among *Rhinopithecus*, *P. nemaeus* and *N. larvatus*, and the placement of *S. entellus*. These have also been the relationships most difficult to resolve in previous analyses of colobine genera (Zhang and Ryder, 1998; Li et al., 2004; Xing et al., 2005; Sterner et al., 2006; Whittaker et al., 2006; Osterholz et al., 2008; Karanth et al., 2008; Ting et al., 2008). In all trees, the two Chinese snub-nosed monkeys (*R. roxellana* and *R. bieti*) form a robustly supported clade with the Tonkin snub-nosed monkey (*R. avunculus*) (ML and NJ BS = 100%, PP = 1.00), which in turn is most closely related to *P. nemaeus* and

Table 3
Characterization of mitochondrial genes examined in the present study.

Genes	Alignment length	Parsimony-informative sites	Nucleotide composition				Ti/Tv	Best fit model	Among-site rate variation		Pairwise distance (%)
			A	T	G	C			I	α	
ND1	957	226	0.300	0.298	0.115	0.287	4.947	TVM + I + G	0.5484	3.6431	14.9
ND2	1044	279	0.354	0.284	0.083	0.279	3.424	TrN + I + G	0.4228	1.5559	17.8
COX1	1554	327	0.273	0.325	0.159	0.243	4.311	GTR + I+G	0.5684	1.5216	12.9
COX2	684	142	0.314	0.296	0.139	0.251	4.558	TrN + G	0	0.1867	13.6
ATP8	210	64	0.381	0.298	0.065	0.255	2.937	TrN + I + G	0.3912	0.9438	18.1
ATP6	681	181	0.315	0.300	0.106	0.278	4.441	TrN + I + G	0.482	2.7421	17.4
COX3	784	192	0.279	0.316	0.139	0.267	6.342	HKY + I + G	0.5734	3.7662	15.3
ND3	346	93	0.320	0.317	0.102	0.261	5.262	HKY + I+G	0.4618	2.4733	19.1
ND4L	297	71	0.304	0.341	0.112	0.243	4.986	K81uf + I + G	0	0.3116	16.7
ND4	1378	356	0.317	0.307	0.100	0.276	4.491	TrN + I + G	0.5589	8.0614	16.1
ND5	1806	483	0.323	0.303	0.105	0.270	4.38	HKY + I + G	0.4671	1.5987	18.3
ND6	525	125	0.221	0.415	0.289	0.075	6.061	HKY + I + G	0.5332	1.3472	15
CYTB	1135	304	0.298	0.299	0.114	0.289	4.591	HKY + I + G	0.505	1.2894	17.7
12SrRNA	952	117	0.348	0.234	0.182	0.236	3.55	GTR + I + G	0.6081	0.8859	7.6
16SrRNA	1595	237	0.359	0.241	0.174	0.226	2.798	GTR + I + G	0.4262	0.4819	9.2
tRNA	1555	203	0.348	0.280	0.150	0.222	5.846	GTR + I + G	0.4666	0.5404	8.1
D-loop	1204	336	0.294	0.286	0.134	0.286	2.5468	TVM + I + G	0.346	1.1386	18.3
Combined data	16,781	3779	0.323	0.288	0.128	0.261	3.764	GTR + I + G	0.5166	1.4545	14.5

Note: Ti = transition; Tv = transversion; I = proportion of invariable sites; and α = gamma distribution shape parameter.

N. larvatus (ML and NJ BS = 100%, PP = 1.00). All inferred trees also show a sister taxon relationship between *T. obscurus* and *P. melalophos* (ML BS = 100%, NJ BS = 97%, PP = 1.00).

In the Bayesian analysis (Fig. 1a), *S. entellus* is the sister taxon to all other Asian colobines, and the *T. obscurus*/*P. melalophos* clade and the *Rhinopithecus*/*P. nemaeus*/*N. larvatus* clade are sister groups (PP = 0.95). Within the latter clade, *P. nemaeus* and *N. larvatus* are more closely related (PP = 1.00). In the NJ analysis (Fig. 1b), the *T. obscurus*/*P. melalophos* clade is sister to the rest of the Asian colobines, and *S. entellus* is the sister taxon to the *Rhinopithecus*/*P. nemaeus*/*N. larvatus* clade (BS = 77%), in which *P. nemaeus* and *N. larvatus* are more closely related (BS = 95%). In the ML analysis (Fig. 1c), *S. entellus* is more closely related to the *T. obscurus*/*P. melalophos* clade (BS = 60%). Within the *Rhinopithecus*/*P. nemaeus*/*N. larvatus* clade, *Rhinopithecus* and *P. nemaeus* have a closer affinity (BS = 65%). Although the Bayesian tree seems more likely because of its higher support on all branches (PP ≥ 0.95), the addition of nuclear DNA sequence (beyond Ting et al.'s, 2008 dataset) would aid in resolving the relationships among the colobine genera.

3.3. Positive selection analysis

Because the CODEML likelihood analysis may be sensitive to the tree topology used, the three tree topologies inferred from the different tree-building methods (Fig. 1a–c) were all used in the positive selection analysis.

Table 4 displays the results of positive selection tests. Identical conclusions were obtained regardless of the tree topology used. In the analyses of branch-specific models, the ω ratio calculated in the M0 model is 0.06538, suggesting that most mitochondrial genes in the sampled colobines have evolved under strong functional constraints, which is in accordance with the known functional significance of mitochondria as a respiration chain necessary for OXPHOS and electron transport. Interestingly, both "two-ratio" and "free-ratios" (M1) models fit the data significantly better than the one-ratio model (M0) ($P<0.05$), indicating that the mitochondrial protein-coding genes have been subject to different selection pressures in the Colobinae, especially between the Chinese snub-nosed monkeys and other colobines.

When we conducted LRTs based on the branch-site models for the Chinese snub-nosed monkeys, our analyses suggest that there is significant evidence of positive selection along the branch to *R. roxellana* ($0.01<P<0.05$). Residues 533 and 3307, which are located in the *NADH2* (corresponds to site 215) and *NADH6* (corresponds to site 86) genes respectively, were inferred as positively selected sites for the *R. roxellana* branch with high posterior probabilities of >95% (Table 4).

4. Discussion

Mammalian mitochondrial genomes include 13 protein-coding genes, and their products are necessary for oxygen usage and energy metabolism (Boore, 1999; Xu et al., 2007; Luo et al., 2008). An increasing number of cases of adaptive evolution in mitochondrial genes have been reported in artiodactyls, perissodactyls, humans and pikas living in high altitude environments (Torroni et al., 1994; Mishmar et al., 2003; Ruiz-Pesini et al., 2004; Xu et al., 2005; Di Rocco

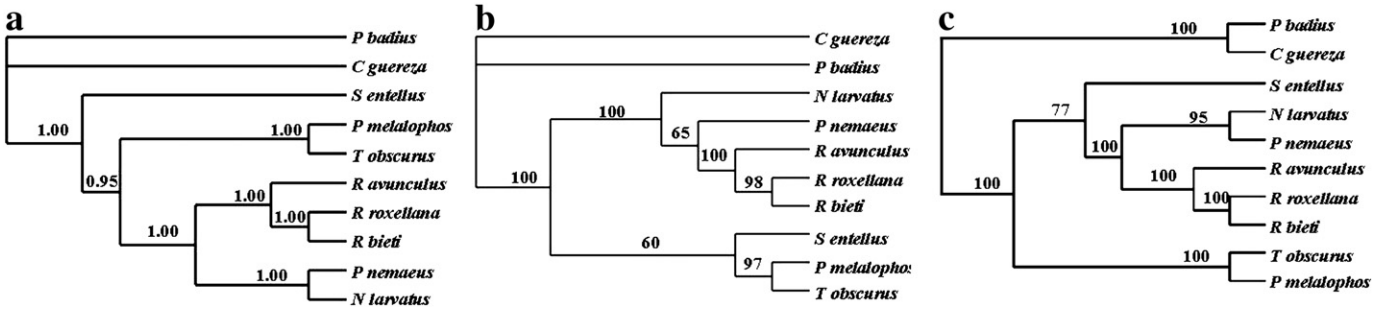

Fig. 1. Phylogenetic trees inferred from (a) Bayesian analysis, (b) NJ analysis, and (c) ML analyses. Posterior probabilities and bootstrap supports are indicated above the nodes.

Table 4
CODEML analyses of selection on mitochondrial genes in the Colobinae.

Trees	Models	ln L	Parameter estimates	2ΔL	Positively selected sites
	Branch-specific models				
Bayesian tree (Fig. 1a)	M0	−47106.02026	$\omega = 0.06538$		
	Two-ratio	−47101.92036	$\omega1 = 0.08760$ $\omega0 = 0.06384$	(Two-ratio versus M0) 8.199814**	
	M1	−47089.39747		(M1 versus M0) 33.245586**	
NJ tree (Fig. 1b)	M0	−47104.46526	$\omega = 0.06541$		
	two-ratio	−47100.36542	$\omega1 = 0.08763$ $\omega0 = 0.06387$	(Two-ratio versus M0) 8.199684**	
	M1	−47088.47364		(M1 versus M0) 31.983234*	
ML tree (Fig. 1c)	M0	−47106.65971	$\omega = 0.06578$		
	Two-ratio	−47102.72148	$\omega1 = 0.0876$ $\omega0 = 0.06427$	(Two-ratio versus M0) 7.876476**	
	M1	−47091.90107		(M1 versus M0) 29.517294*	
	Branch-site models (the branch to R. roxellana)				
Bayesian tree (Fig. 1a)	Null model	−46543.52924	$p_0 = 0.89758$ $p_1 = 0.07776$ $p_{2a} = 0.02270$ $p_{2b} = 0.00197$ $\omega_0 = 0.03388$ $\omega_1 = 1$ $\omega_2 = 1$		
	Model A	−46541.11551	$p_0 = 0.91505$ $p_1 = 0.07908$ $p_{2a} = 0.00540$ $p_{2b} = 0.00047$ $\omega_0 = 0.03398$ $\omega_1 = 1$ **$\omega_2 = 6.16586$**	(Model A versus null model) 4.827462*	533 (0.976) 3307 (0.982)
NJ tree (Fig. 1b)	Null model	−46539.5779	$p_0 = 0.89792$ $p_1 = 0.07762$ $p_{2a} = 0.02252$ $p_{2b} = 0.00195$ $\omega_0 = 0.03388$ $\omega_1 = 1$ $\omega_2 = 1$		
	Model A	−46537.14626	$p_0 = 0.91527$ $p_1 = 07891$ $p_{2a} = 0.00536$ $p_{2b} = 0.00046$ $\omega_0 = 0.03397$ $\omega_1 = 1$ **$\omega_2 = 6.20591$**	(Model A versus null model) 4.863292*	533 (0.976) 3307 (0.982)
ML tree (Fig. 1c)	Null model	−46545.63992	$p_0 = 0.89760$ $p_1 = 0.07823$ $p_{2a} = 0.02223$ $p_{2b} = 0.00194$ $\omega_0 = 0.03404$ $\omega_1 = 1$ $\omega_2 = 1$		
	Model A	−46543.26715	$p_0 = 0.91475$ $p_1 = 0.07953$ $p_{2a} = 0.00527$ $p_{2b} = 0.00046$ $\omega_0 = 0.03414$ $\omega_1 = 1$ **$\omega_2 = 6.18166$**	(Model A versus null model) 4.745554*	533 (0.976) 3307 (0.982)

The bold form of the items means the signature of positive selection, as evidenced by ω larger than 1.
* $0.001 < p < 0.01$.
** $p < 0.01$.

et al., 2009; Xu et al., 2007; da Fonseca et al., 2008; Luo et al., 2008; Hassanin et al., 2009; Ning et al., 2010). In the present study, the mitochondrial protein-coding genes of the Colobinae, including 2 Chinese snub-nosed monkeys and 8 other colobines, were analyzed in the first attempt to understand the genetic basis of high-altitude adaptation in non-human primates. Our study adds to the growing evidence of adaptive evolution in the mitochondrial genome of high-altitude species. We found signatures of positive selection in one Chinese snub-nosed monkey, *R. roxellana*, which may indicate adaptation to physiological hypoxia and cold stress in this species. In addition, we provide valuable information on the potentially important adaptive amino acid replacements. Two residues in the *NADH2* and *NADH6* genes were found to be under positive selection (533 and 3307, respectively) with high posterior probabilities in all analyses. The two residues are M and W, respectively, in *R. roxellana*, whereas in the other colobines examined here, they correspond to A/T and F. To test if these two residues identified are also present in other *R. roxellana* individuals, we sequenced the *NADH2* and *NADH6* genes in two additional *R. roxellana* individuals collected from Gansu Province at an elevation of 4000 m and found the same residues in these two positive selection sites (data not shown), indicating that these two positively selected sites are most likely fixed in this species. In addition, we also surveyed the amino acid changes in these two positions across the other mammalian orders, and found that although amino acid M identified in ND2 of the present study is also observed in some species of Cetacea, Artiodactyla and Rodents, amino acid W identified in ND6 here is not observed in any of the other mammalian order species examined, showing the uniqueness of this substitution. It is possible that amino acid W is more likely to be involved in the energy metabolism change of *R. roxellana*.

Interestingly, the NADH dehydrogenase complex has been considered important in the adaptive evolution of the mammalian mitochondrial genome in previous studies (Xu et al., 2007; da Fonseca et al., 2008; Ning et al., 2010). For example, *NADH6* was found to be under positive selection in high altitude living Tibetan horses (Xu et al., 2007; Ning et al., 2010). NADH dehydrogenase is the first and largest enzyme complex in the mitochondrial oxygen-respiration chain. It receives electrons from the oxidation of NADH and provides electrons for the reduction of quinone to quinol (da Fonseca et al., 2008). The residue substitutions that occur in this complex are thought to interfere with the efficiency of the proton-pumping process (da Fonseca et al., 2008), which then influences metabolic performance (Hassanin et al., 2009).

Interestingly, further examination of the two positively selected residues found in this study reveals that both are located in the trans-membrane domains. This result is in contrast to that from da Fonseca et al. (2008). In their study of adaptive evolution in mammalian mitochondrial genomes, signatures of adaptive variation in the NADH dehydrogenase complex have been suggested to be confined solely to the loop regions. Characterizing the substitutions found under positive selection in this study through the use of functional assays would be important to testing how changes to them might affect metabolism.

Although the functional changes brought by these two positively selected residues were not tested here, we predict that mitochondrial genes, specifically *NADH2* and *NADH6*, play a role in *R. roxellana's* adaptation to a high altitude environment. Surprisingly, no evidence for positive selection was found in *R. bieti*, another Chinese snub-nosed monkey examined here. It seems that the adaptive response to metabolic physiology from mitochondrial proteins described for the other high-altitude mammals is not present in *R. bieti*. Besides the *R. bieti* individual analyzed here, we also sequenced all 13 mt protein-coding genes of another *R. bieti* individual collected from Weixi district (3580 m) in Yunnan Province and found no evidence of the positive selection as well (GenBank accession number HQ287798). The unexpected absence of positive selection observed in the *R. bieti* mitochondrial genes is intriguing, especially because *R. bieti* inhabits a higher altitudinal distribution than *R. roxellana*. A possible explanation is that a different adaptive genetic basis for high altitude living exists in *R. bieti*. Signatures of positive selection and functionally associated mutations in *R. bieti* may perhaps be detected in nuclear genes that are related to energy and oxygen metabolisms, such as *leptin*, *hemoglobin*, *myoglobin*, *transforming growth factor-β* (*TGF-β*) and *transcription factor hypoxia-inducible factor* (*HIF*) genes, etc. Indeed, previous analyses of nuclear DNA have detected adaptive variation in the *leptin* gene of

plateau pikas (Yang et al., 2008), the *hemoglobin* gene of high altitude living deer mice (Storz et al., 2007) and Andean dabbling ducks (McCracken et al., 2009), the *myoglobin* gene of Tibetans (Wu et al., 2009), and the *HIF* gene of human Andean populations (Bigham et al., 2009).

More information on the structure, function, and evolution of mitochondrial and nuclear genomes in Chinese snub-nosed monkeys is still required to reveal the exact molecular mechanisms underlying the adaptation of these monkeys to high altitude. Our study establishes a necessary foundation for these experimental investigations. It will be interesting to test the physiological roles of the *NADH2* and *NADH6* genes in *R. roxellana* and the functional effects of 2 positively selected amino acid substitutions. In addition, nuclear genes and more sequence data from Chinese snub-nosed monkey individuals are needed for further study of non-human primate adaptation to high altitude environments.

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